Formation of Enzymes of Autotrophic Metabolism During Heterotrophic Growth of *Alcaligenes eutrophus*

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Alcaligenes eutrophus strain H16 formed key enzymes of autotrophic metabolism during heterotrophic growth. The formation of the soluble and membrane-bound hydrogenases, ribulose-5-phosphate kinase and ribulosebisphosphate carboxylase were investigated. In addition, selected enzymes shared by autotrophic and heterotrophic carbon metabolism were examined. Key enzymes of autotrophic metabolism were not detected during exponential growth on succinate, pyruvate or acetate, but were found at intermediate activities in cells grown on fructose, gluconate or citrate. Growth with succinate at a suboptimal pH of 7.7 resulted in a decreased growth rate and a marked increase of enzyme activities. Oxygen-limited growth with succinate also led to a derepression of the synthesis of the hydrogenases and the key enzymes of the Calvin cycle. During growth on glycerol or formate, the activities of these enzymes were comparable with those found under autotrophic conditions with H₂ and CO₂.

The results indicate that both the hydrogenases and the key enzymes of the Calvin cycle were formed under conditions of limited availability of energy. Molecular hydrogen was not required for the formation of the hydrogenases. The regulation of the gluconeogenic enzymes, common to both autotrophic and heterotrophic carbon metabolism, was more balanced. An increase of enzyme activities was observed under autotrophic conditions, in accord with the physiological role of these enzymes under autotrophic and heterotrophic growth conditions.

INTRODUCTION

Alcaligenes eutrophus is an obligate aerobic, facultative chemolithoautotrophic micro-organism. During autotrophic growth on carbon dioxide and hydrogen, the latter substrate is activated by a soluble NAD⁺-linked hydrogenase (EC 1.12.1.2) and a membrane-bound hydrogenase unable to reduce pyridine nucleotides. Carbon dioxide is fixed via the Calvin cycle; its initial reactions are catalysed by ribulose-5-phosphate kinase (EC 2.7.1.19) and ribulosebisphosphate carboxylase (EC 4.1.1.39).

There have been several reports on the appearance of the autotrophic key enzymes in hydrogen-oxidizing bacteria during heterotrophic growth. Schatz & Bovell (1952) found 5 to 25% of the autotrophic hydrogen uptake rate with whole cells of *Hydrogenomonas facilis* grown on glucose, tryptose or lactate. This result could not be repeated by Wilson *et al.* (1953) who found complete repression of hydrogen uptake in cells grown heterotrophically on the above substrates. However, Wilson *et al.* (1953) reported that significant amounts of hydrogenase were present during growth on lactate provided the partial pressure of oxygen was reduced to 5% (5 kPa). Wilde (1962) applied these conditions to *Alcaligenes eutrophus* strain H16 (formerly *Hydrogenomonas eutropha* strain H16) but found no derepression of

hydrogenase activity. However, she observed high hydrogenase activities in cells of *A. eutrophus* grown on glutamate, pyruvate, succinate, malate or acetate (Wilde, 1962). With pyruvate as carbon source a similar result was obtained by Schink & Schlegel (1978). In contrast, Eberhardt (1966) did not find hydrogenase activity in succinate-grown cells, but he found intermediate activities in cells grown on fructose, as also reported by Rittenberg (1969) and Friedrich *et al.* (1979).

During growth of *A. eutrophus* on fructose, ribulosebiphosphate carboxylase was also present at intermediate activities, as reported by Kuehn & McFadden (1968), Rittenberg (1969), and Stukus & DeCicco (1970), but no activity was detected during growth on succinate (Rittenberg, 1969) or pyruvate (Stukus & DeCicco, 1970).

To extend the limited knowledge on the formation of the key enzymes of the Calvin cycle and in view of the contradictory results concerning the formation of hydrogenase, we have re-examined the influence of growth conditions on the formation of key enzymes of autotrophic metabolism and selected enzymes of the central carbon metabolism. We have studied the effects of growth rate, aeration, time of harvest, treatment of the cells after harvest, and assay conditions of the enzymes. The present study defines the conditions under which the key enzymes of autotrophic metabolism are formed or are not formed. These results are a necessary basis for investigating the regulatory mechanisms that enable *A. eutrophus* to grow autotrophically.

METHODS

Organism. *Alcaligenes eutrophus* strain H16 (ATCC 17699, DSM 428) was used throughout this study.

Media and growth conditions. The medium of Schlegel *et al.* (1961) was used for precultures and cultures up to volumes of 300 ml for autotrophic and heterotrophic growth in 1 l baffled Erlenmeyer flasks. For enzyme studies, cells were precultivated in 10 ml medium in 100 ml Erlenmeyer flasks, then transferred to 250 ml medium. When this culture reached about 1 g dry wt cells l⁻¹ it was used as inoculum for 10 l medium in a 12 l fermenter (B. Braun, Melsungen, F.R.G.). When cultures were grown autotrophically with H₂ and CO₂ or with organic acids, conditions were as described by Friedrich *et al.* (1979). With fructose or glycerol as carbon and energy source the extended culture technique was used in the following way. Acidification of the medium due to ammonia uptake was used to titrate a 1.0 M-NaOH solution containing 1.0 M-fructose or 1.76 M-glycerol. The ratio of carbon source to NaOH was based on the average nitrogen content of *A. eutrophus* [17.6 g N (100 g dry wt cells)⁻¹] and the yield coefficient of $Y_{\text{fructose}} = 0.45$ and $Y_{\text{glycerol}} = 0.50$. The fermenter was equipped with a steam sterilizable oxygen electrode (type G2L3; Biolaftite, Stuttgart, F.R.G.). The dissolved O₂ partial pressure was recorded and not allowed to drop below 10 kPa (0.1 atm) during growth on organic substrates and was kept between 2 and 8 kPa during autotrophic growth with H₂ and CO₂. The O₂ content of the exit gas of the fermenter was determined with a paramagnetic O₂ analyser (Magnos 2T; Hartmann & Braun, Frankfurt, F.R.G.), and the exit CO₂ concentration with an infrared analyser (Uras 7N). The results were recorded with a two-channel recorder.

Growth was followed turbidimetrically at 436 nm. O₂-limited growth was achieved by decreasing the O₂ transfer rate by reducing the air flow rate from 4 to 1.5 l min⁻¹ and reducing the impeller rotation rate from 600 to 260 rev. min⁻¹.

The substrate shift from pyruvate to formate was done as described by Friedrich *et al.* (1979).

About 1 g wet wt cells was needed to obtain sufficient quantities of particulate hydrogenase and the membrane-bound cytochromes. Samples of 100 to 300 ml culture were taken and immediately cooled to 0 °C; the bacteria were collected by centrifugation (Christ Junior 2, Osterode/Harz, F.R.G.) for 15 min at 5000 rev. min⁻¹, washed once with ice-cold 0.9% (w/v) NaCl and stored frozen at -20 °C. Cell-free extracts and membrane fractions were prepared at 0 °C according to the procedure described by Friedrich *et al.* (1979).

Enzyme assays. Enzyme activities were determined at 30 °C and expressed as units (mg protein)⁻¹. One unit (U) of enzyme activity was defined as the utilization of 1 μmol substrate min⁻¹. The soluble hydrogenase (hydrogen:NAD⁺ oxidoreductase; EC 1.12.1.2), particulate hydrogenase and ribulose-5-phosphate kinase (EC 2.7.1.19) activities were determined as described by Friedrich *et al.* (1979). Ribulosebiphosphate carboxylase (EC 4.1.1.39) activity was measured by ribulose biphosphate-dependent incorporation of ¹⁴CO₂ into acid-stable product as described by Bowien *et al.* (1976). The following enzyme activities were measured spectrophotometrically at 340 nm. Fructose-1,6-bisphosphatase (EC 3.1.3.11) was assayed according to Abdelal & Schlegel (1974). Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13) was determined in a reaction mixture containing (total vol. 0.60 ml) 50 mM-Tris/HCl buffer (pH 7.6), 1 mM-Fe(NH₄)₂SO₄, 1 mM-cysteine hydro-

chloride, 1 U glycerol-3-phosphate dehydrogenase (EC 1.1.1.8), 3 U triosephosphate isomerase (EC 5.3.1.1), 0.25 mM-NADH, 2 mM-fructose-1,6-bisphosphate and an appropriate amount of enzyme. Assay mixtures were incubated anaerobically by sparging the cuvettes with pure nitrogen, thus avoiding the oxidation of ferrous iron. 3-Phosphoglycerate kinase (EC 2.7.2.3) was assayed in a mixture containing (total vol. 1.0 ml) 80 mM-Tris/HCl buffer (pH 7.4), 5.5 mM-MgSO₄, 0.4 mM-EDTA, 10 U glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), 0.25 mM-NADH, 2.5 mM-ATP, 8 mM-3-phosphoglycerate and enzyme. Glyceraldehyde-3-phosphate dehydrogenase activity was determined in an identical system except that 10 U 3-phosphoglycerate kinase was added instead of glyceraldehyde-3-phosphate dehydrogenase.

Cytochrome identification. Quantitative determination of membrane-bound cytochromes *aa*, *b* and *c* as well as soluble cytochrome *c* was done as described by Probst & Schlegel (1976). The soluble flavohaemoprotein was determined as described by Probst *et al.* (1979).

Immunochemical analysis. Antisera against purified ribulosebiphosphate carboxylase and soluble hydrogenase were prepared as previously described (Bowien & Mayer, 1978; Schink & Schlegel, 1980). The presence of ribulosebiphosphate carboxylase and hydrogenase antigen in cell extracts was determined by immunodiffusion according to Ouchterlony (1953).

Protein was determined by the Lowry method.

RESULTS

Enzyme activities during different growth phases in batch culture

The activities of enzymes of autotrophic metabolism in *Alcaligenes eutrophus* were followed during autotrophic and heterotrophic growth in batch culture. Ammonia was chosen to restrict the final amount of growth in order to allow conditions comparable to those of autotrophic growth where the gaseous substrates have to be continuously transferred to the medium. In order to supply the substrate continuously to heterotrophically grown cultures the extended culture technique was chosen to keep the organic substrate concentration relatively constant. Growth ceased at about 3 g dry wt cells l⁻¹ due to complete exhaustion of ammonia in the medium. The surplus of the carbon source, however, allowed an increase in cell mass due to the formation of intracellular poly- β -hydroxybutyrate (results not shown).

Figure 1 shows the growth of *A. eutrophus* with glycerol as carbon and energy source and the enzyme activities determined. It is evident that in this heterotrophic culture both the

Fig. 1. Enzyme activities during growth of *Alcaligenes eutrophus* on glycerol. Cells were grown in a 121 fermenter (see Methods) with 0.2% (w/v) NH₄Cl and 0.5% (w/v) glycerol. Glycerol was added continuously according to growth and consumption. Appropriate culture samples were taken to yield about 1 g wet wt and processed as described in Methods. Turbidity (○), soluble hydrogenase (●), membrane-bound hydrogenase (□), ribulose-5-phosphate kinase (■), ribulosebiphosphate carboxylase (△), glyceraldehyde-3-phosphate dehydrogenase (▲), fructose-1,6-bisphosphate aldolase (▽).

Table 1. *Specific activities of enzymes of autotrophic metabolism of Alcaligenes eutrophus grown on various carbon sources*

Carbon source	Specific growth rate (h ⁻¹)	Specific activity [U (mg protein) ⁻¹]			
		Hydrogenase		Ribulose-5-phosphate kinase	Ribulose-bisphosphate carboxylase
		Soluble	Particulate		
CO ₂ /H ₂	0.22	0.916	0.387	0.210	0.258
Formate	0.17	0.480	0.343	0.268	0.264
Glycerol	0.14	0.518	0.290	0.242	0.216
Fructose	0.33	0.190	0.065	0.045	0.036
Gluconate	0.33	0.189	0.047	0.017	0.061
Citrate	0.38	0.068	0.005	0.034	0.026
Pyruvate	0.46	0.015	0.005	0.001	0.001
Succinate	0.46	0.010	0.002	0.001	0.003
Acetate	0.46	0.005	0.002	0.001	0.001

soluble NAD⁺-linked and the membrane-bound hydrogenases were produced at activities up to 0.6 U (mg protein)⁻¹ (Fig. 1*b*), which were similar to the activities found in autotrophically grown bacteria. However, the specific activity of the hydrogenase did not remain constant during the exponential growth phase but decreased gradually. A similar decrease of both hydrogenase activities was observed during autotrophic growth with H₂ and CO₂ but not during heterotrophic growth with fructose.

The activities of ribulose-5-phosphate kinase and ribulosebisphosphate carboxylase (Fig. 1*c*) were similar to those found in bacteria grown autotrophically with either H₂ and CO₂ or formate as carbon and energy source (Friedrich *et al.*, 1979). Again, there was a slight decrease in activity as the bacterial mass increased with time. Ribulosebisphosphate carboxylase was not rapidly inactivated when the culture reached the stationary growth phase caused by depletion of ammonia from the medium. Kuehn & McFadden (1968) reported a rapid inactivation of the enzyme when the carbon source fructose was exhausted. Activities of representative enzymes of gluconeogenesis and glycolysis – fructose-1,6-bisphosphate aldolase and glyceraldehyde-3-phosphate dehydrogenase – did not vary significantly during growth (Fig. 1*d*). This was also observed when cells were grown autotrophically with H₂ and CO₂ or heterotrophically with fructose or pyruvate.

For further investigations, bacteria were harvested in the mid-exponential growth phase when the biomass was approximately 1 g dry wt cells l⁻¹. This enabled a comparison to be made between the enzyme activities with different carbon and energy sources.

Enzyme activities during growth on different substrates

During heterotrophic growth the key enzymes of autotrophic metabolism exhibited specific activities which depended on the carbon substrate (Table 1). On glycerol the enzyme activities were as high as those found when cultures were grown autotrophically with H₂ and CO₂. *Alcaligenes eutrophus* also grows autotrophically with formate where ribulose-5-phosphate kinase and ribulosebisphosphate carboxylase are essential for growth, but the hydrogenases are not. The latter enzymes, however, were present at high activities during growth on formate (Table 1) (see also Friedrich *et al.*, 1979).

On substrates such as fructose, gluconate or citrate, only low activities of the hydrogenases were observed. With succinate, pyruvate or acetate only trace activities of the key enzymes of autotrophic metabolism were detected.

Effect of growth rate

The growth rates and activities of the key enzymes of autotrophic metabolism during growth on different substrates are listed in Table 1. The activities of the enzymes were

Table 2. *Specific activities of enzymes of autotrophic metabolism during optimal and suboptimal growth of Alcaligenes eutrophus*

Alcaligenes eutrophus was grown in a 12 l fermenter and samples were taken during exponential growth and processed as described in Methods.

Growth conditions			Specific growth rate (h ⁻¹)	Specific activity [U (mg protein) ⁻¹]			
Carbon source	Temp. (°C)	pH		Hydrogenase		Ribulose-5-phosphate kinase	Ribulose-bisphosphate carboxylase
				Soluble	Particulate		
Succinate	30	7.0	0.46	0.005	0.005	0.002	0.003
Succinate	17	7.0	0.15	0.044	0.002	0.002	0.001
Succinate	30	7.7	0.07	0.612	0.002	0.082	0.058
Fructose	30	7.0	0.32	0.108	0.030	0.077	0.041
Fructose	30	7.7	0.13	0.124	0.004	0.022	0.017

Fig. 2. Enzyme activities of *Alcaligenes eutrophus* during oxygen-limited growth on succinate. Turbidity (○), oxygen partial pressure (●), membrane-bound hydrogenase (□), soluble hydrogenase (■), ribulosebisphosphate carboxylase (Δ), ribulose-5-phosphate kinase (▲).

apparently inversely correlated with the growth rates. In order to distinguish between a substrate-specific and a growth rate-specific effect, two substrates were chosen and the growth rates were decreased by applying suboptimal growth conditions.

Alcaligenes eutrophus was grown with succinate as carbon source at 17 °C giving a specific growth rate of 0.15 h⁻¹ compared with 0.46 h⁻¹ at 30 °C. At 17 °C the hydrogenase activity was 5 to 7% of that of autotrophically grown cells (Table 2). In a different experiment at 30 °C, at a constant pH of 7.7, the specific growth rate was 0.07 h⁻¹. Under these conditions, the soluble hydrogenase was present at a high activity and the key enzymes of the Calvin cycle were found at intermediate activity.

Since a heterotrophic substrate supplies the cell with carbon and energy it was of interest to know if the decrease in growth rate and the concomitant increase in activities of the key enzymes of autotrophic metabolism were due to carbon or energy limitation. Energy limitation can be achieved by limiting the supply of either the electron donor or the electron acceptor. Under heterotrophic growth conditions the choice was restricted to the limitation of the electron acceptor – oxygen. Figure 2 shows the growth of *A. eutrophus* on succinate in extended culture with oxygen as the growth-limiting substrate. Under this condition, the

Fig. 3. Rate of formation of the key enzymes of autotrophic metabolism after a substrate shift from pyruvate to formate. The substrate shift and determination of enzyme activity were done as described in Methods. Formate was added at zero time. Ribulosebiphosphate carboxylase (○), soluble hydrogenase (●), ribulosebiphosphate carboxylase in the presence of $200 \mu\text{g}$ chloramphenicol ml^{-1} (□), soluble hydrogenase in the presence of $200 \mu\text{g}$ chloramphenicol ml^{-1} (■).

culture did not grow exponentially but arithmetically because of the constant oxygen transfer rate within the fermenter; thus the specific growth rate continuously decreased. After the onset of oxygen limitation, the activity of the soluble hydrogenase significantly increased as, after a short delay, did the activity of the particulate hydrogenase. Both enzymes reached similar activities to those in autotrophically grown cells within the duration of the experiment. Ribulose-5-phosphate kinase and ribulosebiphosphate carboxylase were also produced, but at only 30% of the activity in autotrophically grown cells.

Coordination of enzymes of autotrophic energy and carbon metabolism

As has been shown before (Friedrich *et al.*, 1979), ribulose-5-phosphate kinase and ribulosebiphosphate carboxylase appeared to be synthesized coordinately. Neither enzyme was present in pyruvate-grown cells (Table 1).

The appearance of the energy-generating and carbon-assimilating enzymes during oxygen-limited growth indicated that the formation of the hydrogenases and the carbon dioxide-assimilating enzymes was not strictly coordinate (Fig. 2). The activities of two indicator enzymes of autotrophic metabolism – ribulosebiphosphate carboxylase and the soluble hydrogenase – were therefore followed in a transition experiment. Pyruvate-grown cells were shifted down to formate as sole source of carbon and energy (Fig. 3). Only 30 min after the addition of formate to the medium, ribulosebiphosphate carboxylase activity was detected and thereafter increased at a constant rate. The soluble hydrogenase was formed after a lag of more than 2 h. This result was further support for the non-coordinate control of the carbon-assimilating and energy-generating enzymes.

Immunochemical analysis of enzyme formation

Double immunodiffusion assays were performed with crude extracts from cells grown on succinate, pyruvate, fructose and glycerol or under autotrophic conditions with H_2 and CO_2 . Antisera against two representative enzymes of autotrophic metabolism – ribulosebiphosphate carboxylase and the soluble NAD^+ -linked hydrogenase – showed cross-reactions with cell extracts except those derived from cells grown on succinate or pyruvate. Thus, the enzyme activities determined truly reflect the presence or absence of the two indicator proteins.

Table 3. *Specific activities of some gluconeogenetic enzymes of Alcaligenes eutrophus grown on various carbon sources*

Growth conditions and extract preparations were identical to those of Table 1.

Carbon source	Specific activity [U (mg protein) ⁻¹]			
	Fructose-1,6-bisphosphatase	Fructose-1,6-bisphosphate aldolase	Glyceraldehyde-3-phosphate dehydrogenase	3-Phosphoglycerate kinase
CO ₂ /H ₂	0.268	0.172	2.43	1.36
Formate	0.186	0.201	1.20	1.19
Glycerol	0.177	0.198	2.54	1.87
Fructose	0.154	0.175	1.77	1.37
Citrate	0.032	0.113	0.755	0.824
Pyruvate	0.051	0.177	1.74	1.31
Succinate	0.059	0.114	0.649	0.788

Activities of selected enzymes of the central carbon metabolism

Fructose-1,6-bisphosphatase, fructose-1,6-bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase function in the Calvin cycle during autotrophic growth and also in the gluconeogenetic and glycolytic pathways during heterotrophic growth. Therefore, the influence of different substrates on the activities of these enzymes was examined (Table 3). Although the magnitude of the variations in their activities was not as pronounced as observed for the enzymes of autotrophic metabolism, the overall pattern was similar (Table 1). The enzymes showed highest activities during autotrophic growth with H₂ and CO₂ or heterotrophic growth with glycerol. With formate, fructose, gluconate or pyruvate the enzymes were also present at a relatively high activity. Bacteria grown on succinate, acetate or citrate showed significantly lower activities, probably representing the basal activity of the enzymes. Although there was some variation in the enzyme activities in different experiments, the results for individual experiments always exhibited the activity pattern described above. This is compatible with the different physiological role of these enzymes under autotrophic and heterotrophic growth conditions. While all carbon must flow through these essential Calvin cycle activities during autotrophic growth, only that fraction of carbon necessary for cell wall synthesis has to pass this route in cells growing heterotrophically on gluconeogenetic substrates, such as succinate. It has recently been shown that the gluconeogenetic carbon requirement of *Escherichia coli* is about 5 % of its total catabolic and anabolic needs (Irani & Maitra, 1977).

Cytochrome content

No qualitative difference was found in either the membrane-bound cytochromes *aa*₃, *b* or *c* or the cytoplasmic cytochrome *c* or the oxygen-binding flavohaemoprotein in cells grown under autotrophic or heterotrophic conditions, thus confirming the results of Probst & Schlegel (1976). There was also no significant quantitative difference in the content of membrane-bound cytochromes under the different growth conditions (results not shown). The concentration of soluble flavohaemoprotein, however, was about fivefold higher in autotrophically grown cells than in those grown heterotrophically (Table 4). The reason for this observation is not known. It was suggested (Probst *et al.*, 1979) that the soluble flavohaemoprotein, because of its high affinity to oxygen, functions as an oxygen store providing a constant oxygen tension to the cell. In fact, during autotrophic growth either with formate or H₂ and CO₂ the oxygen demand is about six- to eightfold higher than during heterotrophic growth on, for example, fructose or pyruvate.

Table 4. *Cytoplasmic cytochrome content of Alcaligenes eutrophus grown on various carbon sources*

Cytochrome *c* was identified and quantified as described by Probst & Schlegel (1976) and flavohaemoprotein as described by Probst *et al.* (1979).

Carbon source	Cytochrome content [$\mu\text{mol (g protein)}^{-1}$]	
	Flavohaemoprotein	Cytochrome <i>c</i>
CO ₂ /H ₂	0.035	0.270
Formate	0.038	0.145
Pyruvate	0.009	0.152
Fructose	0.006	0.150

DISCUSSION

The results presented in this paper provide evidence that *Alcaligenes eutrophus* strain H16 produces the key enzymes of autotrophic energy generation and CO₂ fixation during growth on different organic substrates. Three groups of substrates could be distinguished: (i) with formate or glycerol these enzymes were formed at activities comparable with those found under autotrophic growth conditions with H₂ and CO₂ as sources of energy and carbon; (ii) fructose, gluconate or citrate allowed the production of intermediate enzyme activities up to 20% of the autotrophic values; (iii) during growth on pyruvate, succinate or acetate the formation of the enzymes was completely repressed. These results are in accord with those of a previous study (Stukus & DeCicco, 1970) with the *A. eutrophus* type strain, which demonstrated the total repression of ribulosebiphosphate carboxylase by pyruvate or acetate and a partial repression of this enzyme by fructose or glutamate. The fact that the soluble as well as the membrane-bound hydrogenase was present in heterotrophically grown cells suggested that the synthesis of these enzymes was regulated by a repression/derepression mechanism at the transcriptional level. Since no H₂ was produced heterotrophically under the experimental conditions employed (unpublished result), molecular hydrogen is not considered as an inducer of the hydrogenase system. At present, no conclusion can be drawn regarding the regulation of the CO₂-fixing system. CO₂ cannot be excluded as inducer since it is always present in the cell. However, repression/derepression has been suggested as a control mechanism for the synthesis of ribulosebiphosphate carboxylase in *Pseudomonas oxalaticus* (Dijkhuizen & Harder, 1979).

The growth rate supported by a particular substrate depends on its quality either as carbon or as energy source. For *A. eutrophus* there was an inverse relationship between the specific growth rate on different substrates and the specific activity of the key enzymes of autotrophic metabolism. The substrates which supported fast growth – pyruvate, succinate or acetate – exerted a severe repression on the synthesis of these enzymes. These results resemble those reported for *P. oxalaticus* whose CO₂-fixing enzyme system is also repressed by substrates that support fast growth (Blackmore *et al.*, 1968; Dijkhuizen *et al.*, 1978). However, our results indicate that the autotrophic enzymes of *A. eutrophus* are not simply formed in response to the growth rate. A decrease in the growth rate on succinate caused by a decrease in temperature to 17 °C, or on fructose at pH 7.7, did not result in high enzyme activities. On the other hand, a decrease in the growth rate on succinate caused by limitation of the electron acceptor oxygen led to the formation of the autotrophic enzymes, as did growth on succinate at pH 7.7 when the rate of uptake of this substrate was presumably restricted. Therefore, a limited supply of energy rather than of carbon appears to be responsible for the formation of the key enzymes of autotrophic metabolism in *A. eutrophus*. This conclusion is consistent with earlier observations of Kuehn & McFadden (1968) who found that ribulose-biphosphate carboxylase was present at high concentrations in cells of the *A. eutrophus* type

strain and *Pseudomonas facilis* grown on fructose with a low aeration rate. Furthermore, hydrogen uptake was markedly increased in *P. facilis* grown on lactate under oxygen starvation (Wilson *et al.*, 1953).

The physiological and enzymic results indicate an apparent correlation between the formation of enzymes of H_2 oxidation and CO_2 assimilation. However, since nothing is known about their genetic interrelationship it is too early to speculate about a coordination of enzyme synthesis on a molecular basis. Further studies with mutants altered in the regulation of the respective enzymes should elucidate this question.

The immunochemical analysis showed that the various activities of the autotrophic enzymes correlated with the respective enzyme proteins present in the cells. In addition, substrate shift-down experiments from pyruvate to formate in the presence of chloramphenicol confirmed the assumption that new protein synthesis is primarily involved in the regulation of the autotrophic enzymes. Similarly, Rittenberg (1969) found that the *A. eutrophus* type strain showed a marked increase in specific activity of hydrogenase at the end of the exponential growth phase when cultured on a variety of organic substrates. The increase was not observed if cultures were treated with chloramphenicol before reaching the stationary phase.

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