JOURNAL OF BACTERIOLOGY
Vol. 88, No. 3, p. 624-629 September, 1964

EVIDENCE FOR THE PRESENCE OF CERTAIN TRICARBOXYLIC ACID CYCLE ENZYMES IN *THIOBACILLUS THIOPARUS*[1]

ROBERT C. COOPER[2]

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

Received for publication 20 April 1964

ABSTRACT

COOPER, ROBERT C. (Michigan State University, East Lansing). Evidence for the presence of certain tricarboxylic acid cycle enzymes in *Thiobacillus thioparus*. J. Bacteriol. 88:624–629. 1964.—Various tricarboxylic acid cycle enzymes appear to be present in *Thiobacillus thioparus*. Cell-free extracts of *T. thioparus* were active for a number of tricarboxylic acid cycle enzymes, including aconitase, isocitric dehydrogenase, and malic dehydrogenase. Tests for the presence of fumarase and the condensing enzyme, citrogenase, were inconclusive. Citrate was shown to be active in the metabolism of *T. thioparus*, but the actual mechanism involved in its formation was not clear. The enzyme, isocitratase, appeared to be absent. Evidence for the presence of succinic dehydrogenase was found in experiments with whole cells. From these results, it would appear that *T. thioparus* has a terminal respiration pathway similar to that found in many heterotrophic microorganisms.

The basic metabolic patterns of chemosynthetic bacteria are most probably similar in many respects to those found in heterotrophic microorganisms.

A number of studies of *Thiobacillus* were carried out, in which certain intermediates of glycolysis and various cofactors common to heterotrophic metabolism were isolated. Starkey (1925) demonstrated that glucose was utilized by *T. thiooxidans*. Nicotinic acid, pantothenic acid, biotin, riboflavine, thiamine, and pyridoxine were isolated from *T. thiooxidans* by O'Kane (1942). LePage and Umbreit (1943*a*, *b*) isolated a number of organic compounds from *T. thiooxidans*, including adenosine triphosphate, fructose 1,6-diphosphate, phospho-glyceric acid, fructose 6-phosphate, glucose 6-phosphate, and glucose 1-phosphate, and obtained presumptive evidence for the presence of coenzyme I. They concluded that the internal metabolism of the autotroph is markedly similar to that of the heterotroph. Rittenberg and Grady (1950) succeeded in inducing a mutant of *T. thiooxidans* which required yeast extract for growth. Skarzynski, Klimek, and Szczepokowski (1956) and Aubert et al. (1958) demonstrated the presence of cytochromes in various species of thiobacilli. The formation of oxalacetic acid from phosphoenol pyruvic acid and carbon dioxide was shown by Suzuki and Werkman (1958) in their studies on carbon dioxide fixation by *T. thiooxidans*.

The purpose of the present work is to give some evidence that the chemosynthetic organism, *T. thioparus*, has a terminal oxidative system similar to the tricarboxylic acid cycle found in many heterotrophic microorganisms.

MATERIALS AND METHODS

The culture of *T. thioparus* ATCC 8158 used in this study was obtained from the American Type Culture Collection. The organism was grown in a medium containing: $Na_2S_2O_3 \cdot 5H_2O$, 10 g; $(NH_4)_2SO_4$, 0.1 g; K_2HPO_4, 2.0 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $MnSO_4 \cdot 2H_2O$, trace; $FeSO_4 \cdot 7H_2O$, trace; and 1,000 ml of water. The thiosulfate, ammonium sulfate, and a mixture of the remaining salts were each autoclaved separately and mixed aseptically after cooling (Society of American Bacteriologists, 1957).

Stock cultures of *T. thioparus* were maintained on solid medium with the above formulation, with 2% agar added.

To obtain a large number of cells for the various determinations made in this study, a New Brunswick fermentor was used. A temperature of 30 C, an aeration rate of 6 liters per min, and an agitation speed of 350 rev min were maintained in the fermentor. The 3 liters of medium contained

[1] This paper was taken in part from a thesis submitted to the Graduate School, Michigan State University, in partial fulfillment of the requirements for the Ph.D. degree.

[2] Present address: School of Public Health, University of California, Berkeley.

in each unit of the fermentor were inoculated with bacteria which had been washed from 48-hr agar slants. The cells were allowed to grow until all the thiosulfate, as measured by iodine titration, had been consumed (usually about 40 hr). The pH was adjusted to 7.0 when necessary, by use of sodium carbonate.

At the end of the growing period, the cells were harvested in a Sharples continuous centrifuge. The resultant material was a mixture of elemental sulfur and cells. The sulfur-cell mixture was washed with 0.1 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.4) and was centrifuged in the cold (5 C) at 20,000 $\times$ *g* for 15 min. The sulfur packed on the bottom of the centrifuge tube and the reddish-brown cells on top of the sulfur. These cells were carefully removed with a small spatula, washed repeatedly with tris buffer, and centrifuged in the cold.

If fresh material was not required, the cells were acetone-dried by the method of Umbreit, Burris, and Stauffer (1957) and stored in glass-stoppered vials at -15 C until ready for use.

Cell-free extracts were obtained by suspending the material in cold, 0.1 M tris buffer (pH 7.4) and exposing it to sonic oscillation for 15 min at approximately 5 C. The resulting material was collected and centrifuged in the cold for 1 hr at 20,000 $\times$ *g*. The supernatant fluid was poured off and used as the crude cell extract. The extract was clear and light red in color.

Respiration studies were conducted by standard Warburg respirometer techniques.

The reduction of nicotinamide adenine dinucleotide phosphate (NADP) in the presence of cell-free extracts and the substrates citrate, cis-aconitate, and isocitrate, respectively, was quantitated to determine the enzymatic activity of *T. thioparus* on these substrates by the method of Ochoa (1955). The reactions were carried out at room temperature in spectrophotometer cuvettes containing 0.1 M tris buffer (pH 7.4), 0.5 μmole of NADP, 0.1 μmole of Mn^{++}, 1.0 ml of cell-free extract, and 5 μmoles of substrate. The NADP reduction was followed by measuring the absorption of ultraviolet light at 340 mμ.

The production of α-ketoglutarate by cell-free extracts of *T. thioparus* in the presence of isocitrate was determined by reacting 1 ml of cell-free extracts with 10 μmoles of isocitric acid in the presence of 0.1 μmole of Mn^{++}, 0.1 M tris buffer (pH 7.4), 3 μmoles of arsenite, and 2 μmoles of NADP. Phosphotungstic acid was added to stop the reaction. The precipitate formed was filtered, and the filtrate was tested for α-ketoglutarate by the method of Cavallini and Frontaili (1953), in which any keto acids present are converted to the corresponding dinitrophenylhydrazone and chromatograms are made of the resulting compounds.

Isocitratase activity was measured by use of the same procedure as for α-ketoglutarate, except that arsenite was omitted and the reaction was carried out in a nitrogen atmosphere. The method of Cavallini and Frontaili (1953) was used to determine the presence of the dinitrophenylhydrazone derivative of glyoxylic acid.

To demonstrate succinic dehydrogenase activity, whole fresh cells of *T. thioparus* were examined for their ability to reduce methylene blue in the presence of sodium succinate. The test employed Thunberg tubes containing 2 ml of 0.04 M sodium succinate, 0.1 ml of 1:1,000 methylene blue, 2 ml of 0.1 M phosphate buffer (pH 7.4), and 1 ml of cell suspension prepared by washing 48-hr agar slants. The tubes were incubated at 30 C. Malic dehydrogenase determinations were made by using 1 ml of cell-free extracts in the presence of 10 μmoles of L-sodium malate, 0.1 M tris buffer (pH 7.4), and 1.5 μmoles of nicotinamide adenine dinucleotide (NAD). NAD reduction was measured by ultraviolet light absorption according to the method of Mehler et al. (1948).

Fumarase activity determinations were made with cell-free extracts by the method of Racker (1950), in which the production of L-malate from fumarate was followed by measuring the change in optical density to ultraviolet light (240 to 300 mμ) as fumarate is removed. Cell-free extract (1 ml) was added to a cuvette containing 10 μmoles of L-malate and 0.1 M phosphate buffer (pH 7.4). The reaction proceeded at room temperature.

All measurements of ultraviolet absorption were made with a Beckman model DU spectrophotometer.

The enzymatic production of citric acid from either acetate, pyruvate, or oxalacetate, or in combinations of these acids, was determined by reacting 1 ml of cell-free extract in Warburg vessels with 10 μmoles of substrate and 0.1 M tris buffer (pH 7.4). These reactions were carried out with agitation at 30 C for 60 min.

Citric acid determinations were made by the

TABLE 1. *Average ratio of oxygen utilization in the presence of various tricarboxylic acid intermediates to endogenous oxygen utilization*

Substrate	Substrate/ endogenous
Pyruvate	1.37
Isocitrate	1.15
α-Ketoglutarate	1.20
Succinate	1.23
Fumarate	1.37
Malate	1.03
Citrate	1.34
Oxalacetate	1.30

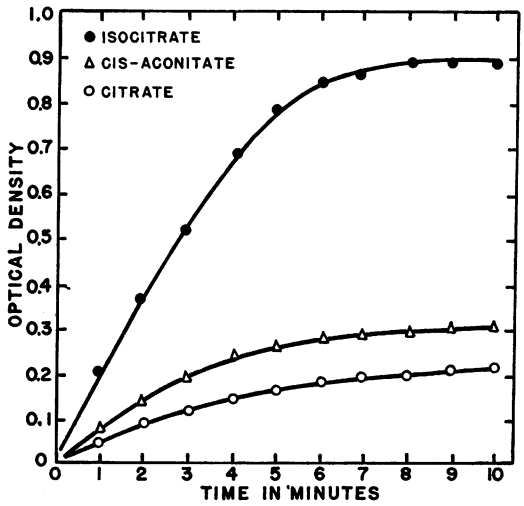

FIG. 1. *Reduction of nicotinamide adenine dinucleotide phosphate by cell-free extracts of Thiobacillus thioparus in the presence of citrate cis-aconitate and isocitrate at room temperature.*

method of Ettinger, Goldbaum, and Smith (1952), in which citrate is converted to penta bromoacetone. A red color, whose intensity is proportional to the citrate concentration, is developed when the bromoacetone is extracted with pyridine.

RESULTS

A number of attempts were made to determine oxygen uptake by whole cells and cell-free extracts of *T. thioparus* in the presence of tricarboxylic acid intermediates. No oxygen uptake was observed with the cell-free extracts and only a small amount with whole cells. One of the problems encountered with this organism was the presence of elemental sulfur which always accompanied the whole cells and made endogenous rates quite high. On the average, whole cells in the presence of substrate took up 1.25 times as much oxygen as those without substrate (Table 1). Because of the high endogenous rate, the significance of the slight oxygen uptake in the presence of substrate is not clear. These inconclusive results directed attention to determination of individual reactions with the use of both cell-free extracts and whole cells.

The activity of cell-free extracts of *T. thioparus* on citrate, *cis*-aconitate, and isocitrate as measured by NADP reduction is shown in Fig. 1. The amount and rate of NADP reduction is greatest in the presence of isocitrate and becomes increasingly smaller with *cis*-aconitate and citrate. These results indicate the presence of the enzyme, isocitric dehydrogenase, in cell-free extracts of *T. thioparus*. The isocitric dehydrogenase appears to be NADP-specific, because no activity could be demonstrated when NAD was substituted. Supplementing with 2 μmoles of Fe^{++}, a known co-factor for aconitase in other organisms, had no effect on these results.

The results of experiments to demonstrate the production of α-ketoglutarate by cell-free extracts of *T. thioparus* are tabulated in Table 2. These data also indicate the presence of isocitric dehydrogenase in *T. thioparus*, since α-ketoglutarate is the product of the reaction of isocitrate, NADP, and cell-free extract. This is indicated by the production of a spot which has the same R_F value as does the 2,4-dinitrophenylhydrazone of α-ketoglutarate. No hydrazone derivative of glyoxalate was found under aerobic or anaerobic conditions, which would indicate that isocitratase was not present. The spots giving R_F values of 0.65, 0.66, and 0.69 are due to excess phenylhydrazone.

The tests for succinic dehydrogenase activity were qualitative. Whole-cell suspensions that had to be used contained elemental sulfur, and photometric measurements of methylene-blue reduction were impossible. In Thunberg tubes, the cells took 45 min to reduce completely the methylene blue, whereas the control without succinate was reduced in 135 min. These results would indicate the presence of succinic dehydrogenase in these cells, since the dye is reduced three times faster in the presence of succinate. The longer endogenous reduction is most probably due to the presence of elemental sulfur which is being

oxidized and which in turn is reducing the methylene blue. As might be expected, the same test with the use of acetone powders and cell-free extract was negative.

The reduction of NAD in the presence of L-malate and cell-free extracts is illustrated in Fig. 2. The amount of NAD reduction was quite small, a total of 0.108 μmoles in 5 min; however, the overall reaction was relatively rapid. The reaction rate at zero time was estimated as a change in optical density of 0.4 units per min or 0.19 μmoles of NAD reduced per min. No activity was obtained with this system in the presence of NADP, indicating that the malic dehydrogenase of these cells is NAD-specific.

TABLE 2. *Formation of α-ketoglutarate by the action of cell-free extracts of Thiobacillus thioparus on isocitric acid*

Determination	R_F values of the 2,4-dinitrophenylhydrazone	
Standards		
α-Ketoglutarate	0.33	—
Glyoxalate	0.40	0.58
Reactions with		
Isocitrate (aerobic)		
Test	0.31	0.69
Control	—	0.65
Isocitrate (anaerobic)		
Test	—	0.66
Control	—	0.66

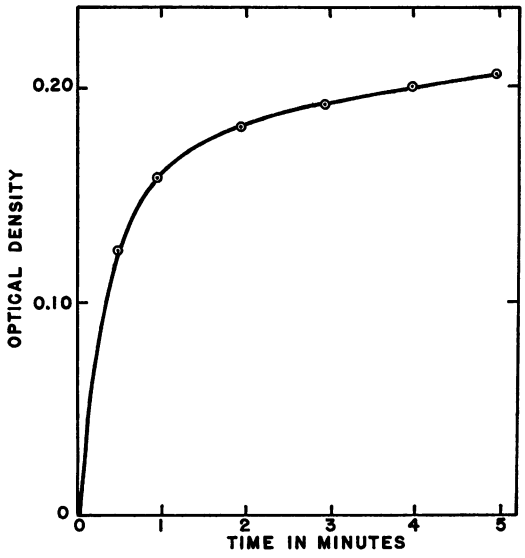

FIG. 2. *Reduction of nicotinamide adenine dinucleotide by cell-free extracts of Thiobacillus thioparus in the presence of L-malate at room temperature.*

TABLE 3. *Production of citric acid in the presence of cell extracts and acetate, and of oxalacetate and pyruvate*

Reaction	Citrate produced
	μg
Acetate control	0
Oxalacetate control	6.24
Pyruvate control	9.18
Cell extract control	0
Acetate and cell extract	0
Oxalacetate and cell extract	55.05
Pyruvate and cell extract	12.10
Acetate, oxalacetate, and cell extract	47.71
Oxalacetate, pyruvate, and cell extract	58.72

Attempts to demonstrate fumarase activity in this organism were fruitless. The method used was the same as for aconitase, because both compounds absorb light between 240 and 300 mμ. The cell-free extracts of *T. thioparus* absorbed highly at these wavelengths, and this absorption might mask fumarase activity if such activity were very small.

A series of experiments were carried out to find evidence for the production of citric acid from acetate, pyruvate, and oxalacetate. The data from one such experiment are presented in Table 3. These experiments indicated that citrate was formed by cell extracts of *T. thioparus* in the presence of oxalacetate, never in the presence of acetate alone, and only slightly in the presence of pyruvate. Attempts to show the presence of the condensing enzyme, citrogenase, by demonstrating an increase in citrate production when pyruvate or acetate plus oxalacetate were added to the cell-free extract, were uncertain in that the quantitative results varied widely from one experiment to another; and, in the majority of cases, the citrate produced in the presence of combinations of the aforementioned compounds and cell-free extracts exceeded, only slightly, that produced in the presence of only oxalacetate and cell-free extract.

DISCUSSION

Respiration studies carried out in the presence of tricarboxylic acid cycle intermediates were

not conclusive. There was some oxygen uptake by whole cells in the presence of tricarboxylic acid cycle intermediates; however, the endogenous respiration was high because of carryover sulfur. The amount of oxygen uptake in the presence of intermediates over that of the endogenous was small and never approached theoretical values. *T. thioparus*, being an autotroph, normally does not metabolize organic materials; however, Vogler, LePage, and Umbreit (1942) presented some evidence that the addition of C_4 dicarboxylic acids enhances the respiration rate of the autotroph *T. thiooxidans* on thiosulfate. The slight increase in respiration seen in these experiments might be explained in terms of Vogler's observations.

Cell-free extracts of *T. thioparus* did not possess any respiratory activity against these organic intermediates. This result does not necessarily mean that the cells are incapable of utilizing these compounds. The author noted in other experiments that the amount of oxygen uptake in the presence of thiosulfate oxidation is reduced tenfold by breaking up the cells. This reduction is most probably also reflected in the loss of activity against the organic intermediates.

Since the respiration studies gave no evidence for the presence of tricarboxylic acid cycle enzymes, more direct methods were employed.

Isocitric dehydrogenase determinations were quite successful. It was by far the most active enzyme determined. Aconitase could not be demonstrated directly, but it was shown that NADP is reduced, at different rates, in the presence of citrate and *cis*-aconitate as well as isocitrate. The established pathway for these reactions in other organisms is:

$$\text{citrate} \xrightleftharpoons{\text{aconitase}} \textit{cis}\text{-aconitate} \xrightleftharpoons{\text{aconitase}} \text{isocitrate}$$

If such a system existed in *T. thioparus*, one might expect that isocitrate would show a greater dehydrogenase activity than does citrate or *cis*-aconitate. From the data, such seems to be the case, since the cell-free extract reduced 27% of the NADP present in the presence of citrate, 31% in the presence of *cis*-aconitate, and 86.4% in the presence of isocitrate. This latter result indicates that the concentration of NADP used was not the limiting factor in these reactions. These reactions were all NADP-specific. More evidence for the presence of isocitric dehydrogenase was found by the demonstration that α-ketoglutarate is the product of this dehydrogenase activity.

From the experiments performed, it would appear that the enzyme, isocitratase, is not involved, since glyoxylate could not be demonstrated when isocitrate and cell-free extracts were reacted together under anaerobic conditions. These negative results are not conclusive, because there may be certain co-factors or conditions necessary in this particular organism of which we are unaware.

Succinic dehydrogenase activity was measured qualitatively by the Thunberg technique; it would be reasonable to assume that the succinate arises from α-ketoglutarate, although this latter reaction was not measured. Since cell-free extracts were unable to oxidize succinate, one would assume that the dehydrogenase is associated with the cell wall, as is common in other bacteria.

In the typical tricarboxylic acid cycle, succinic dehydrogenase activity results in the formation of fumarate; however, fumarase activity could not be demonstrated in cell-free extracts of this organism; yet, malic dehydrogenase activity could be shown. This latter reaction was not very great in terms of the amount of NAD reduced, but the reaction was positive and specific for NAD. From these experiments, one would assume fumarate to be an intermediate, since both succinate and malate are oxidized by this organism and fumarate is normally the bridge compound between these two.

Attempts to show the presence of the condensing enzyme, citrogenase, were not conclusive. Citrate was produced by cell-free extracts in the presence of oxalacetate, slight amounts were produced in the presence of pyruvate, and none in the presence of acetate. Attempts to show the citrogenase enzyme by demonstrating an increase in citrate production in the presence of pyruvate and oxalacetate, or acetate and oxalacetate, were not conclusive since, in the majority of cases, it seems that as much citrate is produced from oxalacetate and cell-free extract as when oxalacetate is in combination with pyruvate or acetate; however, these results do indicate that citrate is apparently an intermediate in these cells.

From these results, it seems reasonable to conclude that this organism has a terminal respiration which is very similar to that found in aerobic-heterotrophic microorganisms. The data obtained

appear to agree, in most particulars, with the well-established tricarboxylic acid cycle. The weakest points in this conclusion are to be found in the lack of direct evidence for the enzymes, aconitase and fumarase, and the inconclusive results in determining the condensing enzyme, citrogenase.

ACKNOWLEDGMENT

The author wishes to express his appreciation to W. L. Mallman for his guidance during the course of this study.

LITERATURE CITED

AUBERT, J. P., C. MILHAUND, C. MONCEL, AND J. MILLET. 1958. Existence, isolement et propriétés physicochimiques d'un cytochrome C. de *Thiobacillus denitrificans*. Compt. Rend. **246**:1616–1619.

CAVALLINI, D., AND N. FRONTAILI. 1953. Determinazione chromatografica quantitative dei chetoacidi. Ric. Sci. Suppl. **23**:605–608.

ETTINGER, R. H., L. R. GOLDBAUM, AND L. H. SMITH. 1952. A simplified photometric method for the determination of citric acid in biological fluids. J. Biol. Chem. **199**:531–536.

LEPAGE, G. A., AND W. W. UMBREIT. 1943*a*. Phosphorylated carbohydrate esters in autotrophic bacteria. J. Biol. Chem. **147**:263–271.

LEPAGE, G. A., AND W. W. UMBREIT. 1943*b*. The occurrence of adenosine 3-triphosphate in autotrophic bacteria. J. Biol. Chem. **148**: 255–260.

MEHLER, A. H., A. KORNBERG, S. GRISOLIA, AND S. OCHOA. 1948. The enzymatic mechanisms of oxidation reductions between malate or isocitrate and pyruvate. J. Biol. Chem. **174**:961–977.

OCHOA, S. 1955. Isocitric dehydrogenase system (TPN) from pig heart, p. 699–704. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.

O'KANE, D. J. 1942. The presence of growth factors in the cells of autotrophic sulfur bacteria. J. Bacteriol. **43**:7.

RACKER, E. 1950. Spectrophotometric measurements of enzymatic formation of fumaric and cis-aconitic acids. Biochim. Biophys. Acta **4**:211–214.

RITTENBERG, S. C., AND R. P. GRADY. 1950. Induced mutants of Thiobacillus thiooxidans requiring organic growth factors. J. Bacteriol. **60**:509–510.

SKARYNSKI, B., R. KLIMEK, AND T. W. SZCZEPOKOWSKI. 1956. Cytochrome in *Thiobacillus thioparus*. Bull. Acad. Polon. Sci. Classe III **4**:299.

SOCIETY OF AMERICAN BACTERIOLOGISTS. 1957. Manual of microbiological methods. McGraw-Hill Book Co., Inc., New York.

STARKEY, R. L. 1925. Concerning the carbon and nitrogen nutrition of Thiobacillus thiooxidans, an autotrophic bacterium oxidizing sulphur under acid conditions. J. Bacteriol. **10**:165–195.

SUZUKI, I., AND C. H. WERKMAN. 1958. Chemoautotrophic carbon dioxide fixation by extracts of *Thiobacillus thiooxidans*. I. Formation of oxalacetic acid. Arch. Biochem. Biophys. **76**:103–111.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. Manometric techniques, 3rd edition. Burgess Publishing Co., Minneapolis.

VOGLER, K. G., G. A. LEPAGE, AND W. W. UMBREIT. 1942. Studies on the metabolism of autotrophic bacteria. I. The respiration of *Thiobacillus thiooxidans* on sulphur. J. Gen. Physiol. **26**:89–102.