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Influence of Light Intensity on Reductive Pentose Phosphate Cycle Activity during Photoheterotrophic Growth of *Rhodospirillum rubrum*[1]

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ABSTRACT

Light intensity during growth affects the proportion of carbon dioxide fixed by the reductive pentose phosphate cycle relative to that incorporated via C_4 acids in acetate phototrophs of *Rhodospirillum rubrum*. With cells grown at high light intensity (9000 lux) the specific activities of ribulose-1,5-diphosphate and propionyl CoA carboxylases were increased compared with cells grown at low light intensity (1500 lux), although pyruvate carboxylase activity was unaltered.

Kinetic experiments with cells assimilating acetate at high light intensity showed that when the cells had been grown at high light intensity there was a rapid incorporation of $^{14}CO_2$ into phosphate esters compared with cells grown at low light intensity and fixing $^{14}CO_2$ while assimilating acetate at low light intensity. The percentage of the total radioactivity present in phosphate esters plotted against time gave a negative slope for high light conditions compared with a positive slope for low light conditions. High light-grown cells assimilating acetate at high light intensity showed the greatest combined rate of $^{14}CO_2$ fixation via the reductive pentose phosphate cycle and C_4 acids, and this corresponded to the shortest mean generation time. When cells were grown at high light intensity and allowed to assimilate $^{14}CO_2$ at high light intensity but in the stationary phase, the pattern of $^{14}CO_2$ fixation resembled that for low light-grown cells assimilating acetate and fixing $^{14}CO_2$ at low light intensity, showing that both acetate assimilation and high light intensity were necessary for the rapid incorporation of $^{14}CO_2$ via the reductive pentose phosphate cycle.

The importance of the reductive pentose phosphate cycle in the carbon metabolism of autotrophic micro-organisms is well established (20). The enzymes of the cycle have been shown to be present in the Athiorhodaceae (2, 12), Thiorhodaceae (8, 9), and Chlorobacteriaceae (22). Growth of *Chromatium* (8, 9) or *Rhodospirillum rubrum* (2) in the light in the presence of an organic carbon source resulted in the repression of ribulose-1,5-diP carboxylase. Pulse-labeling experiments with *R. rubrum* grown photoheterotrophically on acetate-carbon dioxide or malate-carbon dioxide showed that the entry of carbon into phosphate esters was much slower than into organic and amino acids (1, 2), suggesting that during photoheterotrophic growth the contribution of the reductive pentose phosphate cycle to over-all carbon metabolism was significantly decreased. The present paper reports that light intensity during photoheterotrophic growth is an important factor that can regulate the contribution of the reductive pentose phosphate cycle to over-all carbon assimilation.

MATERIALS AND METHODS

Rhodospirillum rubrum (NCIB 8255) was grown on acetate in the light as described by Porter and Merrett (18). Cells were grown photoheterotrophically in completely filled 250-ml medical flat bottles with the medium of Ormerod *et al.* (14), in which sodium acetate (20 mM) replaced malic acid as the organic carbon source. The light intensity was either 1500 lux (low light) or 9000 lux (high light) provided by using banks of Osram 15W daylight tubes. Cells were harvested at the mid-exponential phase of growth corresponding to a dry weight of 0.3 mg/ml. When investigating the necessity of CO_2 for photoheterotrophic growth on acetate, cells were grown in Dreschel bottles and bubbled with either O_2 free N_2 or N_2 containing 5% CO_2 (v/v) at a rate of 5 l/hr.

Preparation of Cell Extracts and Enzyme Assays. Cells were harvested by centrifugation at 5000*g* for 10 min, washed by resuspending in buffer, and followed by a further centrifugation at 5000*g* for 10 min. Suspensions of freshly grown organisms (10 mg dry wt/ml in the requisite buffer) were subjected to four bursts of 45 sec duration of high frequency sound (15–18 kc/sec) separated by cooling periods of 30 sec in an M.S.E. ultrasonic disintegrator. Cell extracts were centrifuged at 10,000*g* for 5 min at 2 C. The supernatant solutions thus obtained were used for assays, except for the assay of β-carboxylation enzymes when the extract was given an additional centrifugation at 100,000*g* for 30 min at 2 C to remove a particulate NADH oxidase fraction. Extracts used in ribulose-1,5-diP carboxylase assays were prepared in 0.1 M potassium phosphate buffer, pH 7.3, whereas those for propionyl-CoA carboxylase and β-carboxylation enzyme assays were prepared in 0.1 M tris-HCl buffer, pH 8.0. Protein was determined by the method of Lowry *et al.* (13). Phosphoenolpyruvate carboxylase was assayed by the method of Cánovas and Kornberg (7), pyruvate carboxylase by the malate dehydrogenase coupled method (16) and ribulose-1,5-diP carboxylase by the method of Jakoby

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et al. (10). Propionyl-CoA carboxylase was assayed by determining propionyl-CoA enhanced $^{14}CO_2$ fixation in cell extracts. The reaction mixture contained 50 μmoles of tris-HCl buffer pH 8.0, 3 μmoles of ATP, 3 μmoles of $MgCl_2$, 3 μmoles of GSH, 0.5 μmole of propionyl-CoA, and 10 μmoles of $NaH^{14}CO_3$ containing 0.1 μc and 0.1 ml of extract in a total volume of 1.0 ml. Controls lacking propionyl-CoA or containing boiled enzyme were also run. The reaction was started by the addition of ^{14}C-bicarbonate and at intervals reaction mixtures were stopped by adding 1 ml of ethyl alcohol-8 N acetic acid (1:1 v/v). Precipitated protein was centrifuged and radioactivity determined by counting aliquots in a Packard liquid scintillation counter, quench correction being determined by the channels ratio method (5).

Experiments with $NaH^{14}CO_3$. Cells were harvested by centrifugation, washed in dilute phosphate buffer, and then resuspended in distilled water (2 mg dry wt/ml). Cell suspension (20 ml) in a rapid sampling apparatus (23), maintained at 30 C and aerated with O_2 free N_2, was illuminated at the same intensity as during growth. At zero-time 45 μmoles of sodium acetate in 1 ml were added followed after 10 min by 88 nmoles $NaH^{14}CO_3$ containing 200 μc. Samples of 2 ml were collected at intervals, aqueous ethanol soluble compounds were extracted, separated by chromatography, located by autoradiography, and identified as described previously (19). Special emphasis was placed on the identification of citramalic acid which was distinguished from α-oxoglutaric, methylmalonic, malic, and citric acids by ascending paper chromatography using Blundstone's solvent (3). It was further identified by cochromatography in one direction with authentic DL-citramalic acid using Blundstone's solvent (3) or 95:5 (v/v) 1-butanol-water-ethanol-water-diethylamine (80:10:20:1 v/v). Radioactive compounds on chromatograms were counted with a Nuclear-Chicago Model 1032 chromatogram scanner, at least 2000 counts being recorded for each spot.

Degradation of ^{14}C-Glutamic Acid. ^{14}C-Glutamic acid was eluted from chromatograms, 200 μmoles of carrier L-glutamic acid was added, and the specific radioactivity of an aliquot determined by persulfate combustion (11). $^{14}CO_2$ released was precipitated as $Ba^{14}CO_3$ and collected and weighed. ^{14}C content was determined by adding 2 N H_2SO_4 to the sample in an evacuated flask and trapping the $^{14}CO_2$ released in 0.2 ml of 1 M hydroxide of Hyamine in a detachable center well. The center wells were transferred to vials containing scintillation fluid, and radioactivity was determined by scintillation counting, efficiency being determined by the channels ratio method (5). Aliquots of ^{14}C-glutamic acid solution were pipetted into the main compartment of Warburg flasks containing 1 ml of 0.5 M acetate buffer, pH 5.0, 0.2 ml of CO_2 free 2.5 N NaOH in the center well, and 0.5 ml of L-glutamate decarboxylase (1.6 units) in the sidearm. After gassing with N_2 and equilibrating at 37 C, sidearm contents were tipped, the reaction was allowed to proceed for 1 hr, then 0.2 ml 10 N H_2SO_4 was introduced into each flask to ensure complete release of $^{14}CO_2$. Center well contents were sampled and $^{14}CO_2$ determined, while the flask contents were neutralized and aliquots counted in Bray's scintillator (4).

Chemicals and Substrates. Radiochemical compounds were obtained from the Radiochemical Center, Amersham, United Kingdom. Ribulose-1-diP (tetrasodium salt), ATP (sodium salt), phosphoenolpyruvate, CoA, DL-citramalic acid, malate dehydrogenase, and L-glutamate decarboxylase (type I from *E. coli*) were purchased from Sigma (London) Chemical Co. Ltd., U.K. Propionyl CoA was purchased from P. L. Biochemicals, Milwaukee.

RESULTS

Carbon Dioxide Fixation and Growth Rate. When bubbled with CO_2-free N_2, cells did not grow on acetate, demonstrating an absolute requirement for carbon dioxide during photoheterotrophic growth on acetate. The rate of carbon dioxide incorporation into the soluble fraction of the cells was determined by both the growth conditions for the cells and experimental conditions used to determine assimilation (Table I), most rapid assimilation occurring with exponential phase high light cells.

Pyruvate, Propionyl CoA, and Ribulose 1,5-diP Carboxylases. Although Rinne *et al.* (21) detected an ATP-dependent pyruvate carboxylase, later reports suggested that the β-carboxylation enzyme in *R. rubrum* was P-enolpyruvate carboxylase (6). Pyruvate carboxylase was readily demonstrated in extracts of acetate phototrophs but P-enolpyruvate carboxylase was not present. Addition of acetyl CoA increased the reaction rate, showing that the enzyme is similar to that of *Rhodopseudomonas spheroides* where the partially purified enzyme had an absolute requirement on acetyl CoA for activity (17). The specific activity of pyruvate carboxylase was unaltered by light intensity during growth; in contrast the specific activity of ribulose 1,5-diP carboxylase was greater in extracts from cells grown at high light intensity (Table II). Propionyl CoA carboxylase was present and again the specific activity was greater in extracts from high light grown cells (Table II).

Products of $^{14}CO_2$ Fixation. When *R. rubrum* grown at low light intensity, already metabolizing acetate, was supplied with a pulse of $NaH^{14}CO_3$, citramalate was the earliest product labeled, closely followed by glutamate at low light intensity (Fig. 1). Although after 300 sec over 50% of the total counts in the soluble fraction of the cells were in phosphate esters, the rate of incorporation into these compounds was slow, showing that the reductive pentose phosphate cycle was making only a limited contribution to carbon assimilation under these conditions. With high light-grown cells from the earliest sample, a far greater proportion of the total ^{14}C fixed was present in sugar phosphates (Fig. 2). The percentage of the total ^{14}C present in sugar phosphates was decreasing from the earliest sample showing that under these conditions the reductive pentose phosphate pathway was a major pathway of carbon

Table I. *Rate of CO_2 Uptake by Acetate Phototrophs under Different Conditions*

Growth Conditions	CO_2 Uptake	Mean Generation Time
	cpm/mg dry wt·hr × 10^{-2}	*hr*
Exponential phase cells, low light	0.3	40.6
Exponential phase cells, high light	6.0	17.2
Stationary phase cells, high light	0.9	

Table II. *Effect of Light Intensity during Growth on the Specific Activities of Some Carboxylating Enzymes in R. rubrum*

	Specific Activity		
	Ribulose-1,5-diP carboxylase	Pyruvate carboxylase	Propionyl CoA carboxylase
	μmoles product formed/mg protein·hr		
Low light	0.28	0.48	0.50
High light	0.68	0.47	1.20

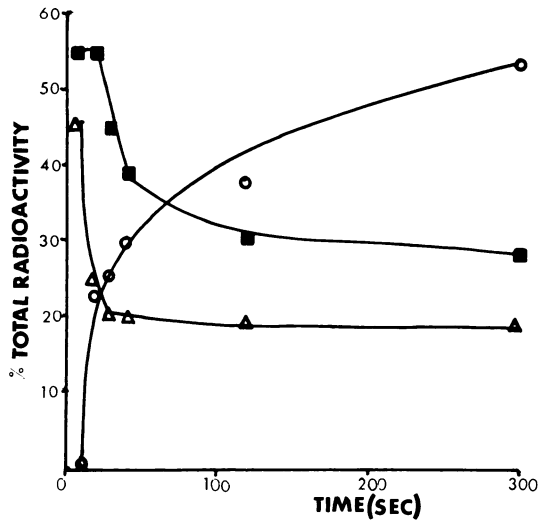

FIG. 1. Kinetics of $NaH^{14}CO_3$ incorporation at low light intensity by cell suspensions of *R. rubrum* grown on acetate at low light intensity. Conditions as in "Materials and Methods." ○: phosphate esters; ■: glutamate; △: citramalate.

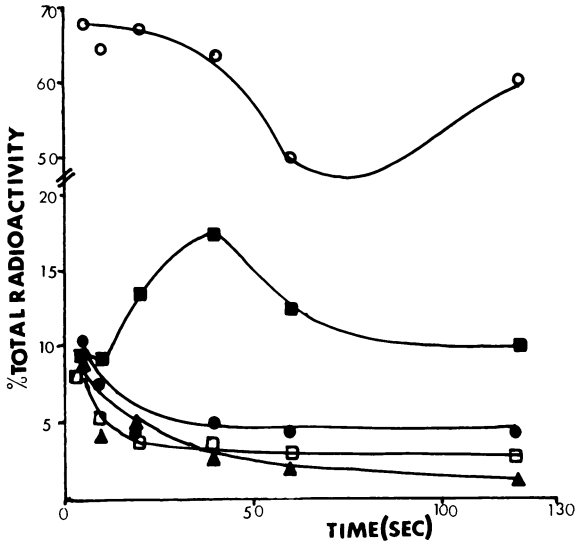

FIG. 2. Kinetics of $NaH^{14}CO_3$ incorporation at high light intensity by cell suspensions of *R. rubrum* grown on acetate at high light intensity. Conditions as in "Materials and Methods." ○: phosphate esters; ●: malate; ■: glutamate; ▲: citrate; □: aspartate.

assimilation. Malate, citrate, and aspartate all rapidly became labeled under these conditions, showing that carbon dioxide was also being incorporated directly at the level of C_4 dicarboxylic acids (Fig. 2). Resting cultures of high light-grown cells incorporated less $^{14}CO_2$ into sugar phosphates compared with growing cells, but incorporation into malate, aspartate, and citrate was altered little (Fig. 3). Total counts incorporated into the soluble fraction of the cells under the various conditions are given in Figure 4.

Labeling of Glutamate. Degradation of glutamate from low light cells gave over 96% of ^{14}C in the C_1 position of glutamate compared with 62% for high light-grown cells (Table III).

DISCUSSION

The products of $^{14}CO_2$ assimilation in *R. rubrum* were regulated by both conditions during growth and the experimental conditions employed during the determination of carbon dioxide incorporation, the major difference being that the contribution of the reductive pentose phosphate cycle relative to the C_4 dicarboxylic acid pathway was decreased in resting cells or

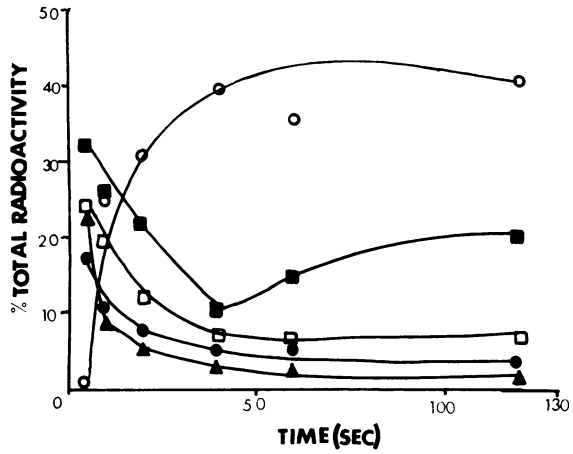

FIG. 3. Kinetics of $NaH^{14}CO_3$ incorporation at high light intensity by stationary phase cells of *R. rubrum* grown on acetate at high light intensity. Experimental conditions as in "Materials and Methods." ○: phosphate esters; □: aspartate; ■: glutamate; ▲: citrate; ●: malate.

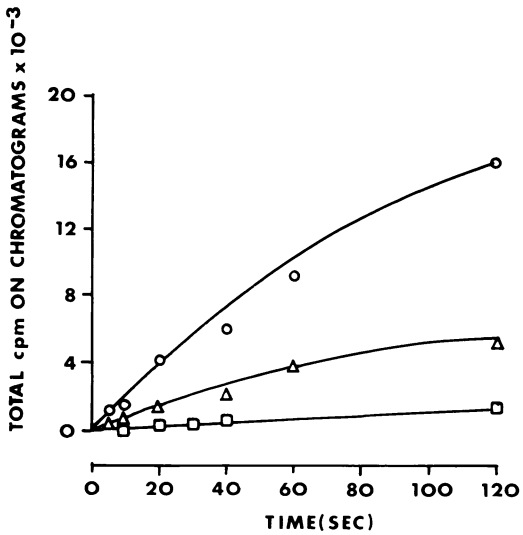

FIG. 4. Uptake of $NaH^{14}CO_3$ by cells of *R. rubrum* under different conditions. Experimental conditions as in "Materials and Methods." ○: high light-grown exponential phase cells; △: high light-grown stationary phase cells; □: low light-grown exponential phase cells.

Table III. *Degradation of ^{14}C-Glutamate Formed during the Assimilation of $NaH^{14}CO_3$ by Cells of R. rubrum Growing Photoheterotrophically on Acetate*

Sample	Glutamate					
	Total dpm	μmoles	Yield of C-1	dpm C-1	dpm C-2 to C-5	Total radioactivity in C-1
			%			%
Low light, exponential phase cells	10,150	28.5	76.3	7470	2750	96.5
High light, exponential phase cells	915	20.2	53.5	270	765	62.8

cells grown at low light intensity. The two enzymes that may lead to the incorporation of carbon dioxide directly into C_4 dicarboxylic acids are pyruvate carboxylase and propionyl CoA carboxylase (Table II). A mutant of *R. spheroides* lacking pyruvate carboxylase was still able to grow on acetate in the light (16), so the absolute requirement for carbon dioxide during photoheterotrophic growth on acetate is not for pyruvate carboxylation. Pulse-labeling high light-grown cells with $^{14}CO_2$ resulted in glutamate, citrate, aspartate, and malate all being labeled after 20 sec (Fig. 2), whereas only glutamate was labeled in low light-grown cells (Fig. 1). The specific activity of pyruvate carboxylase was unaffected by light intensity but the specific activity of propionyl CoA carboxylase was increased in cells grown at high light intensity (Table II), as also occurs with other enzymes metabolizing C_4 dicarboxylic acids (19). The increased specific activity of propionyl CoA carboxylase would explain the increased $^{14}CO_2$ incorporation into malate, citrate, and aspartate at high light intensity and further show that propionyl CoA carboxylase activity regulates $^{14}CO_2$ incorporation at the C_4 dicarboxylic acid level. Propionate has been shown to function as an endogenous carbon dioxide acceptor in *R. rubrum* grown photoheterotrophically on acetate (15).

The carboxylation of pyruvate with $^{14}CO_2$ and subsequent glutamate formation via the tricarboxylic acid cycle gives glutamate labeled exclusively in the C_1 position. The further metabolism of propionyl CoA via methylmalonyl CoA and succinate would give oxaloacetate labeled exclusively in the carboxyl carbon atoms. One ^{14}C would be lost during the decarboxylation of isocitrate, and the conversion of α-ketoglutarate to glutamate would result in the other ^{14}C exclusively in the C_1 position of glutamate. Thus, the proportion of the total ^{14}C present in the C_1 of glutamate represents the contribution of reactions catalyzed by propionyl CoA and pyruvate carboxylases to over-all carbon dioxide assimilation. Degradation of glutamate labeled during $^{14}CO_2$ kinetic experiments at low light intensity showed that the bulk of ^{14}C was in the C_1 position of glutamate (Table III), whereas with experiments at high light intensity more of the total ^{14}C was in carbon atoms C_2 to C_5 of glutamate (Table III). Thus, virtually all the ^{14}C incorporated into glutamate at low light intensity results from propionyl CoA carboxylase activity or β-carboxylation reactions, whereas at high light intensity other reactions are important. The other pathway by which ^{14}C could rapidly enter carbon atoms 2 and 3 of oxaloacetate is fixation by the reductive pentose phosphate cycle followed by metabolism of the products via the glycolytic pathway. Increased specific activity of ribulose-1,5-diP carboxylase, rapid incorporation of carbon dioxide via the reductive pentose phosphate cycle, and degradation of labeled glutamate all support an influx of ^{14}C fixed via the reductive pentose phosphate cycle into tricarboxylic acid cycle intermediates under these conditions.

Besides increased specific activity of ribulose-1,5diP carboxylase in high light-grown cells, it may be argued that at high light intensities ATP levels would be increased, so favoring regeneration of ribulose-1,5-diP. However, in resting cells, *i.e.*, not assimilating acetate, at high light incorporation of $^{14}CO_2$ into reductive pentose phosphate cycle intermediates resembled exponential phase cells at low light, showing that the primary effect of light was not on photosynthetic phosphorylation. Thus both high light intensity and acetate assimilation are required to give greatest growth rate (Table II) and this results from an active reductive pentose phosphate cycle and CO_2 incorporation via C_4 acids.

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