MICROBIOLOGICAL DISSIMILATION OF TRICARBALLYLATE AND *TRANS*-ACONITATE[1]

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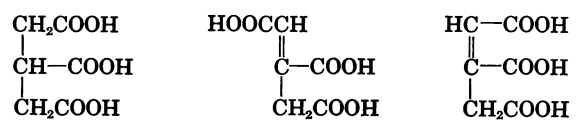
ABSTRACT

ALTEKAR, W. W. (National Chemical Laboratory, Poona, India) AND M. R. RAGHAVENDRA RAO. Microbiological dissimilation of tricarballylate and *trans*-aconitate. J. Bacteriol. **85:**604–613. 1963.—Two fluorescent pseudomonads capable of metabolizing tricarballylate and *trans*-aconitate were isolated by the soil-enrichment culture technique. These and some other species of bacteria were tested for their ability to utilize for growth the salts of many di- and tricarboxylic acids. Alloisocitrate and mesaconate were not utilized by any of the ten strains tested; only two strains grew on tricarballylate and itaconate. *trans*-Aconitate was utilized by many strains which had not been previously exposed to this compound. The resting cells of two strains could adapt to oxidize two acids (tricarballylate and *trans*-aconitate), and this induction was chloramphenicol-sensitive. The tricarballylate-grown cells were simultaneously induced to oxidize *trans*-aconitate and other tricarboxylates, whereas the *trans*-aconitate-grown cells were not induced to oxidize tricarballylate, and their subsequent induction was inhibited by chloramphenicol. This was confirmed by experiments with lyophilized cells and with enzymatic tests on cell-free extracts. The "permease systems" for these acids tended to be inactivated in older cells, and the ability of the cells to resynthesize them seemed to depend upon their age. Aconitate isomerase was present in cell-free extracts and cells grown on *trans*-aconitate or tricarballylate. But tricarballylate dehydrogenase was present only in tricarballylate-grown cells. The cell-free extracts of the two organisms contained the enzymes of the Krebs cycle and isocitritase. These enzymes are most probably operative during growth on and oxidation of these two acids as sole carbon sources.

Tricarballylic and *trans*-aconitic acids occur in nature in unripe beet root, barley, maize, and sugar beet as minor products (Nelson and Mottern, 1931; Dalgliesh, Long, and Tyler, 1952; Roberts and Martin, 1954; Vavruch, 1954; Miller and Swain, 1960). *trans*-Aconitic acid occurs to the extent of 4 to 5% in cane molasses (Roberts and Martin, 1954). A preliminary report (Gray and Brooks, 1954) on isolation of tricarballylate-oxidizing microorganisms and on their ability to oxidize the Krebs cycle intermediates and tricarballylate appeared almost 10 years ago. No other reports on the biological degradation of these two acids appeared in the literature until Rao and Altekar (1961, 1962) reported the presence of two new enzymes, aconitate isomerase and tricarballylate dehydrogenase, in the microorganisms capable of degrading *trans*-aconitate and tricarballylate, respectively. Soon afterwards, Gray and Raulett (1962) also reported the presence of tricarballylate dehydrogenase in a tricarballylate-oxidizing pseudomonad. The present communication deals with the ability (or otherwise) of many microorganisms to grow on these acids as the sole carbon source, their inductive patterns towards



I *Tricarballylic acid*

II *trans-Aconitic acid*

III *cis-Aconitic acid*

[1] N.C.L. Communication no. 534.

these two acids, and, finally, the presence in cell-free extracts of the enzymes of the tricarboxylic acid cycle and of the glyoxylate pathway.

MATERIALS AND METHODS

Bacteriological. The microorganisms capable of metabolizing *trans*-aconitate and tricarballylate were obtained by the soil-enrichment culture technique. The fluorescent pseudomonads obtained by the above method were employed in the experiments. These differed slightly in morphology, but were quite distinct physiologically (for example, in enzyme content and details of adaptive pattern). The other microorganisms were obtained from the National Collection of Industrial Microorganisms, National Chemical Laboratory, Poona. The microorganisms were grown in a chemically defined medium containing (g/100 ml) tricarballylic acid or *trans*-aconitic acid (neutralized with KOH) or other organic compounds as the sole carbon source, 0.5; ammonium nitrate, 0.2; potassium dihydrogen phosphate, 0.1; magnesium sulfate heptahydrate, 0.05; and ferric chloride, 0.001. For growth experiments, microorganisms were first grown in a complex medium consisting of 0.5% each of glucose, peptone (Armour), yeast extract (Oxoid), and K_2HPO_4. After 24 hr of incubation in this medium at 28 C on a shaker, they were centrifuged, washed aseptically, and suspended in an equal volume of 0.9% sterile saline. One drop of this suspension was used as inoculum for 50 ml of various media in 250-ml flasks. The flasks were incubated at 28 C on a shaker. Growth was measured at the end of 24 hr in a Klett-Summerson colorimeter (with a 660-mμ filter), using washed cells except in the case of *Aerobacter aerogenes* which does not settle well. Dried cells were prepared by lyophilization of a dense suspension of cells (10 g of cell paste plus 10 ml of water). Cell-free extracts were prepared by suspending 5 g of wet cells in 50 ml of cold phosphate buffer (0.02 M, pH 7.0). In the case of *trans*-aconitate-grown cells, 5 mM cysteine was added. The cell suspension was subjected to sonic oscillation in a Raytheon magnetostrictive oscillator (10 kc, 250 w) for 15 min. The cell debris was removed by centrifugation in the cold at 18,000 $\times$ *g* for 20 min.

Oxidation of substrates by cells was followed by the conventional manometric procedures (Umbreit, Burris, and Stauffer, 1957). Each Warburg vessel contained (in a final volume of 3 ml) 3 to 10 mg of nonproliferating ("resting") or lyophilized cells, 150 μmoles of phosphate buffer (pH 7), 20 μmoles of substrate, and 0.2 ml of 20% KOH in the center well. When used, chloramphenicol concentration was 50 μg per ml.

Enzymatic. Aconitate isomerase and tricarballylate dehydrogenase activities were determined as described previously (Rao and Altekar, 1961, 1962). The following enzymatic activities were determined: condensing enzyme according to Ochoa, Stern, and Schneider (1951); aconitase by citrate formation from *cis*-aconitate (Dickman and Cloutier, 1951) and according to Racker (1950); α-ketoglutarate dehydrogenase and succinate dehydrogenase by reduction of 2,6-dichlorophenol-indophenol (Bonner, 1955); fumarase according to Racker (1951); isocitrate and malate dehydrogenases spectrophotometrically (Ochoa, 1948; Mehler, Kornberg, and Ochoa, 1948); isocitritase by glyoxylate formation (Smith and Gunsalus, 1954); and malate synthetase according to Ajl and Wong (1956). All enzymatic units are represented as μmoles of substrate transformed per min per g of dry cells.

Chemical. Citric acid was assayed according to Natelson, Pincus, and Lugovoy (1948); isocitrate by pig heart isocitrate dehydrogenase (Ochoa, 1948); and glyoxylate according to Friedmann and Haugen (1943). Protein was estimated either by the micro-Kjeldahl method or according to Warburg and Christian (1941). DL-Isocitric lactone and *p*-chloromercuribenzoate (PCMB) were gifts from Sigma Chemical Co., St. Louis, Mo. *trans*-Aconitic acid was prepared from citrate according to Bruce (1943), *cis*-aconitic anhydride according to Anschutz and Bertram (1904), and glyoxylate according to Radin and Metzler (1955); tricarballylic acid was prepared by reduction of *trans*-aconitate (Allen, Wyatt, and Henze, 1939) and also by the procedure of Clarke and Murray (1941). Nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from the Sigma Chemical Co. The other chemicals used were commercial samples.

RESULTS

Growth. The ability of some species of bacteria to utilize for growth the various tricarboxylic

TABLE 1. *Growth on different carbon sources*

Organism	Complex medium*	Glucose	Citrate	*cis*-Aconitate	*trans*-Aconitate	DL-Isocitrate	Tricarballylate
Bacillus subtilis ATCC 6633	430	196	35	5	40	125	0
Aerobacter aerogenes NCTC 418	520	160	120	140	140	105	0
Pseudomonas fluorescens A.3.12	440	60	145	145	220	190	0
"*P. trans*," local isolate	435	100	150	240	160	115	185
"*P.* TCL," local isolate	425	132	95	220	110	155	110

* Consisted of glucose, 0.5%; peptone, 0.5%; yeast extract, 0.5%; and dipotassium hydrogen phosphate, 0.5% (final pH 7). Inoculum was grown in the complex medium for 24 hr on a shaker, aseptically centrifuged, washed, and suspended in an equal volume of sterile saline (0.9%). One drop of this was added to each 250-ml flask containing 50 ml of media. Figures represent 24-hr Klett readings.

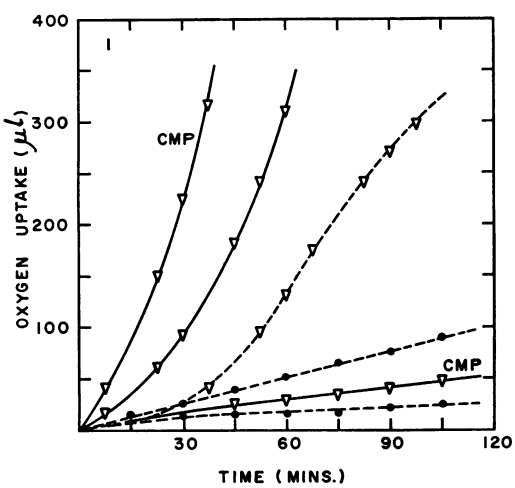


FIG. 1. *Effect of age on the oxidation of trans-aconitate by "resting cells (5.4 mg dry wt/flask) of Pseudomonas fluorescens A.3.12 grown on trans-aconitate. Solid line = 6-hr culture; broken line = 24-hr culture;* ● *= endogenous; CMP = chloramphenicol.*

acid salts as carbon sources in a chemically defined medium was tested; results are given in Table 1. While all strains tested were capable of growth on glucose, none grew on mesaconate and alloisocitrate. Most strains seemed capable of growth on *trans*-aconitate, although *Bacillus subtilis* was not efficient in this respect. Tricarballylate was a less favored substrate, being utilized by only two strains. Itaconate did not promote growth of most organisms; *Pseudomonas aeruginosa* ATCC 9027 and *P. fluorescens* A.3.12 and 8049 showed feeble growth. The feeble growth observed after 24 hr in some cases may be due to the fact that the organisms grown in the complex and rich medium containing yeast extract, peptone, and glucose were inoculated after aseptic washing into the simple, chemically defined media. No fluorescent pigment was produced on glucose, whereas with most of the organic acid salts there was pigment formation. Since *B. subtilis* did not seem to grow well on citrate or *cis*- and *trans*-aconitate and not at all on tricarballylate as the sole carbon source, it was not used in further experiments.

Induced oxidations of trans-aconitate and tricarballylate. Nonproliferating cells of cultures grown on *trans*-aconitate oxidized *trans*-aconitate without a lag, and this oxidation was not inhibited by chloramphenicol. When grown on any other substrate except tricarballylate, however, there was a lag in the oxidation of *trans*-aconitate and induction was inhibited by chloramphenicol. Except the two cultures isolated by the enrichment method on *trans*-aconitate and tricarballylate, resting cells of the other cultures grown in *trans*-aconitate seemed incapable of adapting to tricarballylate. Some of the representative data are shown in Fig. 1 and 2. The adaptive patterns seemed typical. The organisms incapable of growth on tricarballylate lacked the inductive oxidative capacity. Citrate-grown cells oxidized other substrates after a lag in the absence of chloramphenicol. It should be mentioned here that the age of the cells was important for the oxidation of *trans*-aconitate and tricarballylate by strains of pseudomonads (Fig. 2b). For example, cells harvested in the (late) stationary phase of growth on *trans*-aconitate oxidized *trans*-aconitate only after a lag period and attained a high rate of oxidation. This induction was inhibited by chloramphenicol. Apparently the permease system was destroyed and it was synthesized during this induction, as is indicated

by the results and also from experiments on lyophilized cells and cell extracts (*see below*). The ability of cells grown on tricarballylate to oxidize tricarballylate progressively diminished with age, and the cells did not seem to regain this ability when shaken with tricarballylate; cells harvested during the early log phase did, however, show inductive ability. Another peculiar feature of tricarballylate-grown cells seemed to be their chloramphenicol-insensitive ability to oxidize *trans*-aconitate without any lag.

Oxidation with lyophilized cells. In the above cases of nonoxidation of *trans*-aconitate and tricarballylate by the late stationary-phase cells grown on the respective substrates, the permease systems appeared to be destroyed or inactivated, as is indicated by the results of experiments on lyophilized cells (Table 2). In the case of *P. fluorescens* A.3.12 only, the lyophilized cells grown on *trans*-aconitate were found incapable of oxidizing *trans*-aconitate. The reason for such behavior is not clear, unless one makes the seemingly improbable assumption that aconitate isomerase in this organism is far more labile than in others. The oxidation of *trans*-aconitate does not seem to be specifically inductive, as all organisms capable of growth on *trans*-aconitate seemed to oxidize *trans*-aconitate to some extent, irrespective of the growth substrate. Citrate-grown cells also oxidized *trans*-aconitate, but *cis*-aconitate-grown cells were even better. From the rates of oxidation (isocitrate > *cis*-aconitate > *trans*-aconitate or tricarballylate), it would appear that these are oxidized via the Krebs cycle. It is not clear why isocitrate was not oxidized by lyophilized cells of *A. aerogenes*. Tricarballylate and *cis*-aconitate-grown cells were simultaneously adapted to *trans*-aconitate also, although the converse was not true. According to the sequential induction hypothesis (Stanier, 1947), *trans*-aconitate therefore qualifies as a product of oxidation of tricarballylate. But it has been shown that the above assumption is not correct in this instance, since tricarballylate has been shown to be dehydrogenated directly to *cis*-aconitate (Rao and Altekar, 1962). Further, it has been found more recently that dehydrogenation of tricarballylate is particle-bound and can be separated from aconitate isomerase activity (*unpublished data;* Gray and Raulett, 1962). The simultaneous induction to *trans*-aconitate cannot be attributed to any *trans*-aconitate impurity in tricarballylate, since the

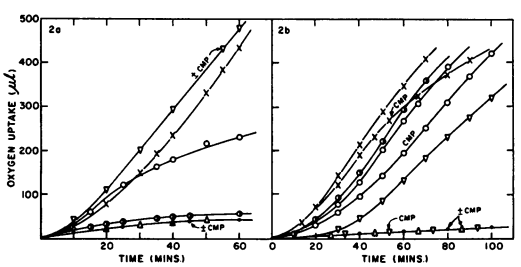


FIG. 2. *Oxidation of tricarboxylates by "resting" cells of (2a) Aerobacter aerogenes (5.4 mg dry wt/flask) and (2b) Pseudomonas fluorescens (5.4 mg dry wt/flask). Symbols for substrates:* ● = *endogenous;* ○ = *cis-aconitate;* ▽ = *transaconitate;* △ = *tricarballylate;* × = *citrate;* ◑ = DL-*isocitrate; CMP = chloramphenicol.*

TABLE 2. *Oxidation of tricarboxylic acids by lyophilized cells*

Organism	Growth substrate	Endogenous Q_{O_2}	Q_{O_2}*				
			Citrate	*cis*-Aconitate	*trans*-Aconitate	DL-Isocitrate	Tricarballylate
"*P. trans*"	Citrate	16	37	29	3	70	<Endogenous
	cis-Aconitate	5	30	31	13.5	42	<Endogenous
	trans-Aconitate	17	27	25	24	42	<Endogenous
	Tricarballylate	14	20	27	25	44	19
"*P.* TCL"	Citrate	5	31	37	4	33	<Endogenous
	trans-Aconitate	9	10	16	12	22	<Endogenous
	Tricarballylate	16	19	32	26	28	22
Aerobacter aerogenes	*trans*-Aconitate	9	1	4	4	=Endogenous	<Endogenous
Pseudomonas fluorescens A.3.12	*trans*-Aconitate	4	5	8	0.1	16	<Endogenous

* These represent values after making correction for the endogenous respiration.

latter does not contain any *trans*-aconitate; even permanganate-treated and repeatedly recrystallized samples of tricarballylic acid induced the formation of aconitate isomerase.

Formation of aconitate isomerase. Tricarballylate-grown cells oxidized *trans*-aconitate (Table 2, Fig. 3), and this oxidation was not chloramphenicol-sensitive. However, this induction of aconitate isomerase was not peculiar to tricarballylate. Cells grown on citrate and *cis*-aconitate (but not on glucose or acetate) could oxidize *trans*-aconitate, although at much lower rates, even in the presence of chloramphenicol (Fig 3). Since tricarballylate, besides *trans*-aconitate, seems to be the inducer of aconitate isomerase, a few experiments were conducted to ascertain its mode of action.

Cultures were grown in a medium containing citrate or *trans*-aconitate as the sole carbon source, and the ability of the organisms (both resting cells and lyophilized cells) to oxidize *trans*-aconitate was tested under various conditions. It is clear (Table 3) that tricarballylate inhibited the oxidation of *trans*-aconitate by "resting" as well as by lyophilized cells. The extent of inhibition seemed to depend upon the relative concentrations of the two acids (salts). The inhibition by tricarballylate of the oxidation of *cis*-aconitate and isocitrate was slight (10%) and perhaps not significant. However, preliminary experiments have indicated that the formation of citrate from *trans*- or *cis*-aconitate by cell-free extracts was not inhibited by tricarballylate even at high concentrations (20 mM).

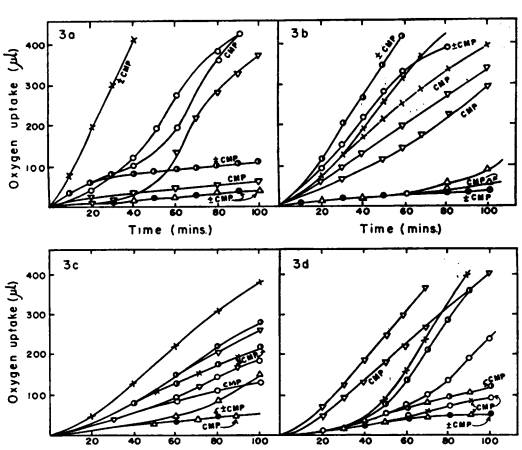


FIG. 3. *Oxidation of tricarboxylates by "resting" cells of "P. trans" grown on different substrates: citrate (3a), cis-aconitate (3b), trans-aconitate (3c), and tricarballylate (3d). Cells in 3a and 3c, 5.4 mg dry wt/flask; in 3b and 3d, 4.2 mg dry wt/flask. Symbols as in Fig. 2.*

TABLE 3. *Effect of tricarballylate on the oxidation of trans-aconitate*[a]

Condition	Resting cells[b]		Lyophilized cells
	1 hr	2 hr	
Endogenous	3.5	3.5	6.4
Endogenous + CMP[c]	3	3	—
trans-A	125	—	31.5
trans-A + CMP	104	125	—
TCL	4	15	6.0
TCL + CMP	6	4	—
trans-A + TCL	73	113	17.6
trans-A + TCL[d]	36	114	10.7
trans-A + TCL + CMP	49	100	—
trans-A + TCL[d] + CMP	21	40	—

[a] Figures represent Q_{O_2} values.

[b] Cells grown in *trans*-aconitate (*trans*-A) medium.

[c] Chloramphenicol.

[d] Tricarballylate (TCL) concentration: 20 mM.

The possibility was considered that tricarballylate might act as an "inactive or nonsubstrate inducer," as in the case of the induction of β-galactosidase by the nonsubstrate inducer methyl thio-β-galactoside (Monod, Cohen-Bazir, and Cohn, 1951). *P. fluorescens* A.3.12 was grown in a medium with succinate as the sole carbon source and with 0.5% K-tricarballylate as the "inactive inducer." The results obtained with the resting cells after 24 hr of growth are graphically represented in Fig. 4. Growth in the presence of tricarballylate did not obviously result in the induction of the enzymes responsible for the uptake of and initial attack on *trans*-aconitate, for the oxidation of *trans*-aconitate by these resting cells could be induced by *trans*-aconitate and inhibited by chloramphenicol. A rather unexpected observation was that even the adaptation to *trans*-aconitate was inhibited partially at 0.067 mM, and completely at 20 mM, by tricarballylate.

Enzyme analyses. The cell-free extracts contained an active NADH-oxidase; hence NAD^+-linked oxidations could not be demonstrated spectrophotometrically. But these were tested by coupling with 2,6-dichlorophenol-indophenol. In such coupled systems, NAD^+, $NADP^+$, flavin

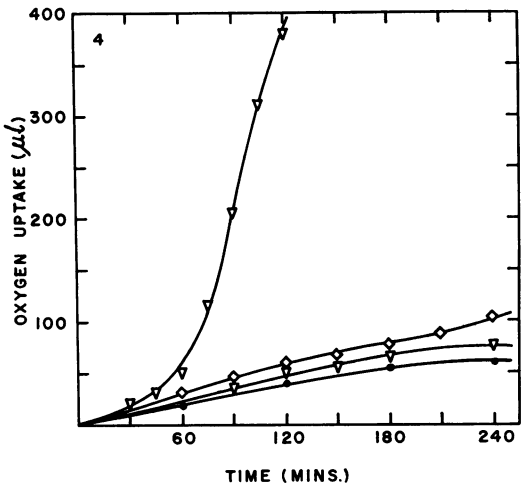


FIG. 4. *Oxidation of trans-aconitate by "resting" cells of Pseudomonas fluorescens A.3.12 (3 mg dry wt/flask) grown on succinate and tricarballylate. Symbols:* ● = *endogenous;* ▽ = *trans-aconitate;* ◇ = *trans-aconitate* + *tricarballylate.*

adenine dinucleotide (FAD), and flavin mononucleotide (FMN) did not influence the rate of dehydrogenation of tricarballylate.

Tricarballylate dehydrogenase, aconitate isomerase, and aconitase. It has already been reported (Rao and Altekar, 1961, 1962) that both tricarballylate and *trans*-aconitate are first converted into *cis*-aconitate, which then can be metabolized via the Krebs cycle or other pathways. The enzymatic content of cell-free extracts of the tricarballylate-grown cells ("*P.* TCL") is shown in Table 4. The results were qualitatively similar in the case of *trans*-aconitate-grown cells ("*P. trans*"). When the various strains of bacteria mentioned above were grown on citrate as the sole carbon source, their content (units per g of dry weight) of aconitate isomerase and aconitase in the cell-free extracts was as follows: "*P. trans*," 0.05 and 2.7; *P. fluorescens* A.3.12, 0.05 and 2.0; *A. aerogenes* (contained plenty of mucilage), 0.03 and 0.23. However, these were devoid of tricarballylate dehydrogenase. It appeared that a high concentration of *trans*- or *cis*-aconitate in the growth media favored an abundant formation of aconitate isomerase. The fact that tricarballylate-grown cells do contain significant amounts of aconitate isomerase has yet to be explained. Perhaps the structural requirements for aconitate isomerase formation by the competent strains are met with by tricarballylate also. Aconitase is present in much greater amounts than aconitate isomerase or tricarballylate dehydrogenase so that it is not a limiting enzyme. Both aconitase and aconitate isomerase of these organisms seem to require ferrous ions and a reducing agent for full activity.

Isocitrate dehydrogenase. Isocitrate dehydrogenase (Fig. 5) of these organisms was $NADP^+$-specific and was quite inactive with NAD^+ in the dye reduction tests also. Mg^{2+} or Mn^{2+} were necessary for the activity. There

TABLE 4. *Content* of tricarballylate dehydrogenase, aconitate isomerase, and aconitase of cell-free preparations of tricarballylate-grown cells*

Growth substrate	Tricarballylate dehydrogenase	Aconitate isomerase	Aconitase
Citrate	0	0.1	7.2
cis-Aconitate	0	1.0	1.7
trans-Aconitate	0	1.6	2.1
Tricarballylate	5.4	0.37–0.75	5.4–1.5

* Figures represent μmoles transformed per min per g of dry cells. The activities vary significantly with age, storage, etc.

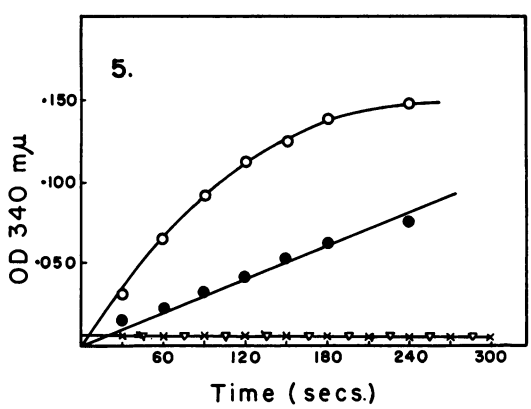


FIG. 5. *Isocitrate dehydrogenase. The test system contained potassium phosphate buffer (pH 7.4), 100 μmoles;* Mg^{2+}, *5 μmoles; nicotinamide adenine dinucleotide phosphate (*$NADP^+$*), 0.1 μmole;* DL *K-isocitrate, 10 μmoles; and, when added, ethylenediaminetetraacetate (EDTA), 6 μmoles; and protein of "P. trans" extract, 6 mg. Water was added to a final volume of 3 ml. Blank contained no* $NADP^+$. *Symbols:* ○ = $NADP^+$; × = *nicotinamide adenine dinucleotide;* ● = *no* Mg^{2+}; ▽ = *EDTA.*

was no reduction of $NADP^+$ by glyoxylate or succinate under the above conditions.

α-Ketoglutarate dehydrogenase system. The oxidation of α-ketoglutarate as well as tricarballylate and *trans*-aconitate by resting cells was inhibited (50%) by arsenite. The cell-free preparations were much less active in this oxidation. The rate of dehydrogenation of α-ketoglutarate was enhanced by NAD^+ in "*P. trans*" and $NADP^+$-specific in "*P.* TCL." The cell-free extracts, however, seemed to contain bound pyridine nucleotides (Fig. 6).

Succinate dehydrogenase system (SDH) and fumarase. SDH and fumarase were present in the cell extracts (Fig. 7). The SDH activity, however, seemed to be enhanced by the addition of $NADP^+$ (and NAD^+), which may be due merely to the simultaneous dehydrogenation of malate formed by the coupled action of SDH and fumarase. It may be mentioned that the SDH activity was also present in the soluble fraction (110,000 × *g* supernatant) of the sonic extracts. An active fumarase was present in the sonic extracts.

Dehydrogenation of malate. There was an active

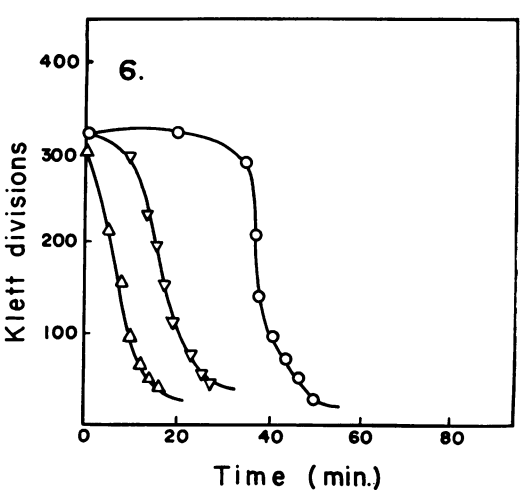


FIG. 6. *α-Ketoglutarate dehydrogenase. The test system contained, in a Thunberg tube, 200 μmoles of potassium phosphate buffer (pH 7.5); 5 μmoles of Mg^{2+}; 0.05 μmole of $NADP^+$ or NAD^+; 3 mg of protein in "P. trans" extract. The side arm contained 3 μmoles of 2,6-dichlorophenol-indophenol and 20 μmoles of K-α-ketoglutarate. Water was added to bring the final volume to 5 ml. The contents of side arm were tipped after the tube was evacuated and filled with hydrogen.* ○ = *no addition;* △ = $NADP^+$; ▽ = NAD^+.

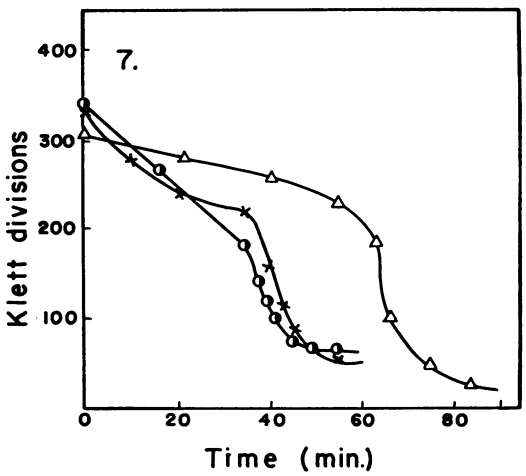


FIG. 7. *Succinate dehydrogenase system. The test system was the same as that of α-ketoglutarate dehydrogenase, the substrate used being K-succinate.* △ = *no addition;* × = $NADP^+$; ◑ = NAD^+.

TABLE 5. *Isocitritase activity of cell extracts*

Omission from reaction mixture*	Glyoxylate formed (μmoles)	
	a	b
None	0.67	1.14
Mg^{2+}	0.35†	0.32†
GSH	0.54†	—
Mg^{2+} and GSH	0.45	0.12
Mg^{2+} (EDTA added)	0.1†	0.12†
(EDTA added)	0.69	1.14
GSH (PCMB added)	0	0
PCMB added	0.57	—
Mg^{2+}, GSH, and (PCMB and EDTA added)	0	0

* The reaction mixture contained in 3 ml the following (in μmoles): tris(hydroxymethyl)aminomethane (pH 7.6), 200; $MgCl_2$, 10; glutathione (GSH), 10; enzyme, protein, 7 mg (≃20 mg of dry "*P.* TCL" cells); DL-isocitrate-K, 10; and, when added, ethylenediaminetetraacetate (EDTA), 6; and *p*-chloromercuribenzoate (PCMB), 3. Incubation for 10 min at 30 C. Reaction stopped by addition of 0.5 ml of 80% trichloroacetic acid, and glyoxylate assayed according to Friedmann and Haugen (1943). Enzyme had been dialyzed overnight against (a) 0.02 M phosphate (pH 7) and (b) 0.6% EDTA in 20 mM phosphate (final pH 7) for 20 hr and then against 20 mM phosphate (pH 7) for 4 to 6 hr.

† The color is stable and the ratios of 420:520 mμ readings are approximately 1.9, unlike in other cases; hence, not likely to be glyoxylate.

reduction of $NADP^+$ by malate in the presence of cell extracts (Fig. 8). This, however, seemed to require metal ions. $NADP^+$ but not NAD^+ enhanced the rate of dye reduction. The rate of oxidation of NADH by the extracts was not significantly affected by oxalacetate; NADH oxidase was highly active. Under strictly anaerobic conditions, better results could perhaps be expected. From the above, it is not clear whether the dehydrogenation of malate observed here is due to the malic enzyme or to malate dehydrogenase.

Isocitritase. Isocitritase was also present in the extract. The formation of glyoxylate from isocitrate (Table 5) by the cell extracts was enhanced by Mg^{2+} and glutathione. The activation of isocitritase by glutathione in the absence of Mg^{2+} is due to its ferrous iron content.

Malate synthetase. Tests for this enzyme did not give unequivocal results. There was a pronounced disappearance of glyoxylate but tests (enzymatic tests with pig heart malate dehydrogenase and chromatography) indicated the formation of only traces of malate.

The data regarding the ability of the cells to oxidize tricarballylate and *trans*-aconitate and the content of the various enzymes are tabulated in Tables 6 and 7. It is clear from the data that the amounts of enzymes in cell-free extracts, except in some cases, are not commensurate with the rates of oxidation of the two acids by nonproliferating or lyophilized cells.

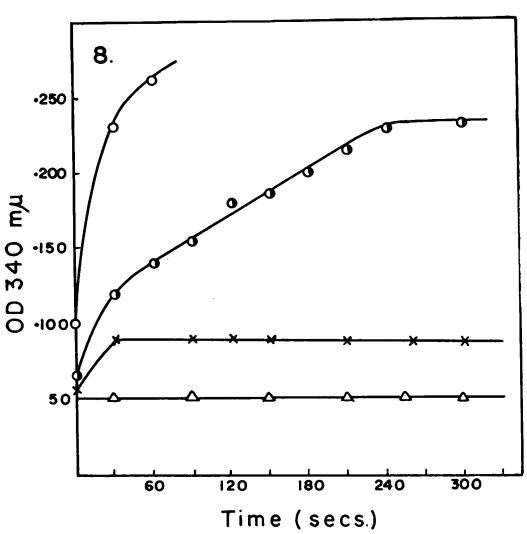


FIG. 8. *"Malic dehydrogenase." The test system contained 200 μmoles of tris(hydroxymethyl)aminomethane (pH 9.0); 5 μmoles of Mg^{2+}; 0.05 μmole of $NADP^+$ or NAD^+; 20 μmoles of* DL*-malate; 1.15 mg of protein in "P. trans" extract; and water to a final volume of 3 ml. Ethylenediaminetetraacetate (EDTA) concentration used was 6 μmoles in 3 ml.* ◑ = $NADP^+$; ○ = $NADP^+ + Mg^{2+}$; × = $NADP^+$ + *EDTA;* △ = NAD^+.

TABLE 6. *Oxidation of trans-aconitate and tricarballylate by nonproliferating and lyophilized cells**

Acid	Cells			
	trans-Aconitate		Tricarballylate	
	Resting	Lyophilized	Resting	Lyophilized
trans-Aconitate	50	18	50	1.9
Tricarballylate	0	0	16–25	19

* Figures represent μmoles of oxygen consumed per min per g of dry cells.

TABLE 7. *Content of Krebs cycle enzymes in cell extracts**

Enzymes	Cell extract from	
	trans-Aconitate-grown "*P. trans*"	Tricarballylate-grown "*P.* TCL"
Tricarballylate dehydrogenase	0	4–5.4
Aconitate isomerase	0.4–5.0	0.37
Aconitase	1.6–5.0	2.7–5.4
Isocitrate dehydrogenase	38	64
α-Ketoglutarate dehydrogenase system	3.8	11–21
Succinate dehydrogenase system	6.2	8.0
Fumarase	86	82
Dehydrogenation of malate	15	18–24
Condensing enzyme (citrogenase)	0.83	2.4
Isocitritase	0.9	3.4–6
Malate synthetase	Traces	Traces

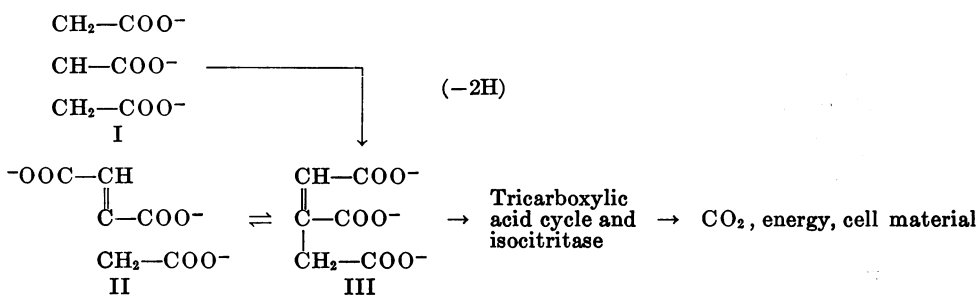
* Figures represent μmoles of substrate transformed, product formed, or dye decolorized per min per g of dry cells.

DISCUSSION

The ability to degrade tricarballylate, alloisocitrate, itaconate and mesaconate does not appear to be as widespread among microorganisms as in the case of *trans*-aconitate.

Organisms grown on tricarballylate or *cis*-aconitate are simultaneously adapted to *trans*-aconitate also. These seem to be instances of nonsubstrate induction. The induction of aconitate isomerase here reminds one of that of β-galactosidase by thiomethyl galactoside, which is also a nonsubstrate inducer. Tricarballylate interferes with the oxidation of *trans*-aconitate, perhaps by preventing the entry into the cell and not by competing for same sites on aconitate isomerase, for tricarballylate does not inhibit the aconitate isomerase activity. Further, the capacity of tricarballylate to induce formation of aconitate isomerase seems restricted only to organisms capable of metabolizing it. No aconitate isomerase activity is evident in cells of *P. fluorescens* A.3.12 when grown in a medium containing succinate and tricarballylate as sole carbon sources (Fig. 3). The induction of aconitate isomerase in such nonproliferating cells is inhibited or abolished by chloramphenicol and tricarballylate. The mode of action of tricarballylate seems to be different from that of methyl thio-β-galactosides. Whether derivatives of tricarballylate (such as methyl derivatives, etc.) have a similar effect is yet to be investigated. In the case of *cis*-aconitate, however, it seems to be a question of induction by the product of reaction as in the case of amylase induction by maltose (Rao and Sreenivasaya, 1946; Shu and Blackwood, 1951; Fukumoto, Yamamoto, and Tsuru, 1957). These cross-adaptations require more detailed examination. Another noticeable fact is that the ability of the resting cells to oxidize the salts of the two acids depends greatly upon their age at harvest. The older cells are markedly less active and sometimes even show a chloramphenicol-sensitive induction towards the same substrate on which they were grown. Apparently the permease systems for these salts are quite labile and are formed once again on shaking the resting inactive cells with the substrate, since the lyophilized cells in these cases do contain aconitase isomerase.

It is evident from the enzymatic analyses of the cell-free extracts that the extracts contain all of the enzymes of the tricarboxylic acid cycle and also isocitritase. But the amounts of many of the enzymes present are too low to explain the rates of oxidation of these two acids. Whether the enzymes have been quantitatively extracted or whether they are inactivated during sonic treatment has not been ascertained. In the case of the condensing enzyme and malate synthetase, a teleological explanation may be that they are not essential for cell growth since citrate and dicarboxylic acids are formed from these acids, and hence these two enzymes are formed in low concentration or not at all. The Krebs cycle enzymes and also isocitritase would, of course, be necessary to derive from the tricarboxylic acids the other cell components such as sugars, amino acids, etc. Although the presence of the enzymes of the Krebs cycle is not a proof that these two acids are metabolized by this pathway alone, the operation of the cycle for this purpose is highly probable.



Probable route of dissimilation

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