

Phototrophic Growth of *Rhodopseudomonas gelatinosa* on Citrate; Accumulation and Subsequent Utilization of Cleavage Products

CHRISTINE SCHAAB, FRIEDRICH GIFFHORN, SIEGFRIED SCHOBERTH,
NORBERT PFENNIG, and GERHARD GOTTSCHALK

Institut für Mikrobiologie der Universität Göttingen and Institut für Mikrobiologie der Gesellschaft für Strahlen- und Umweltforschung mbH München in Göttingen

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Excretion of acetate, fermentation of citrate, citrate lyase, utilization of citrate
by *Rhodopseudomonas gelatinosa*

Rhodopseudomonas gelatinosa grows on citrate anaerobically in the light. Under aerobic conditions citrate cannot be utilized. Citrate is rapidly fermented by *Rps. gelatinosa* in the dark, and this fermentation is associated with limited growth (one generation). Anaerobically in the light citrate is decomposed much faster than its catabolic products can be used for the synthesis of cellular material. Therefore, acetate and malate are accumulated in the medium. The acetate concentration in the medium after complete consumption of citrate is higher than the original citrate concentration. Acetate is formed by the citrate lyase reaction and by partial degradation of oxaloacetate. After exhaustion of citrate, growth of *Rps. gelatinosa* continues at the expense of acetate.

A few species of the *Rhodospirillaceae* are able to utilize citrate. Abundant growth on this substrate has been reported for some strains of *Rhodopseudomonas gelatinosa*¹ while strains of *Rps. sphaeroides* and *Rps. acidophila* grow only poorly with citrate as carbon source².

The present study was undertaken to examine the reactions involved in citrate breakdown by *Rps. gelatinosa*. It will be shown that the capacity of this microorganism to cleave citrate under anaerobic conditions is extraordinary and that cleavage products are excreted into the medium in substrate amounts.

Methods and Material

Rps. gelatinosa strain SMG 149 was grown in the following medium: KH_2PO_4 , 1 g; NH_4Cl , 0.4 g; NaCl , 0.4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g; trace element solution, 1 ml; yeast extract, 0.5 g; sodium citrate (dihydrate), 1 g water to 1000 ml. The pH was 6.7. Cells were grown in screw-cap bottles (100 or 500 ml) or in 5-l flasks at 1500–3000 Lux.

The optical density of the cultures at 650 nm was measured with a Zeiss PM 4 spectrophotometer in cuvettes of 1 cm light path at room temperature. Protein was determined according to BEISENHERZ et al.³ after extraction of the pigments with acetone (two times with 4 ml).

Requests for reprints should be sent to Prof. Dr. G. GOTTSCHALK, Institut für Mikrobiologie d. Univ., D-3400 Göttingen, Grisebachstr. 8.

Abbreviation. SMG, Sammlung für Mikroorganismen, Göttingen.

Citrate, malate and lactate were determined enzymatically⁴. Acetate was determined either by gas chromatography or as acetyl hydroxamate⁵. Gas chromatographic analyses were carried out with an apparatus of Perkin-Elmer, model 900; conditions: glass column, 1.83 m long, filled with Porapak Q-S; temperature at injection port, 240°C; oven temperature, 180°C; carrier gas, N_2 ; detection, FiD. The separation of citrate, malate and acetate was done as described previously⁶. The solvent systems used for descending paper chromatography were: propanol-1 : Ammonia : water (6 : 3 : 1) and butanol-2 : formic acid : water (6 : 1 : 2)⁷. Radioactivity was determined in a Packard Tri-carb scintillation spectrometer (model 3325). The scintillation fluid of BRAY⁸ was used.

Enzymes and Chemicals. Acetate kinase, EC 2.7.2.1 (170 U/mg); phosphotransacetylase, EC 2.3.1.8 (1000 U/mg); malate dehydrogenase, EC 1.1.1.37 (1100 U/mg); citrate lyase, EC 4.1.3.6 (8 U/mg) and citrate synthase, EC 4.1.3.7 (100 U/mg) were purchased from Boehringer, Mannheim.

$4\text{-}^{14}\text{C}$ -aspartate and $1,5\text{-}^{14}\text{C}$ -citrate were products of the New England Nuclear Corp., Boston, and $1\text{-}^{14}\text{C}$ -acetic anhydride and (U)- ^{14}C -aspartate of the Radiochemical Centre, Amersham. $1\text{-}^{14}\text{C}$ -, $5\text{-}^{14}\text{C}$ - and $1,2,3,6\text{-}^{14}\text{C}$ -citrate were prepared as described previously⁹.

Results

Rps. gelatinosa was grown on citrate anaerobically in the light and the increase of turbidity and of cell protein and the consumption of the substrate were measured. As shown in Fig. 1 growth in the early logarithmic phase was associated with a rapid and complete consumption of citrate. After ex-

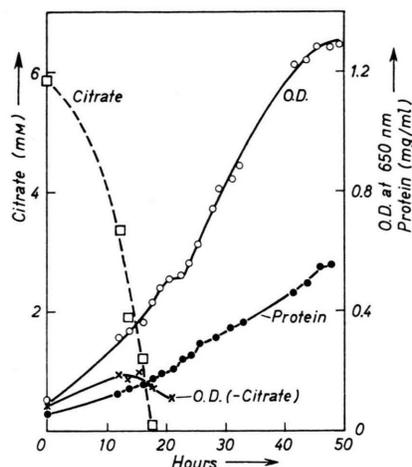


Fig. 1. Growth of *Rps. gelatinosa* on citrate. A 5-l flask containing 3 l of medium was inoculated with 500 ml of a 24 hrs old culture of *Rps. gelatinosa* which had been grown on citrate. The flask was filled up to the neck with sterile medium and incubated at 27 C (light intensity: 2400 Lux; slow agitation by a magnetic stirrer). Aliquots were removed by nitrogen pressure and analyzed for the concentration of citrate, the optical density and the protein content.

haustion of the substrate, growth continued following a short lag period. By variation of the concentration of citrate in the medium it was examined whether the second growth phase correlated to the amount of citrate originally present. Fig. 2 demon-

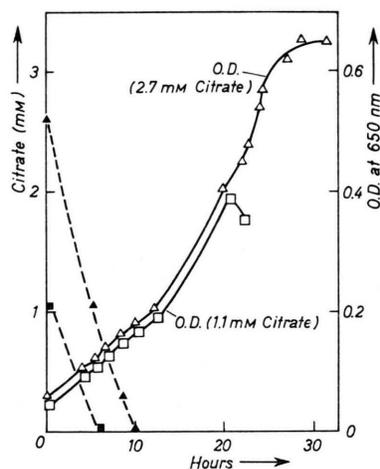


Fig. 2. Dependence of growth of *Rps. gelatinosa* on the concentration of citrate. Experiments were carried out as described in Fig. 1. Dotted lines, citrate concentrations.

strates that this was the case. In the second growth phase the increase of the optical density was 0.29 with 1.1 mM citrate, 0.48 with 2.6 mM citrate and

0.82 with 5.5 mM citrate (Fig. 1). High concentrations of citrate (40 mM) were not completely degraded (30 to 35 mM) and a second growth phase could not be observed.

After exhaustion of citrate growth originated most likely from the assimilation of conversion products accumulated in the medium during citrate degradation. In order to demonstrate this, *Rps. gelatinosa* was grown with 1,5-¹⁴C-citrate and the decrease of citrate and of radioactivity in the medium was determined (Fig. 3). It can be seen that the

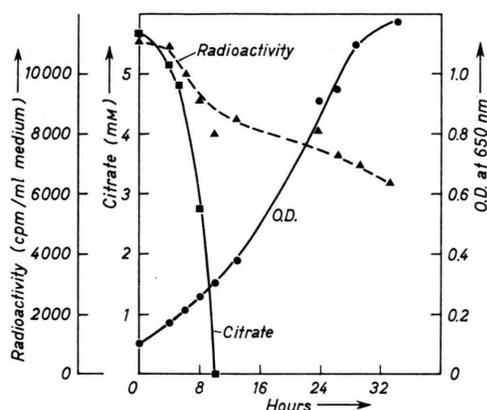


Fig. 3. Decrease of citrate and of the radioactivity added as 1,5-¹⁴C-citrate during growth of *Rps. gelatinosa*. 100-ml culture with a citrate concentration of 5.75 mM, specific activity of citrate, 1950 cpm/ μ mole.

consumption of citrate was not accompanied by a parallel decrease of the radioactivity of the culture medium. After 10 hrs of growth, the medium did no longer contain citrate but still 70% of the original radioactivity were present. The following experiments proved that after the exhaustion of citrate the medium contained acetate and malate as the main components:

- Most of the radioactivity was steam volatile. The steam distillate contained ¹⁴C-acetate which was identified by paper chromatography in propanol-1-ammonia, by its behavior on Dowex-1-formate columns, and by enzymatic conversion to citrate¹⁰. The citrate formed was isolated and its identity was verified by paper chromatography in butanol-2-formic acid.
- From the residue of the steam distillation organic acids were isolated by chromatography on Dowex-1-formate. The radioactive peak eluted with 1 N formic consisted of malate as shown by paper chromatography in two solvent systems.

After ten hours of growth on 1,5-¹⁴C-citrate (Fig. 3), 76% of the radioactivity was present as acetate and 12% as malate in the culture medium. In order to pursue the formation of acetate and malate during growth of *Rps. gelatinosa*, an experiment was performed in which the concentrations of citrate, acetate and malate were measured. Citrate and malate were determined enzymatically and acetate by gas chromatography. Fig. 4 shows that the

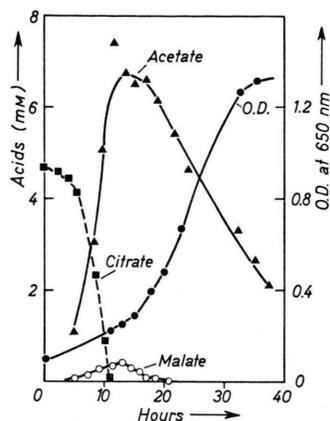


Fig. 4. Formation of malate and acetate and their subsequent utilization during growth of *Rps. gelatinosa* on citrate. The experiment was performed as described in Fig. 1.

breakdown of citrate was associated with the appearance of acetate and malate in the culture medium. The concentration of acetate reached a maximum after 10 hrs of growth and then exceeded the original concentration of citrate. Malate appeared in the medium in small amounts and was preferentially consumed after the exhaustion of citrate.

Since the excretion products were a C₂ and a C₄ compound and could have been formed from citrate by the subsequent action of citrate lyase and malate dehydrogenase it was tested whether these compounds originated from citrate by a stereospecific cleavage reaction. *Rps. gelatinosa* was grown with either 5-¹⁴C- or 1-¹⁴C-citrate for 10 hrs and the labelling of the products accumulated was studied. After 10 hrs of growth citrate had disappeared almost completely but 98% of the radioactivity of 5-¹⁴C-citrate and 38% of the radioactivity of 1-¹⁴C-citrate were still in the culture medium (Tab. I). The radioactivity of 5-¹⁴C-citrate was present in acetate as is evident from chromatography on Dowex-1-formate (Fig. 5). Malate which was eluted with the fractions 12 to 16 was unlabelled. After growth on

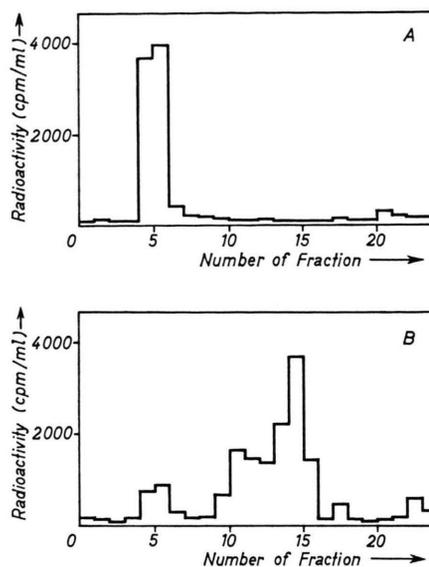


Fig. 5. Labelled compounds in the culture medium after growth of *Rps. gelatinosa* on 1-¹⁴C- and 5-¹⁴C-citrate. 2 ml of the culture medium were applied to a Dowex-1-formate column (1 cm in diameter; bed volume, 5 ml). Fractions 3–8 were eluted with 0.2 N formic acid, fractions 9–14 with 1 N formic acid and further fractions with 4 N formic acid. A Cells grown with 5-¹⁴C-citrate. B Cells grown with 1-¹⁴C-citrate.

| Aliquots removed | 5- ¹⁴ C-citrate (mM) | 5- ¹⁴ C-citrate (cpm/ml) | 1- ¹⁴ C-citrate (mM) | 1- ¹⁴ C-citrate (cpm/ml) |
|------------------|---------------------------------|-------------------------------------|---------------------------------|-------------------------------------|
| Initially | 5 | 5143 | 5 | 13214 |
| After 10 hrs | n. d. ^a | 5060 | n. d. | 4930 |

Table I. Citrate consumption and decrease of radioactivity in the culture medium after growth of *Rps. gelatinosa* on stereospecifically labelled citrate for 10 hours.

^a not detectable

Rps. gelatinosa was grown in 14-ml screw-capped test tubes. The radioactive citrate samples were prepared enzymatically as described in Methods.

1-¹⁴C-citrate only a small portion of the radioactivity was located in acetate. The largest amount resided in malate (Fig. 5 B, fractions 9 to 16). This experiment proved that the degradation of citrate was initiated by a cleavage reaction and that this cleavage proceeded in the same stereochemical manner as that catalyzed by citrate lyase¹¹ and ATP: citrate lyase¹². Furthermore, it was in complete agreement with Fig. 4: Acetate is not utilized as long as citrate is being degraded and C₄-compounds are present; some of the acetate excreted is formed from the C₄-cleavage product.

The effect of L-malate on the breakdown of citrate was studied. Cells grown over several passages on L-malate were used as inoculum for a medium containing 5 mM L-malate and 5 mM citrate. The optical

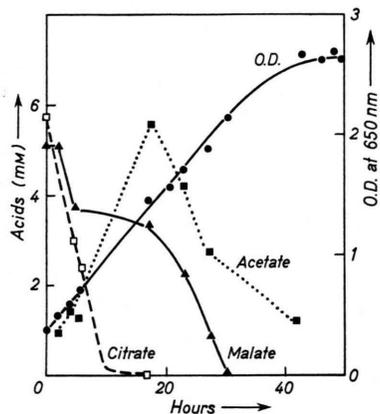


Fig. 6. Utilization of L-malate and citrate by L-malate-grown cells of *Rps. gelatinosa*. Cells grown over several passages on L-malate were used as inoculum. The medium contained 5 mM sodium citrate and 5 mM sodium L-malate. Growth and the concentration of citrate, L-malate and acetate were measured.

density of the culture and the concentrations of citrate, L-malate and acetate were followed (Fig. 6). Despite the precultivation on malate citrate was utilized much faster than malate.

The experiments described so far have been carried out with cells growing photoheterotrophically, and it seemed desirable to study the utilization of citrate under other conditions. *Rps. gelatinosa* did not grow with citrate aerobically. Under anaerobic conditions in the dark, however, citrate was fermented. The fermentative breakdown of citrate by *Rps. gelatinosa* was associated with an increase of the optical density of approximately 0.1 (Fig. 7). To establish that an anaerobic environment was maintained throughout the fermentation period resazurin was added to the medium; it remained colorless. Anaerobic degradation of citrate and growth did also occur when 10^{-4} M sodium azide or potassium cyanide was included in the medium. Moreover, the increase of the optical density with malate as substrate was much smaller excluding the involvement of phototrophic reactions. Since the optical density and the protein content of the culture did not increase further when more than 10 mM of citrate were added it is indicated that anaerobic growth in the dark was limited by one or more unknown factors.

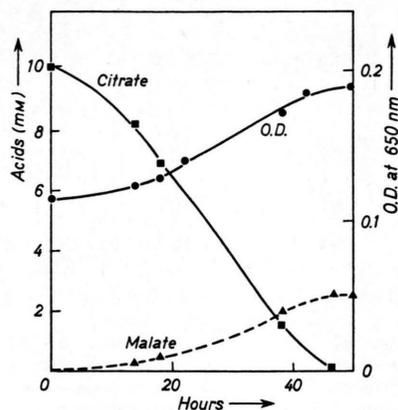


Fig. 7. Degradation of citrate under anaerobic conditions in the dark. The culture medium was given into 1-l bottles, sterilized and freed of oxygen by passing purified nitrogen through it. Cells grown on citrate were used as inoculum. The cultures were kept in the dark and samples were taken with N_2 -pressure.

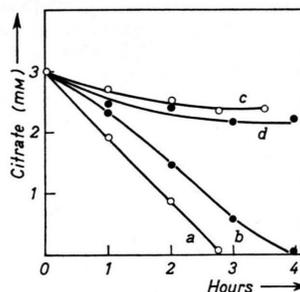


Fig. 8. Degradation of citrate by resting cells of *Rps. gelatinosa*. Conditions of incubation: a) light/anaerobic; b) dark/anaerobic; c) light/aerobic; d) dark/aerobic. Cells grown on citrate were harvested, washed with mineral medium and suspended in growth medium not containing yeast extract and NH_4Cl (1 g of wet weight/100 ml). The aerobic suspensions were incubated on a rotary shaker and the anaerobe ones as described for the growth experiments.

Resting cells of *Rps. gelatinosa* degraded citrate rapidly under anaerobic conditions either in the light or in the dark (Fig. 8). There was only a slow decrease of the citrate concentration when the culture medium was aerated. Oxygen obviously inhibited citrate breakdown. The formation of acetate, malate and of lactate by suspensions of *Rps. gelatinosa* incubated anaerobically in the dark is shown in Fig. 9. A fermentation balance carried out with 1,2,3,6- ^{14}C -citrate is summarized in Tab. II. Carbon and radioactivity were recovered in good yield. The data supported the view that oxaloacetate formed by citrate cleavage was partly oxidized to acetate and partly reduced to malate. Some $NADH_2$ was also consumed by reducing pyruvate to lactate. Since

| Compounds formed or fermented | Amount ($\mu\text{moles/ml}$) | ($\mu\text{moles}/100 \mu\text{moles}$) | Radioactivity (cpm/ml) |
|-------------------------------|---------------------------------|---|------------------------|
| Citrate | 5 | 100 | 11 000 |
| Malate | 1.20 | 24 | 4 100 |
| Lactate | 0.76 | 15.2 | |
| Acetate | 7.75 | 155 | 3 500 |
| Carbon dioxide | 8.60 | 172 | 3 230 |

Table II. Fermentation of 1,2,3,6- ^{14}C -citrate by cell suspensions of *Rps. gelatinosa*.

Carbon recovered=103.3%; Radioactivity recovered=98.4%; Specific radioactivity of acetate: expected 390 cpm/ μmole (only the acetate derived from oxaloacetate is labelled); found, 451 cpm/ μmole ; O/R balance=1.3.

The fermentation was carried out in a 500-ml flask connected with a nitrogen tank and a CO_2 collection unit¹³. 100 ml containing 500 μmoles of 1,2,3,6- ^{14}C -citrate and 1 g (wet weight) cells of *Rps. gelatinosa* were incubated for 8 hrs at 30 C and the carbon dioxide was collected by passing a slow stream of nitrogen through the apparatus. The radioactivity present as sodium carbonate was determined after conversion to hyamin carbonate. Acids were determined as in Methods. ^{14}C -acetate was determined in the steam distillate. Paper chromatography revealed that the residue contained only two radioactive compounds — malate and lactate.

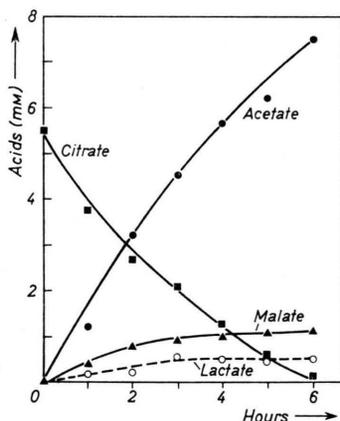


Fig. 9. Fermentation of citrate to acetate, malate and lactate by resting cells of *Rps. gelatinosa*. A cell suspension was incubated with 5.5 mM sodium citrate anaerobically in the dark. The conditions were as in Fig. 8.

in this experiment and in others less malate and lactate were formed than expected the redox balance was not satisfactory. The formation of small amounts of hydrogen gas might be responsible for this.

Discussion

The utilization of citrate by *Rps. gelatinosa* proceeds in a rather uncontrolled manner. Much more citrate is being degraded than can be used for the

synthesis of cellular components. The experiments reveal that acetate, the C_2 moiety produced in the citrate cleavage reaction, is quantitatively excreted and only metabolized after citrate exhaustion. Even a part of the C_4 moiety derived from citrate appears as malate in the medium.

Citrate is cleaved by *Rps. gelatinosa* in the same stereochemical manner as reported for citrate lyase¹¹. When 5- ^{14}C -citrate is administered only acetate becomes radioactive, with 1- ^{14}C -citrate the majority of radio carbon appears in malate. Meanwhile, the citrate lyase of *Rps. gelatinosa* has been demonstrated in high activity in cell-free preparations of this microorganism (unpublished results).

It has not been studied yet whether the capability of *Rps. gelatinosa* to degrade citrate is constitutive. It can only be said that citrate consumption by this bacterium starts rapidly regardless what carbon source was used for phototrophic growth of the inoculum. Cells grown over several passages on L-malate prefer to degrade citrate when both, citrate and malate are administered simultaneously. Under aerobic conditions, however, citrate is not utilized. In the presence of oxygen *Rps. gelatinosa* does not grow on citrate and washed suspensions of this microorganism do not decompose citrate either in the light or in the dark. As has been shown by DAGLEY and DAWES¹⁴ for *Aerobacter aerogenes*, citrate cleavage is abolished by oxygen. In contrast to *A. aerogenes* which metabolizes citrate aerobically via the tricarboxylic acid cycle, *Rps. gelatinosa* is obviously not able to take advantage of this sequence for citrate degradation. Whether this is due to inhibitory effects of citrate as has been observed with other bacteria¹⁵ or to an influence of the sodium concentration as in *Salmonella typhimurium*¹⁶ has not been elucidated yet.

The degradation of citrate by washed cell suspensions allowed to study the reaction balance. One cleavage product, acetate, is quantitatively excreted. Oxaloacetate is partly oxidized to acetate, and NADH_2 formed in the pyruvate dehydrogenase reaction is obviously consumed by reducing part of the oxaloacetate to malate and part of the pyruvate to lactate.

Since citrate is readily fermented by *Rps. gelatinosa*, this microorganism should gain some energy by substrate phosphorylation. Therefore, it is conceivable that *Rps. gelatinosa* is able to grow with citrate anaerobically in the dark. However, growth

under these conditions ceased after approximately one generation. No efforts were made to overcome this cessation by varying the composition of the growth medium. It should be mentioned in this connection that anaerobic growth of *Rhodospirillum rubrum* on pyruvate has recently been demon-

strated¹⁷ and that this microorganism ferments various dicarboxylic acids producing acetate, propionate and CO₂¹⁸.

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Lack of Citrate Lyase — the Key Enzyme of the Reductive Carboxylic Acid Cycle — in *Chlorobium thiosulfatophilum* and *Rhodospirillum rubrum*

NORBERT BEUSCHER and GERHARD GOTTSCHALK

Institut für Mikrobiologie der Universität Göttingen

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Chlorobium thiosulfatophilum, citrate lyase, glutamate synthesis in phototrophs, reductive carboxylic acid cycle, *Rhodospirillum rubrum*

Extracts of *Chlorobium thiosulfatophilum* and of *Rhodospirillum rubrum* have been tested for the presence of citrate lyase under various conditions. This enzyme could not be detected. It is, therefore, concluded that a complete reductive carboxylic acid cycle does not occur in these microorganisms.

The enzymes required for α -ketoglutarate synthesis from oxaloacetate and acetyl-coenzyme A are present in autotrophically grown cells of *C. thiosulfatophilum* and *R. rubrum*. This supports the view that a reaction sequence catalyzing the conversion of α -ketoglutarate into oxaloacetate is unlikely to exist in these bacteria.

A number of anaerobic and phototrophic bacteria contain enzyme systems which catalyze the formation of α -keto acids from acyl-CoA esters by ferredoxin-dependent carboxylations. Extracts of *Rhodospirillum rubrum* and *Chlorobium thiosulfatophilum*, for instance, have been shown to synthesize pyruvate and α -ketoglutarate from acetyl-CoA and

succinyl-CoA, respectively, by reactions of this type¹⁻³. When growing on acetate, pyruvate synthesis from a C₂ compound is of great importance for both microorganisms since they do not contain the key enzymes of the glyoxylate bypath⁴.

The occurrence of pyruvate synthase and α -ketoglutarate synthase in *C. thiosulfatophilum* and *R. rubrum* and the demonstration of other enzymes of the tricarboxylic acid cycle in these bacteria led ARNON and associates to postulate the operation of a reductive carboxylic acid cycle⁵. In its overall

Requests for reprints should be sent to Prof. Dr. G. GOTTSCHALK, Institut für Mikrobiologie der Universität, D-3400 Göttingen, Grisebachstraße 8.

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