The Formation of Isocitratase by the Athiorhodaceae

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SUMMARY

Isocitratase, which cleaves isocitrate to glyoxylate and succinate and is a key enzyme in the glyoxylate cycle, is formed by *Rhodopseudomonas palustris* and *R. capsulatus* when grown on acetate or butyrate either anaerobically in light or aerobically in the dark. Only traces of the enzyme are present in organisms grown on succinate or malate. In contrast, isocitratase is detectable in traces only in *R. spheroides* and *Rhodospirillum rubrum* grown on acetate, butyrate or other substrates. This suggests that the glyoxylate cycle cannot account for net synthesis of cell constituents from acetate or acetate precursors in these latter two organisms.

INTRODUCTION

Many photosynthetic bacteria of the Athiorhodaceae family can grow on bicarbonate + acetate, butyrate or other fatty acids as carbon source (van Niel, 1944), and the catabolism of these organic substrates has been shown by various techniques to proceed via the tricarboxylic acid cycle (Glover, Kamen & van Genderen, 1952; Eisenberg, 1953; Elsdon & Ormerod, 1956). The synthesis of cellular materials during growth demands the occurrence of reactions which effect the net formation of intermediates of the tricarboxylic acid cycle from the growth substrates. In many micro-organisms (reviewed by Kornberg, 1959) this is accomplished via the glyoxylate cycle (Kornberg & Krebs, 1957; Kornberg & Madsen, 1957; 1958), a key enzyme of which, isocitratase (Smith & Gunsalus, 1954, 1955, 1957; Olson, 1954, 1959; Saz & Hillary, 1956), is formed only when such micro-organisms grow on acetate or precursors of acetate (Smith & Gunsalus, 1955; Calley, Dagley & Hodgson, 1958; Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959). The main purpose of this paper is to show that, of the Athiorhodaceae examined, *Rhodopseudomonas palustris* and *R. capsulatus* form isocitratase adaptively when grown on acetate or on precursors of acetate, both anaerobically in light and aerobically in the dark. Since the organisms also contain malate synthetase (Wong & Ajl, 1956) the glyoxylate cycle can operate in these organisms under these conditions. However, although *R. spheroides* and *Rhodospirillum rubrum* also contain malate synthetase, they are virtually devoid of isocitratase under all conditions tested, and must therefore differ in the pathways of biosynthesis from acetate from all other micro-organisms examined.

METHODS

Organisms. The following strains of Athiorhodaceae were used: *Rhodopseudomonas spheroides* NCIB no. 8253, *Rhodospirillum rubrum* NCIB no. 8255, *Rhodopseudomonas capsulatus* and *R. palustris* no. 2.3.11 and no. 2.1.7, respectively, from the collection of Professor C. B. van Niel. Stock cultures of all organisms were maintained as described previously (Lascelles, 1956).

Media. The basal medium used contained (mg./l.): KH_2PO_4 and K_2HPO_4 , 500 each; $(\text{NH}_4)_2\text{HPO}_4$, 800; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200; CaCl_2 , 40; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 4; ferric citrate, 3; nicotinic acid and thiamine hydrochloride, 1 each; biotin, 0.01. For growth of *Rhodopseudomonas palustris*, *p*-aminobenzoic acid ($0.15 \mu\text{g./l.}$) was also added. The basal medium was supplemented as required with DL-malate (20 mM), succinate (10 mM), acetate (20 mM) or butyrate (10 mM), all added as the sodium salts. Sodium bicarbonate (6 mM) was included in all media containing acetate and butyrate as carbon source; the stock solution (0.6 M) was sterilized separately by autoclaving and added aseptically after equilibrating with CO_2 . Media were sterilized by autoclaving at 121° for 10 min.

Growth tests. These were carried out in 10 ml. volumes of media in tubes of 15 mm. diameter. Inoculation was with 0.1 ml. of a 1/100 dilution (about 4×10^6 organisms) of a 24 hr. culture grown anaerobically in light on a medium containing malate, glutamate and yeast extract (Lascelles, 1956). Anaerobiosis was achieved by addition of a few crystals of pyrogallol and a drop of saturated K_2CO_3 to the cotton plug which was forced below the rim of the tubes; they were then sealed with rubber bungs. Tubes were incubated at 32° to 34° at 20 cm. distance from a bank of 60 W. tungsten lamps.

Growth and preparation of organisms for cell-free extracts. Cultures were grown anaerobically in light in 8 oz. medical flat bottles containing 200 ml. of medium and stoppered with a rubber bung. The inoculum and details of incubation were described by Lascelles (1956). Aerobic cultures of *Rhodopseudomonas spheroides* were grown in 500 ml. medium in 2 l. Erlenmeyer flasks; the other organisms were grown in 900 ml. medium in flasks of the same size. Inoculation was with 2–5 ml. of 24 hr. culture developed anaerobically in the light in a medium containing malate, glutamate and yeast extract (Lascelles, 1956). Incubation was at 32° in a gyratory shaker (New Brunswick Instrument Co., New Jersey, U.S.A.) at 180 oscillations/min.

Cultures were harvested during or towards the end of the logarithmic phase of growth, the incubation time varying between 18 and 24 hr. for photosynthetic cultures and from 24 to 48 hr. for aerobic ones. Organisms were harvested by centrifugation, washed with one-half the original culture volume of 0.02 M-potassium phosphate buffer (pH 7.0), and finally suspended in the same buffer (40 mM) to give a concentration of organisms equivalent to 1 to 10 mg. dry wt./ml.

Preparation of extracts. These were prepared by ultrasonic vibration as described before (Lascelles, 1959). The disrupted preparations were spun at 0° for 10 min. at 25,000 g and the supernatant fluid used in the enzyme assays.

Assay of enzymes. Isocitratase and malate synthetase were measured spectrophotometrically by the procedure of Dixon & Kornberg (1959). The activity of

isocitratase is expressed as the specific activity (μ mole glyoxylate formed in the standard assay/hr./mg. protein).

Estimation of protein and bacteriochlorophyll. These were determined as described previously (Lascelles, 1959).

Measurement of growth. This was determined by turbidity measurements with an EEL photoelectric colorimeter (Evans Electroselenium Ltd., Halstead, Essex, England) with filter no. 608. Results are expressed as mg. dry wt. organism/ml. culture and were calculated from calibration curves prepared with suspensions of each organism grown anaerobically in light or aerobically in the dark.

RESULTS

Effect of carbon source on growth and bacteriochlorophyll formation

All the organisms grew anaerobically in light on acetate, butyrate, succinate or malate (Table 1). Bicarbonate was necessary for consistent growth from small inocula on acetate or butyrate but did not affect growth on the dicarboxylic acids. Except for *Rhodopseudomonas spheroides* the carbon source did not greatly influence the level of bacteriochlorophyll in the organisms. In *R. spheroides* considerable variation was found, the pigment concentration in organisms grown on acetate being only one-tenth of that in those grown on malate (Table 1). The same substrates also supported aerobic growth (in the dark) of all the organisms.

Table 1. *Growth and bacteriochlorophyll formation on various carbon sources by Rhodospirillum rubrum, Rhodopseudomonas spheroides, R. capsulatus and R. palustris*

Cultures were incubated anaerobically in light for 72 hr. in 10 ml. volumes as described in the Methods. The concentrations of organic acids were as follows: succinate and butyrate, 10 mM, acetate; DL-malate, 20 mM. In addition, NaHCO₃ (to 6 mM) was added to all tubes after autoclaving. *p*-Aminobenzoic acid (to 1 μ M) was included in the basal medium for *Rhodopseudomonas palustris*.

Organism	Carbon source	Growth (mg. dry wt./ml.)	Bacterio- chlorophyll (μ m-mole/mg. dry wt. organism)
<i>Rhodospirillum rubrum</i>	Succinate	0.68	12.4
	Malate	1.21	13.1
	Acetate	0.67	14.4
	Butyrate	0.12	15.8
<i>Rhodopseudomonas spheroides</i>	Succinate	0.72	5.4
	Malate	1.23	12.0
	Acetate	0.66	1.2
	Butyrate	0.48	4.4
<i>R. capsulatus</i>	Succinate	0.78	22.4
	Malate	0.63	14.8
	Acetate	0.63	16.0
	Butyrate	0.98	10.8
<i>R. palustris</i>	Succinate	0.78	9.1
	Malate	0.98	11.0
	Acetate	0.86	12.8
	Butyrate	1.07	14.0

Isocitratase activity in extracts

Extracts prepared from *Rhodopseudomonas spheroides* grown on acetate or butyrate either anaerobically in light or aerobically in the dark showed only traces of isocitratase. This activity was quite insufficient to contribute significantly to the ability of the organism to utilize these substrates for growth and was no higher than that found in malate-grown organisms. Extracts from organisms which had been successively transferred three times through acetate or butyrate medium were also inactive. Extracts of *Rhodospirillum rubrum* also had negligible isocitratase activity (Table 2).

Table 2. *Isocitratase activity in extracts of Athiorhodaceae*

Extracts were prepared from organisms harvested during the logarithmic phase of growth on the various substrates and were assayed as described in Methods.

Organism	Growth substrate	Isocitratase in culture grown	
		Anaerobic + light (μ mole glyoxylate/hr./mg. protein)	Aerobic
<i>Rhodopseudomonas capsulatus</i>	Acetate	1.90	1.37
	Butyrate	1.58	2.11
	Succinate	0.03	0.04
<i>R. palustris</i>	Acetate	5.4	5.6
	Butyrate	3.4	2.4
	Malate	0.07	—
<i>R. spheroides</i>	Acetate	0.01	0.01
	Butyrate	0.06	0.04
	Malate	0.04	0.03
<i>Rhodospirillum rubrum</i>	Acetate	0.07	0.08
	Malate	0.05	0.04

Isocitratase activity was found in *Rhodopseudomonas capsulatus* and *R. palustris* when grown on acetate and butyrate (Table 2). The specific activity of the isocitratase did not differ significantly in organisms grown anaerobically in light or aerobically in the dark (Table 2). Under both conditions and with both organisms it varied with the physiological age of the organisms at harvesting, reaching a maximum during the logarithmic phase of growth and then declining (Fig. 1).

As found in other organisms (see review, Kornberg, 1959) isocitratase in *Rhodopseudomonas capsulatus* and *R. palustris* was adaptive, being formed only in organisms grown on acetate or butyrate (Table 2). In *R. palustris* addition of succinate to a culture growing on butyrate delayed development of isocitratase; synthesis of this enzyme was resumed after a period during which the added succinate was presumably utilized (Fig. 2). Suppression of isocitratase by succinate has been observed with other organisms (Kornberg *et al.* 1958; Kornberg, Collins & Bigley, 1960).

Malate synthetase. Activity of this enzyme was found in all extracts of all organisms tested under all conditions of growth. Malate synthetase appears to be constitutive in Athiorhodaceae as it is in *Pseudomonas* spp. (Kornberg & Lund, 1959).

Fig. 1. Isocitratase activity of *Rhodospseudomonas palustris* at various stages of growth. Cultures were grown: anaerobically in light (A); aerobically in the dark (B) on butyrate medium. Samples were removed at intervals for determination of isocitratase activity (—●—) and dry wt. (—○—).

Fig. 2. Effect of succinate on the development of isocitratase in cultures of *Rhodospseudomonas palustris*. A culture growing anaerobically in light in butyrate medium was divided at the beginning of the exponential phase. Succinate (to 5 mM) was added to one part and isocitratase activity determined at intervals thereafter (—○—). No addition was made to the other portion (—●—). Total isocitratase activity is expressed as the product of the specific activity and the suspension density and is plotted against the increase in culture density.

DISCUSSION

The tricarboxylic acid cycle, which has been shown to operate in members of the *Athiorhodaceae*, is sufficient as a means of providing both energy and the carbon skeletons of cell constituents when succinate or malate is the sole carbon source; but this cycle alone cannot account for growth on acetate or butyrate. In many types of micro-organisms the required synthesis of intermediates of the tricarboxylic acid cycle is effected via the glyoxylate cycle (see Kornberg, 1959). The ability to

form isocitratase is a prerequisite for the operation of the glyoxylate cycle. It is therefore of some interest that isocitratase was not found in significant amounts in either *Rhodospirillum rubrum* or in *Rhodopseudomonas spheroides* when grown on acetate or butyrate, though it was present in *R. capsulatus* and *R. palustris* grown under the same conditions. The isocitratase-negative organisms did not appear to be at any obvious disadvantage to the isocitratase-positive organisms with respect to growth rate on these substrates, though the amount of bacteriochlorophyll formed in the light by *R. spheroides* on acetate or butyrate was markedly less than that formed by malate-grown cultures. Since *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* cannot use the glyoxylate cycle they must have other means for obtaining net synthesis of tricarboxylic acid cycle intermediates from acetate. The fact that added bicarbonate is necessary for growth of photosynthetic bacteria on fatty acids (van Niel, 1944) may be significant although, since only negligibly small amounts of isotope from added $\text{NaH}^{14}\text{CO}_3$ are incorporated by *R. spheroides* aerobically metabolizing acetate, CO_2 is unlikely to act directly as a main precursor of cellular materials under these conditions (Kornberg, to be published). Moreover, the operation of a reductive pentose cycle (Bassham & Calvin, 1957), which could provide a net synthesis of tricarboxylic acid cycle intermediates, cannot account for aerobic growth of the isocitratase-negative organisms on acetate, since ribulose 1:5-diphosphate carboxylase is formed only by cultures growing anaerobically in light (Lascelles, 1960).

The role of the glyoxylate cycle in the isocitratase-positive organisms (*Rhodopseudomonas palustris*, *R. capsulatus*) cannot be quantitatively assessed from the present information. The enzyme was found only in organisms grown on acetate or butyrate and its formation was suppressed by succinate. Such behaviour is typical of organisms known to use the glyoxylate cycle. On the other hand isocitratase was formed by cultures growing anaerobically in light, where the cycle would not be required for net synthesis of tricarboxylic acid cycle intermediates, since these could be formed via the reductive pentose cycle. It is thus possible that, under these conditions, both biosynthetic routes operate simultaneously.

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