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EXPERIMENTAL ARTICLES

Investigation of the Dark Metabolism of Acetate in Photoheterotrophically Grown Cells of *Rhodospirillum rubrum*

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Abstract——The mechanism of the aerobic dark assimilation of acetate in the photoheterotrophically grown purple nonsulfur bacterium *Rhodospirillum rubrum* was studied. Both in the light and in the dark, acetate assimilation in *Rsp. rubrum* cells, which lack the glyoxylate pathway, was accompanied by the excretion of glyoxylate into the growth medium. The assimilation of propionate was accompanied by the excretion of pyruvate. Acetate assimilation was found to be stimulated by bicarbonate, pyruvate, the C_4-dicarboxylic acids of the Krebs cycle, and glyoxylate, but not by propionate. These data implied that the citramalate (CM) cycle in *Rsp. rubrum* cells can function as an anaplerotic pathway under aerobic dark conditions. This supposition was confirmed by respiration measurements. The respiration of cells oxidizing acetate depended on the presence of CO_2 in the medium. The fact that the intermediates of the CM cycle (citramalate and mesaconate) markedly inhibited acetate assimilation but had almost no effect on cell respiration indicated that citramalate and mesaconate were intermediates of the acetate assimilation pathway. The inhibition of acetate assimilation and cell respiration by itaconate was due to its inhibitory effect on propionyl-CoA carboxylase, an enzyme of the CM cycle. The addition of 5 mM itaconate to extracts of *Rsp. rubrum* cells inhibited the activity of this enzyme by 85%. The data obtained suggest that the CM cycle continues to function in *Rsp. rubrum* cells that have been grown anaerobically in the light and then transferred to the dark and incubated aerobically.

Key words: *Rhodospirillum rubrum*, citramalate cycle, tricarboxylic acid cycle, glyoxylate pathway, acetate.

Many phototrophic bacteria belonging to the family *Rhodospirillaceae* can grow on acetate as a carbon source. Acetate catabolism in such bacteria is due to the functioning of the tricarboxylic acid (TCA) cycle. Growth on acetate as the sole source of carbon is impossible if the C_4-carboxylic acids of the TCA cycle are not replenished. In many purple nonsulfur bacteria, these acids are formed in the glyoxylate cycle, in which acetate is oxidized to glyoxylate that condenses with acetyl-CoA to form malate. The key enzyme of the glyoxylate pathway is isocitrate lyase. However, many of the purple nonsulfur bacteria, including *Rhodospirillum rubrum* and *Rhodobacter sphaeroides*, are able to grow on acetate as the sole source of carbon without isocitrate lyase (another enzyme of the glyoxylate pathway, malate synthase, is present in these bacteria) [1, 2]. Lacking the glyoxylate pathway, the bacteria must have other ways to replenish the pool of the TCA cycle intermediates. We suggested that phototrophically grown *Rsp. rubrum* cells can oxidize acetate to glyoxylate via a novel CO_2-dependent pathway, called the citramalate (CM) cycle (Fig. 1) [3, 4]. Functionally, this cycle is analogous to the glyoxylate cycle.

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In purple nonsulfur bacteria, the role of the TCA cycle depends on the growth conditions. In photoheterotrophically grown cells, the TCA cycle is largely an anabolic (biosynthetic) pathway, while in chemoheterotrophically grown cells, the TCA cycle serves both anabolic and catabolic functions [7–9]. The Calvin cycle, which functions as an anabolic pathway under phototrophic anaerobic conditions, is blocked under chemotrophic aerobic conditions [9]. Therefore, in the dark, the biosynthetic role of the TCA cycle and the role of the anaplerotic of pathways replenishing the TCA cycle intermediates must increase.

This work was undertaken to study the mechanism of the dark aerobic assimilation of acetate in *Rsp. rubrum* cells preliminarily grown anaerobically in the light and to elucidate whether the CM cycle functions as an anaplerotic pathway in such cells.

MATERIALS AND METHODS

The strain *Rhodospirillum rubrum* 2R used in this work was obtained from the culture collection of the Department of Microbiology at the Moscow State University. Experiments were carried out with cells grown photoheterotrophically in media with acetate and bicarbonate [3] to the early exponential growth phase, when

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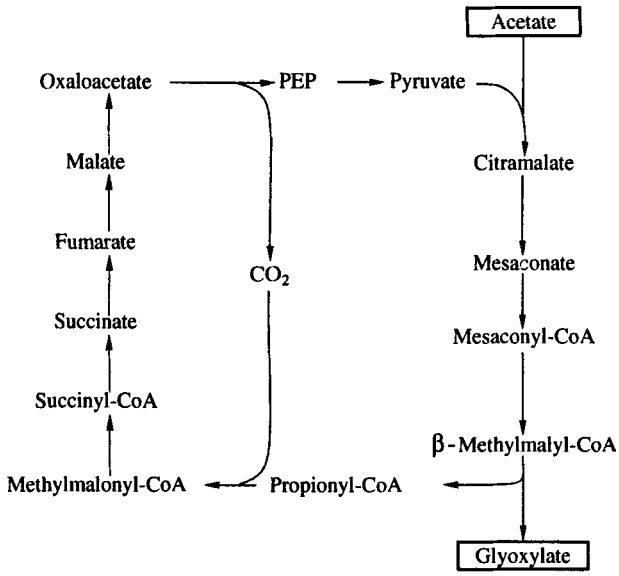

Fig. 1. Putative scheme of the citramalate cycle in *Rsp. rubrum* cells [3, 4]. The sequence acetate + pyruvate ⟶ citramalate ⟶ mesaconate ⟶ mesaconyl-CoA ⟶ methylmalyl-CoA ⟶ propionyl-CoA + glyoxylate was deduced from the scheme given in the publications [5, 6]. PEP is phosphoenolpyruvate.

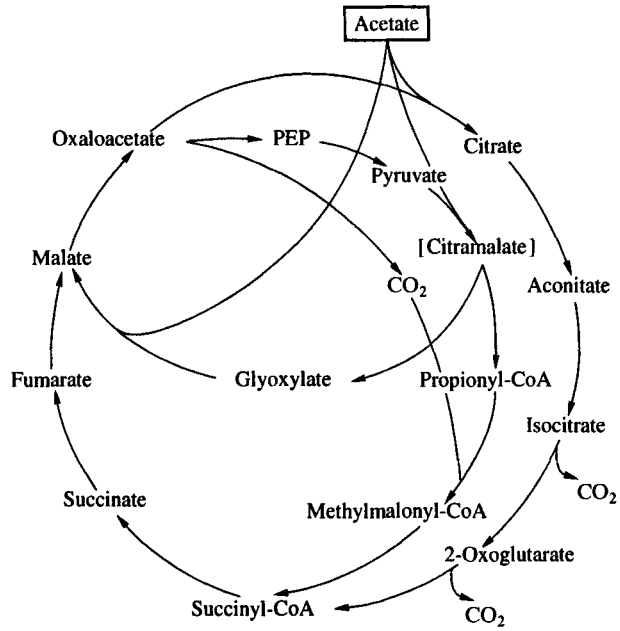

Fig. 2. Relationship belween the CM cycle and the TCA cycle during the dark aerobic assimilation of acetate by *Rsp. rubrum* cells.

the culture density was about 60 mg protein/l. Cells were harvested by centrifugation, washed with a mineral growth medium, and suspended to give a density of 100–200 μg protein/ml.

To study the assimilation of $NaH^{14}CO_3$, 2-(^{14}C)acetate and 2-(^{14}C)propionate, *Rsp. rubrum* cells were aerobically incubated in the dark in flasks shaken at a rotation speed of 180 rpm. To study the effect of bicarbonate on the rate of acetate and propionate assimilation, the evolved CO_2 was absorbed with an alkaline trap filled with triethanolamine. The reaction was started by adding 5 mM $NaH^{14}CO_3$ (0.04 MBq), 5 mM 2-(^{14}C)propionate (0.02 MBq), or 10 mM 2-(^{14}C)acetate (0.02 MBq) cell suspension and stopped, at certain time intervals, by filtering 1 ml of the cell suspension through 0.45-μm-pore-size nitrocellulose filters. The filters with adsorbed cells were dried and counted in an LKB RacBeta 1127 liquid scintillation counter.

To study the excretion of keto acids, cells were incubated in flasks shaken at 180 rpm in the dark at 30°C for six hours in the presence of 1 g/l propionate, mesaconate, or acetate. If required, 1 g/l bicarbonate and the respective inhibitors were added. Cells were then separated from the incubation medium by centrifugation, and keto acids in the supernatant were identified in the form of 2,4-dinitrophenylhydrazones [10] by thin-layer chromatography carried out in *n*-butanol with 4 vol % ammonia.

Cell respiration was measured polarographically using a 1-ml Clark-type oxygen electrode and cell suspensions containing 200–400 μg protein/ml.

The activity of propionyl-CoA carboxylase (EC 6.4.1.3) was measured through the propionyl-CoA–dependent fixation of CO_2 [11]. Cell extracts were prepared as described elsewhere [3].

Protein concentration was measured by the method of Lowry *et al.* [12] using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Experiments showed that the chemotrophic assimilation of acetate by *Rsp. rubrum* cells was stimulated by bicarbonate, pyruvate, the Krebs cycle intermediates, and glyoxylate, but not by propionate (Table 1). The inhibitors of the Krebs cycle—malonate, which inhibits succinate dehydrogenase, and fluoroacetate, which inhibits aconitase—strongly suppressed acetate assimilation. These results are in agreement with our previous data on acetate assimilation under phototrophic conditions [3, 4].

The stimulation of acetate assimilation by bicarbonate and organic acids was due to the CO_2-dependence of the CM cycle (Fig. 1) [3, 4]. Efficient involvement of acetate in metabolism requires that the medium contain either bicarbonate or acetate acceptors. If the CM cycle is active, the following compounds may act as such acceptors (see Fig. 2):

(1) glyoxylate (a product of the CM cycle, which accepts acetate via the malate synthase reaction);

(2) pyruvate (an acceptor of acetate in the citramalate lyase reaction); in the absence of CO_2, pyruvate can be formed from succinate, fumarate, malate, and oxaloacetate via the CM cycle;

Table 1. Aerobic dark assimilation of labeled acetate, propionate, and bicarbonate by *Rsp. rubrum* cells

Compounds added	Assimilation rate, nmol/(min mg protein)
*Acetate	13.9
*Acetate + pyruvate	29.5
*Acetate + propionate	14.8
*Acetate + glyoxylate	55.7
*Acetate + oxaloacetate	37.8
*Acetate + malate	57.8
*Acetate + fumarate	46.8
*Acetate + succinate	58.5
*Acetate + bicarbonate	63.6
*Acetate + bicarbonate + fluoroacetate	11.4
*Acetate + bicarbonate + malonate	15.2
*Acetate + bicarbonate + L-citramalate	15.4
*Acetate + bicarbonate + mesaconate	10.4
*Acetate + bicarbonate + itaconate	4.4
*Propionate;	3.8
*Propionate + bicarbonate	20.8
*Propionate + bicarbonate + itaconate	9.0
*Bicarbonate	0.7
*Bicarbonate + acetate	0.5
*Bicarbonate + propionate	13.7
*Bicarbonate +propionate + itaconate	1.9

Note: Acetate was added at a concentration of 10 mM; bicarbonate, propionate, pyruvate, glyoxylate, malonate, citramalate, and mesaconate, at a concentration of 5 mM; fluoroacetate and itaconate, at a concentration of 1 mM.
*Acetate, * propionate, and * bicarbonate stand for 2-(^{14}C)acetate, 2-(^{14}C)propionate, and Na$H^{14}CO_3$, respectively.

Table 2. Excretion of keto acids by *Rsp. rubrum* cells incubated aerobically in the dark for 6 h

Compounds added	Keto acids, μmol/mg protein
Acetate	0.106
Acetate + fluoroacetate	0.707
Acetate + bicarbonate	0.267
Acetate + bicarbonate + fluoroacetate	0.841
Acetate + bicarbonate + + fluoroacetate + malonate	0.491
Mesaconate	0.165
Mesaconate + fluoroacetate	0.319
Mesaconate + malonate	0.184
Mesaconate + malonate + fluoroacetate	0.212
Propionate	0.158
Propionate + semicarbazide	0.256
Propionate + CO_2	0.232
Propionate + semicarbazide + CO_2	0.291

Note: The concentration of acetate, propionate, and mesaconate in the medium was 10 mM and the concentration of bicarbonate and malonate was 5 mM. Fluoroacetate was added at a concentration of 1 mM; semicarbazide was added at a concentration of 0.1%. Cells incubated in the presence of acetate or mesaconate predominantly excreted glyoxylate; cells incubated in the presence of propionate predominantly excreted pyruvate and 2-oxoglutarate in a ratio of 7 : 3.

(3) oxaloacetate and other C_4-carboxylic acids of the TCA cycle, which can accept acetate via the citrate synthase reaction.

Propionate did not stimulate acetate assimilation, since it cannot accept acetate and is involved in metabolism only if carboxylated.

The fact that the degree of stimulation of acetate assimilation by bicarbonate under chemotrophic conditions (350%, Table 1) is much greater than under phototrophic conditions (about 100% [3]) is indicative of the more significant role of acetate under chemotrophic conditions.

The dependence of acetate metabolism on bicarbonate may be due to the propionyl-CoA carboxylase reaction of the CM cycle (Figs. 1 and 2) [3, 4]. This reaction can also explain the ability of *Rsp. rubrum* cells to utilize propionate for growth [11, 13]. Since acetate-grown cells possess the propionyl-CoA carboxylase activity (6.2 nmol/(min mg protein) [3]), they must be able to assimilate propionate (Table 1). Propionate assimilation, which requires CO_2, was found to be accompanied by the consumption of bicarbonate in equimolar amounts (Table 1). Incubation of *Rsp. rubrum* cells with propionate led to the CO_2-dependent excretion of pyruvate and 2-oxoglutarate in a ratio of 7 : 3 (Table 2). The addition of semicarbazide, known to bind keto acids, to the medium stimulated their excretion, which suggests that the excreted keto acids can be partially utilized by cells. The ability of acetate-grown *Rsp. rubrum* cells to oxidize propionate with the formation of pyruvate provides further evidence for the involvement of this process in acetate metabolism via the CM cycle (Figs. 1 and 2).

Under chemotrophic conditions, the rate of bicarbonate consumption in the presence of acetate and the CO_2/acetate ratio were very low (0.5 nmol/(min mg protein) and ~0.01, respectively). At the same time, in the case of propionate assimilation, the rate of CO_2 fixation and the CO_2/propionate ratio were very high (13.7 nmol/(min mg protein) and ~0.7, respectively) (Table 1). The low rate of bicarbonate consumption by *Rsp. rubrum* cells in the presence of acetate can be explained by the fact that the CM cycle involves the stage of oxalacetate decarboxylation, which follows the propionyl-CoA carboxylase reaction (Figs. 1 and 2). The bicarbonate consumed at the beginning of the cycle

Table 3. Oxygen consumption by *Rsp. rubrum* cells

Compounds added	Oxygen consumption, nmol/(min mg protein)
None (endogenous respiration)	4.3
Acetate	8.5
Bicarbonate	10.9
Acetate + bicarbonate	21.8
Propionate	10.8
Propionate + bicarbonate	20.7
Pyruvate	19.4
Pyruvate + bicarbonate	33.3
Glyoxylate	13.9
Glyoxylate + bicarbonate	14.3
Acetate + propionate	14.4
Acetate + pyruvate	24.9
Acetate + glyoxylate	23.0
Citramalate	8.0
Citramalate + bicarbonate	14.3
Citramalate + acetate + bicarbonate	27.2
Mesaconate	5.3
Mesaconate + bicarbonate	13.7
Mesaconate + acetate + bicarbonate	19.0

Note: All compounds were added at a concentration of 5 mM.

Table 4. Effect of inhibitors on oxygen consumption by *Rsp. rubrum* cells

Compounds added	Oxygen consumption, % of control
Acetate + bicarbonate	100.0
Acetate + bicarbonate + 5 mM malonate	62.8
Acetate + bicarbonate + itaconate	37.1
Acetate + bicarbonate + fluoroacetate	30.0
Acetate + bicarbonate + itaconate + pyruvate	90.6
Acetate + bicarbonate + itaconate + glyoxylate	87.6
Propionate + bicarbonate	100.0
Propionate + bicarbonate + 10 mM malonate	55.6
Propionate + bicarbonate + 15 mM malonate	33.3
Propionate + bicarbonate + itaconate	20.2

Note: Two values of 100% correspond to the rate of oxygen consumption by cells oxidizing acetate + bicarbonate (17.0 nmol/(min mg protein)) and propionate + bicarbonate (20.4 nmol/(min mg protein)). The concentration of acetate, bicarbonate, pyruvate, propionate, and glyoxylate in the medium was 5 mM; the concentration of fluoroacetate and itaconate was 1 mM.

is then excreted into the medium, which explains why the CO_2-dependent assimilation of acetate is characterized by a very low rate of CO_2 fixation. Under phototrophic conditions, the rate of bicarbonate consumption in the presence of acetate (3.1 nmol/(min mg protein)) and the CO_2/acetate ratio (~0.1) [3] are much greater than under chemotrophic conditions. This can be due to the additional fixation of CO_2 in the Calvin cycle [3].

Incubation of *Rsp. rubrum* cells under chemotrophic conditions in the presence of acetate led to the active accumulation of glyoxylate in the medium (Table 2). The accumulation of glyoxylate was stimulated by bicarbonate (because of the CO_2-dependence of the CM cycle) and fluoroacetate, which inhibits glyoxylate metabolism by blocking aconitase (Fig. 2). The addition of malonate slowed down the rate of glyoxylate excretion, since the malonate-sensitive succinate dehydrogenase is involved not only in the TCA cycle, but also in the CM cycle (Figs. 1 and 2). Basically, these effects are analogous to those observed for *Rsp. rubrum* cells incubated anaerobically in the light [3, 4]. However, the degree of stimulation of glyoxylate excretion by fluoroacetate under chemotrophic conditions (about sevenfold) was considerably greater than under phototrophic conditions (about twofold), which confirms the suggestion about the increased role of acetate and anaplerotic pathways in the chemotrophic metabolism of purple nonsulfur bacteria.

Cells grown anaerobically in the light actively consumed oxygen (Table 3), since they retained their ability to synthesize the respiratory chain components under these conditions [14, 15]. Like acetate assimilation, the respiration of *Rsp. rubrum* cells in the presence of acetate was substantially stimulated by bicarbonate (Table 3). Bicarbonate could also stimulate the endogenous respiration of cells. These results can be explained by the fact that poly-β-hydroxybutyric acid, a substrate for endogenous respiration, is abundantly accumulated in cells grown on acetate [16] and is degraded via this acid. The respiration of cells in the presence of acetate was inhibited by malonate and fluoroacetate (Table 4). In the absence of acetate, cell respiration was stimulated by pyruvate, propionate, and glyoxylate by 350, 150, and 220%, respectively (Table 3). Respiration in the presence of propionate and pyruvate was stimulated by bicarbonate, since the metabolism of these acids involves their carboxylation (propionate is carboxylated by propionyl-CoA carboxylase, while pyruvate can be carboxylated either by pyruvate carboxylase or by the PEP synthase/PEP carboxylase system [9]). The respiration of *Rsp. rubrum* cells in the presence of acetate was stimulated by glyoxylate, pyruvate, and, to a lesser degree, by propionate (Table 3). These findings correlate well with data on the assimilation of acetate and propionate and are consistent with

the suggestion about the functioning of the CM cycle in *Rsp. rubrum* cells.

The C_5-dicarboxylic acids of the CM cycle (citramalate and mesaconate, see Fig. 1) were found to substantially inhibit acetate assimilation in *Rsp. rubrum* cells under both phototrophic [3, 4] and chemotrophic conditions (Table 1). The same effect of these compounds on acetate assimilation has been reported for the purple nonsulfur bacterium *Rba. sphaeroides*, which also lacks the glyoxylate pathway [17]. This effect has been attributed to the inhibitory action of citramalate and mesaconate on some reactions of acetate metabolism [17]. However, consideration of the functioning of the CM cycle allows an alternative explanation for the effect of these compounds: they are involved in metabolism instead of acetate and thus dilute the radioactive label of 2-(^{14}C)acetate, which manifests itself as a decrease in the rate of its assimilation. On the other hand, the addition of citramalate and mesaconate to *Rsp. rubrum* cells virtually did not affect their respiration in the presence of acetate and bicarbonate (Table 3). The strong inhibition of acetate assimilation by citramalate and mesaconate and the absence of a noticeable effect of these compounds on cell respiration in the presence of acetate testify to the fact that citramalate and mesaconate are intermediates of acetate assimilation. Furthermore, these acids can serve as substrates for cell respiration (Table 3). These data provide further evidence for the ability of acetate-grown *Rsp. rubrum* cells to synthesize citramalate lyase and mesaconase, which are necessary for the assimilation of citramalate and mesaconate. This inference correlates well with the finding that *Rsp. rubrum* cells excrete glyoxylate when incubated in the presence of mesaconate (Table 2). The excretion of glyoxylate in the presence of mesaconate, as well as of acetate, is stimulated by fluoroacetate (Table 2). The ability of *Rsp. rubrum* cells to metabolize mesaconate is corroborated by the fact that cell extracts incubated with mesaconate, ATP, and CoA produced pyruvate and, in smaller amounts, citramalate, oxaloacetate, 2-oxoglutarate, and acetate (data not presented).

Surprisingly, both acetate assimilation and cell respiration were inhibited by itaconate (Tables 1 and 4), an inhibitor of isocitrate lyase [18] which is routinely used in studies of the glyoxylate pathway in bacteria [19]. Since *Rsp. rubrum* cells are unable to synthesize isocitrate lyase, they must be insensitive to itaconate. If so, the inhibitory action of itaconate on cells could be related to its effect on some other enzymes of the CM cycle. Indeed, itaconate was found to efficiently inhibit propionyl-CoA carboxylase: its addition to cell extracts at concentrations of 0.5 and 5 mM inhibited the activity of this enzyme by 35.7 and 84.5%, respectively. Itaconate drastically inhibited propionate assimilation, as well as the fixation of CO_2 and cell respiration in the presence of propionate (Tables 1 and 4). The inhibition of acetate assimilation and cell respiration by itaconate is a further demonstration of the involvement of propionyl-CoA carboxylase in acetate assimilation in *Rsp. rubrum*.

Thus, the results presented and the relevant data available in the literature can be explained in terms of the functioning of the CM cycle in *Rsp. rubrum* cells. *Rsp. rubrum* cells grown anaerobically in the light do not change the mechanism of acetate assimilation when transferred to chemotrophic conditions. In this case, the TCA cycle retains its key role in acetate metabolism, while the role of the CM cycle considerably increases.

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