

TRICARBOXYLIC ACID CYCLE ACTIVITY IN *ACETOBACTER PASTEURIANUM*¹

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Previous papers from this laboratory (King and Cheldelin, 1952, 1952*a*, 1954; Hauge *et al.*, 1955, 1955*a*) have reported on the oxidation of carbohydrates in *Acetobacter suboxydans*. Neither resting nor disintegrated cells can oxidize acetate or other intermediates of the tricarboxylic acid cycle. These findings have recently been confirmed in another laboratory (Rao and Gunsalus, 1955). From the comparative biochemical viewpoint, it is of interest to study the oxidative behavior of other species of the genus *Acetobacter*. Results reported in this paper demonstrate that *Acetobacter pasteurianum*, in contrast to *A. suboxydans*, can oxidize all the intermediates of the Krebs cycle. Other evidence also indicates the functioning of this cycle in *A. pasteurianum*.

METHODS AND MATERIALS

Acetobacter pasteurianum ATCC 6033 was used. Cells were grown, harvested and lyophilized as reported previously (King and Cheldelin, 1954) for *A. suboxydans*. The lyophilized whole cells will be referred to in this paper as "resting cells." Lyophilized cells were disintegrated in a 10-kc Raytheon sonic oscillator as described in a previous paper (King and Cheldelin, 1956). The product will be referred to as "cell homogenate." The soluble fraction was obtained by centrifuging disintegrated cell mixture for 2-3 hr at about $22,000 \times G$.

DL- α -Lipoic acid was kindly supplied by Dr. Lester J. Reed. All other chemicals were obtained commercially and used without further purification. DL-Isocitric lactone was freshly hydrolyzed before use in a 5 per cent excess of the calculated amount of KOH in a boiling water bath for 15 min, then adjusted to pH 6.0.

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Spectrophotometric determinations of reduced pyridinenucleotides were performed in a Beckman Model B spectrophotometer with 1-cm² Corex cell at $\lambda = 340 \mu\mu$. A Beckman Model DU spectrophotometer with 1-cm² silica cells was used at wave lengths below 300 $\mu\mu$. Oxygen consumption and CO₂ production were determined by the conventional method in a Warburg apparatus. CO₂ retention was corrected by using the effective Bunsen coefficient at pH 6.0. Protein was determined by the biuret method as in a previous paper (King and Cheldelin, 1954). Lipoic acid was determined by a manometric method with *Streptococcus faecalis* strain 10 Cl (Gunsalus *et al.*, 1952). The organism was kindly supplied by Drs. I. C. Gunsalus and D. J. O'Kane. Citrate was determined by a pentabromoacetone method (Perlman *et al.*, 1944).

RESULTS

Oxidation of tricarboxylic acid cycle intermediates. Resting cells of *A. pasteurianum* oxidized all Krebs cycle intermediates completely as shown in table 1. The endogenous oxidation was very small. The initial rate, as well as the time required for complete oxidation, varied with the compounds, probably largely because of permeability differences.

Citrate oxidation by resting cells varied among different batches of resting cells, with some cells showing practically no oxidation. However, preparations of cell homogenates oxidized citrate readily and completely to CO₂ and water as shown by manometric determinations.

These data suggested the overall operation of the tricarboxylic acid cycle in *A. pasteurianum*.

Formation of malate from fumarate. Conversion of fumarate to malate was demonstrated by measuring the latter compounds polarimetrically as its molybdate complex (Wille, 1941). When 1.65 mmoles fumarate were incubated in the presence of 30 mg resting cells in a total volume of 10-ml of 0.1-M phosphate buffer, pH 6.0, under

TABLE 1

Oxidation of tricarboxylic acid cycle intermediates by *Acetobacter pasteurianum*

Substrate	Time	Oxygen Consumption		CO ₂ Formed		Q _{O₂}
		Found	Calculated	Found	Calculated	
		min-utes	atoms/molecule	sub-strate	moles/mole	
Acetate	200	4.0	4	1.9	2	4.5
Pyruvate	200	4.8	5	2.8	3	13.0
Oxalacetate	400	5.1	5	3.6	4	6.8
Citrate		(see text)	9		6	0.2
<i>cis</i> -Aconitate	500	8.4	9	4.5	6	0.7
Isocitrate	450	11.5	9	6.0	6	1.2
α -Ketoglutarate	400	8.0	8	4.9	5	1.9
Succinate	300	7.3	7	3.9	4	2.4
Fumarate	300	6.0	6	4.1	4	1.9
L-Malate	400	5.8	6	3.6	4	3.5

The system contained 10 μ moles substrate (except isocitrate, see below), 10 μ moles MgCl₂, 160 μ moles phosphate buffer, and 20 mg of resting cells; and either 0.2 ml 10 per cent KOH or 0.2 ml water in center well. Total volume, 2.8 ml; pH 6.0; temperature, 29 C. The values of oxygen consumption and of CO₂ formation are net values, corrected for endogenous oxidation. The times listed are the maximum for complete oxidation. 20 μ moles DL-isocitrate were used but the calculation was made on 10 μ moles, assuming that only the natural isomer was oxidized.

For determination of Q_{O₂} (μ atoms oxygen, mg cells/hr during the first 45 min), the system contained 100 μ moles substrate (200 μ moles isocitrate), 20 μ moles MgCl₂, 160 μ moles buffer, and 4 mg resting cells; other conditions were as described above.

nitrogen for 16 hr, an optical rotation of $\alpha_D = 4.16^\circ$ was observed in a 2-dm polarimeter tube. Readings were made after addition of 1.0 ml glacial acetic acid and 9 ml of 20 per cent ammonium molybdate. Under the same conditions no optical activity was observed by omission of either cells or fumarate. This experiment qualitatively demonstrated the presence of fumarase in the cells.

Formation of fumarate and cis-aconitate from L-malate and citrate. The presence of fumarase and *cis*-aconitase was also demonstrated in the soluble fraction from *A. pasteurianum* by Racker's spectrophotometric method (1950). Since pro-

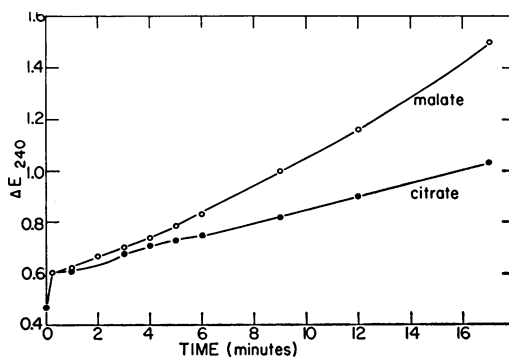


Figure 1. Fumarate and *cis*-aconitate formation from malate and citrate in *Acetobacter pasteurianum*. Each cuvette contained 80 μ moles phosphate buffer and 20 γ (in terms of protein) of the soluble fraction of a cell free extract of *A. pasteurianum*; pH 6.0; total volume 2.6 ml. At zero time 0.1 ml of 0.1-M malate or citrate was added. Both enzyme and substrate blanks were zero.

teins also show high ultraviolet absorption, the concentration of enzymes was kept low. The results are shown in figure 1. In the presence of a great excess of substrates, the formation of fumarate and *cis*-aconitate from malate and citrate, respectively, increased in a nearly linear fashion with time.

Citrate formation. Citrate was rapidly formed from *cis*-aconitate in the presence of *A. pasteurianum* resting cells, but the rate of condensation of acetate or pyruvate with oxalacetate to give citrate was very slow. Addition of adenosine triphosphate, (ATP)², BaCl₂, or monofluoroacetate did not increase the citrate formation. Oxalacetate alone under anaerobic conditions also yielded citrate due to the presence of oxalacetate carboxylase.

The slower formation of citrate from pyruvate and oxalacetate might be due to several reasons. When Krebs cycle intermediates were oxidized in the presence of triphenyl tetrazolium chloride, *cis*-aconitate and citrate were utilized by far the most rapidly (up to 4 times as rapidly as fumarate, 15 times as rapidly as succinate). The most rapid conversion of all was the aconi-

² The following abbreviations were used in this paper: ATP = adenosine triphosphate; CoA = coenzyme A; DPN = diphosphopyridinenucleotide; DPNH = reduced DPN; DPT = diphosphothiamine; TPN = triphosphopyridinenucleotide; Tris = tris (hydroxymethyl) aminomethane.

TABLE 2
Oxidations of α -ketoglutarate and pyruvate by the dialyzed soluble fraction of *Acetobacter pasteurianum*

	ΔE_{340}	
	α -Ketoglutarate	Pyruvate
Complete system.....	1.042	0.485
Complete system + 10 μ g lipoic acid.....	1.005	0.493
No CoA.....	0.093	0.105
No DPT.....	0.173	0.085
No glutathione.....	0.111	0.055
No phosphate.....	0.930	0.470
No substrate.....	0.050	0.050
No enzyme.....	0.010	0.000

The complete systems contained 50 γ CoA, 500 γ DPT, 10 μ moles glutathione, 5 μ moles $MgCl_2$, 200 μ moles tris buffer, 2 mg DPN, 0.1 ml dialyzed cell-free extract containing 1.1 mg protein, and 10 μ moles phosphate. Total volume, 2.9 ml; pH 8.0; temperature 18–20 C. The reaction was started at zero time by addition of 10 μ moles α -ketoglutarate or pyruvate in 0.1 ml. ΔE_{340} values are optical densities at the end of 5 min.

tase reaction. On the contrary, the high activity of oxalacetate decarboxylase (which can also function anaerobically) might deplete the supply of oxalacetate for citrate synthesis.

Malonate inhibition of succinate oxidation. The influence of malonate was tested on succinate oxidation. The degree of inhibition varied with the ratio of the inhibitor to the substrate. About 50 per cent inhibition was observed when 200 μ moles of malonate were added to a system containing 50 μ moles of succinate in the presence of resting cells.

Pyruvate and α -ketoglutarate oxidation. Oxidations of pyruvate and α -ketoglutarate by animal tissues and certain other microorganisms require CoA and lipoic acid (Gunsalus *et al.* 1955, Weinhouse, 1954). In *A. suboxydans* pyruvate is first decarboxylated to acetaldehyde (King and Cheldelin, 1954a), which is in turn oxidized to acetate in the presence of a TPN- or a DPN-linked acetaldehyde dehydrogenase (King and Cheldelin, 1956), with no demonstrable CoA requirement. When these oxidations were studied in the soluble fraction of *A. pasteurianum*, it was found that both were DPN specific, and that CoA and DPT were required. Each

oxidation was measured by the formation of DPNH, as shown in table 2. Attempts to remove lipoic acid by alumina treatment as described by Seaman (1954) failed to demonstrate its requirement. However, manometric assay (Gunsalus *et al.*, 1952) of treated samples with *Streptococcus faecalis* showed that less than 20 per cent of the factor was removed. Apparently lipoic acid is tightly bound to the proteins in this organism in contrast to *Escherichia coli*. These results clearly show that these oxidations in *A. pasteurianum* are different from those in *A. suboxydans*.

DISCUSSION

The results presented above have demonstrated the occurrence of tricarboxylic acid cycle reactions in *A. pasteurianum*. It is interesting to note also that the cytochrome spectra of this organism are different from those of *A. suboxydans* in intact cells (Smith, 1954; Castor and Chance, 1955) and in soluble and solubilized particulate fractions (King and Cheldelin, 1956a). Thus, the biochemical disparity between these two organisms, with respect to substrate oxidizability, terminal pathways and electron transfer, contrasts strongly with their systematic (taxonomic) similarity.

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SUMMARY

Qualitative and quantitative evidence has been presented for extensive tricarboxylic acid cycle activity in *Acetobacter pasteurianum*. The oxidative behavior of this organism contrasts strongly with that of *Acetobacter suboxydans* in several respects, including substrate oxidizability, terminal pathways, and electron transfer.

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