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Derepression of Hydrogenase During Limitation of Electron Donors and Derepression of Ribulosebisphosphate Carboxylase During Carbon Limitation of *Alcaligenes eutrophus*

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Alcaligenes eutrophus did not form the key enzymes of autotrophic metabolism, the soluble and particulate hydrogenases and ribulosebisphosphate carboxylase (RuBPC), during heterotrophic growth on succinate in batch cultures. During succinate-limited growth in a chemostat, high activities of both hydrogenases were observed. With decreasing dilution rate (D) the steady-state hydrogenase activity (H) followed first-order kinetics, expressed as follows: $H = H_{max} \cdot e^{-\alpha \cdot D}$. An identical correlation was observed when autotrophic growth in a chemostat was limited by molecular hydrogen. During autotrophic growth under oxygen or carbon dioxide limitation, the activity of the soluble hydrogenase was low. These data suggested that hydrogenase formation depended on the availability of reducing equivalents to the cells. RuBPC activities were not correlated with the hydrogenase activities. During succinate-limited growth, RuBPC appeared at intermediate activities. During autotrophic growth in a carbon dioxide-limited chemostat, RuBPC was highly derepressed. RuBPC activity was not detected in cells that suffered from energy limitation with a surplus of carbon, as in a heterotrophic oxygen-limited chemostat, nor was it detected in cells limited in carbon and energy, as in the case of complete exhaustion of a heterotrophic substrate. From these data I concluded that RuBPC formation in *A. eutrophus* depends on two conditions, namely, carbon starvation and an excess of reducing equivalents.

The aerobic hydrogen-oxidizing bacteria are facultative chemolithoautotrophs. These bacteria are able to grow on a number of organic substrates, as well as with hydrogen as the electron donor and carbon dioxide as the carbon source. The mechanisms of the responses of these cells to autotrophic growth conditions, which result in the formation of the key enzymes of autotrophic metabolism, are not known. In *Alcaligenes eutrophus* the soluble and membrane-bound hydrogenases are formed in the presence of molecular hydrogen. Also, both of these hydrogenases and the key enzymes of autotrophic carbon dioxide fixation, namely, ribulosebisphosphate carboxylase (RuBPC) and phosphoribulokinase, occur in cells grown heterotrophically in batch cultures (9, 17, 18). High activities of these enzymes have been found in cells grown with glycerol, and intermediate activities have been found in cells grown with fructose or citrate; however, no activity has been found in cells grown with pyruvate or succinate as the growth substrate. Nevertheless, when the uptake of succinate was restricted, the key enzymes of autotrophic metabolism were formed (9). A heterotrophic substrate has two functions (to serve as a carbon source and to serve as an energy source), and the ability of a substrate to serve either function may have accounted for the observed activities. Therefore, this study was performed to define the growth conditions that allowed the preferential function of either carbon metabolism or energy metabolism. This was achieved in chemostat cultures by using limitations of various nutrients.

The principal finding of this investigation was that the hydrogenases of *A. eutrophus* were formed during limited availability of the electron donor and that RuBPC was formed during carbon starvation with an excess energy supply.

MATERIALS AND METHODS

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

Media. Mineral salts media (8) were used for all experiments. For culture volumes up to 300 ml, each component was heat sterilized separately, whereas

larger volumes were sterilized by filtration through type EKS filters (diameter, 25 cm; Seitz, Bad Kreuznach, Federal Republic of Germany). Reservoir media for heterotrophic continuous cultivation and autotrophic continuous cultivation were adjusted to pH 3.5 with 12 M phosphoric acid before filter sterilization. The culture medium was kept at pH 7.0 by using 5 M sodium hydroxide. For succinate limitation the medium contained 0.4% succinic acid and 0.2% ammonium chloride; for heterotrophic oxygen limitation the medium contained 0.8% succinic acid and 0.2% ammonium chloride; and for ammonium limitation the medium contained 0.5% succinic acid and 0.05% ammonium chloride. The other components of the media have been described previously (8).

Chemostat studies. For heterotrophic growth in continuous cultures, a 2-liter Microferm fermentor (New Brunswick Scientific Co., New Brunswick, N.J.) with a working volume of 950 ml was used as the culture vessel. Such cultures were stirred at 500 rpm, aerated at a rate of 380 ml/min, and kept at 30°C. Autotrophic cultures were grown in a 2-liter Biostat fermentor (B. Braun, Melsungen, Federal Republic of Germany) with a culture volume of 970 ml. Cultures in this fermentor were stirred at 500 rpm and temperature controlled (30°C); they were gassed with 82% hydrogen–10% oxygen–8% carbon dioxide (by volume) at a rate of 375 ml/min. The gas mixture was made by using precision piston pumps (H. Wösthoff, Bochum, Federal Republic of Germany). The oxygen partial pressure in the medium was determined with a polarographic oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) that was sterilized chemically with Incidin (Henkel, Düsseldorf, Federal Republic of Germany). The oxygen tension in the culture did not drop below 4 kPa during hydrogen- or carbon dioxide-limited growth. The critical low oxygen partial pressure during autotrophic growth in a chemostat was determined to be 1.5 kPa (7). The gas consumption rate (in liters per hour) during autotrophic growth was determined by using the following equation:

$$\text{gas}_{\text{in}} - \text{gas}_{\text{out}} = \text{gas}_{\text{consumed}} \quad (1)$$

The gas flow rate was determined from the volume of gas that passed the gas meter (Elster & Co., Mainz, Federal Republic of Germany) in 10 min and was expressed as liters per hour. Both heterotrophic and autotrophic chemostat cultures were pH controlled by titrating 5 N sodium hydroxide with a pH stat (Radiometer, Copenhagen, Denmark). The medium was supplied with a peristaltic pump (model Varioperpex 12000; LKB, Bromma, Sweden). Continuous cultures were started from batch cultures in early stationary phase. Growth was followed by measuring the absorbance of the culture; 1 g of dry cell weight per liter corresponded to an absorbance at 436 nm of 4.8, except during ammonia limitation, when 1 g of dry cell weight corresponded to an absorbance at 436 nm of 6.0. The steady state of a culture was reached when the optical density of the culture and the specific activity of hydrogenase remained constant with time, usually after three to five volume changes. To measure the activity of the membrane-bound hydrogenase, relatively large volumes (200 ml) had to be collected. To keep the dilution rate constant after sampling, the necessary volume was not withdrawn from the culture vessel, but the effluent was bypassed and collected in a separate bottle with magnetic stirring and cooling at 0°C. Cells were harvested by centrifugation at 0°C, washed once with ice-cold 0.9% NaCl, and kept at −20°C. The maintenance requirements (m) were calculated from the following equation, as derived by Pirt (16):

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_{\max}} \quad (2)$$

The symbols in this and subsequent equations are described in Table 1. During the steady state in a chemostat,

$$\mu = D = \frac{F}{V} \quad (3)$$

Gas limitation. In a gas-limited chemostat, the steady-state cell concentration (X) (in grams of dry cell weight) was given by the mass transfer rate (MTR_g) of the fermentor and the cellular yield with respect to the gaseous substrate. Therefore, at a constant mass transfer rate, the productivity ($D \cdot X$) was constant over the dilution rate, as given in the following equation:

$$D \cdot X = Y_g \cdot MTR_g \quad (4)$$

Consequently, the steady-state cell concentration changed according to the mean residence time.

The mass transfer rate of a gas depends on its partial pressure, the gas flow rate, and the impeller rotation rate. Controls to evaluate gas limitation were performed by changing the gas partial pressure at a dilution rate of 0.06 h^{-1}. The changes in the steady-state cell concentrations correlated well with the changes in the partial pressures of the gases (data not shown). For heterotrophic oxygen-limited growth, the impeller rotation rate was reduced from 500 to 260 rpm, and the aeration rate was reduced from 380 to 190 ml/min. Autotrophic growth with a gas limitation was done at 360 rpm with a reduced partial pressure of one of the three gasses. The gas flow rate was kept at 375 ml/min. The atmosphere contained (by volume) 12% H_2, 10% O_2, 8% CO_2, and 70% N_2 during hydrogen limitation; during carbon dioxide limitation the atmosphere contained 88% H_2, 10% O_2, and 2% CO_2, and during oxygen limitation it contained 88% H_2, 4% O_2, and 8% CO_2. In these different experiments the partial pressures of the growth-limiting gases were adjusted so that there would be a ratio of H_2 to O_2 to CO_2 of 6:2:1 in order to achieve comparable cell densities and productivities. Hydrogen bacteria usually utilize hydrogen, oxygen, and carbon dioxide in this ratio (19).

Enzyme assays. Cell-free extracts were obtained with a French pressure cell; the soluble and particulate fractions were prepared as described previously (8). In the crude extracts the soluble hydrogenase (EC 1.12.1.2) was assayed as described by Schneider and Schlegel (21), and the membrane-bound hydrogenase was measured by the method of Schink and Schlegel (18). In whole cells the soluble hydrogenase was assayed by the method of Friedrich et al. (6) and RuBPC (EC 4.1.1.39) was assayed by the method of Bowien et al. (1); when whole cells were used to

TABLE 1. Glossary of symbols

Symbol	Description	Units
D	Dilution rate, equal to flow rate divided by volume	h^{-1}
D_{max}	Maximum dilution rate with a given growth-limiting substrate	h^{-1}
F	Flow rate	liters/h
m	Maintenance coefficient	g of substrate per g of dry cell weight per h
V	Volume	liters
Y	Cellular yield	g of dry cell weight per g of substrate
Y_g	Cellular yield of a gaseous substrate	g of dry cell weight per g of gas
Y_{max}	Maximum yield, extrapolated to $D = 0\ h^{-1}$	g of dry cell weight per g of substrate
μ	Specific growth rate	h^{-1}
H	Hydrogenase specific activity	U/mg of protein
H_{max}	Maximum hydrogenase activity extrapolated to $D = 0\ h^{-1}$	U/mg of protein
MTR_g	Mass transfer rate of a gaseous substrate	g of gas per liter of medium per h
X	Steady-state cell concentration	g of dry cell weight per liter of medium
C_f	Carbon dioxide-fixing activity	U/mg of protein

measure this enzyme, 10 μl of 0.3% (wt/vol) cetyltrimethylammonium bromide was included in the assay (B. Bowien, personal communication); 1 U of enzyme activity was defined as 1 μmol of substrate transformed or 1 μmol of product formed at 30°C per min. Protein (either from whole cells or from cell-free extracts) was determined by the Lowry method. Succinate was determined as described by Dorn et al. (5).

Chemicals. Ribulosebisphosphate (disodium salt) was obtained from Sigma Chemical Co., St. Louis, Mo. NAD was purchased from Boehringer, Mannheim, Federal Republic of Germany. All chemicals were of the highest purity available.

RESULTS

Growth limitation under heterotrophic conditions. *A. eutrophus* strain H16 was grown in a batch culture on mineral medium supplemented with pyruvate or succinate. During the lag or exponential growth phase, the key enzymes of autotrophic metabolism were not formed (9). However, when the cells reached the stationary growth phase (caused by depletion of the carbon source in the medium), the soluble NAD^+-reducing hydrogenase appeared and was present at high activities. In contrast, the activities of the membrane-bound hydrogenase and RuBPC did not increase (Fig. 1). When the stationary phase was caused by nitrogen depletion, none of these enzymes was formed (9).

For further experiments on the effects of the availability of the carbon source on the activities of the hydrogenases, *A. eutrophus* was grown in a succinate-limited chemostat. The maximum growth rate was 0.45 h^{-1}, and the maximum yield was 0.39 g of dry cell weight per g of succinic acid, as determined from the double-reciprocal plot of $1/Y$ versus $1/D$ (Fig. 2A). The maintenance requirement was 0.032 g of succinic acid per g of dry cell weight per h, as determined by equation 2. During steady-state succinate-limited growth, the activities of the membrane-bound hydrogenase and the NAD^+-linked hydrogenase were determined. The specific activities of both of these enzymes decreased with increasing dilution rate. The decreases in steady-state activities followed a first-order reaction, as expressed by the following equation:

$$H = H_{max} \cdot e^{-\alpha \cdot D} \quad (5)$$

The correlation factor (α) was 10.5, and this value was determined by using the following equation:

$$\alpha = \frac{\ln H_1 - \ln H_2}{D_1 - D_2} \quad (6)$$

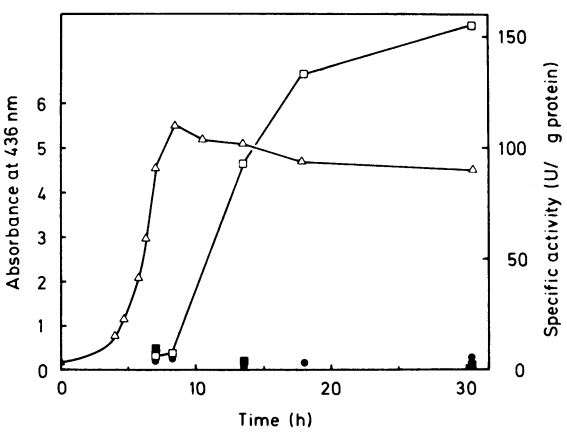

FIG. 1. Formation of hydrogenase activity after the stationary phase. Cells were grown with 0.4% (wt/vol) sodium succinate in a 10-liter fermentor as described in the text. Stationary phase was caused by succinate exhaustion in the medium. Enzyme levels were determined with cell-free extracts. Symbols: △, absorbance at 436 nm; □, soluble hydrogenase; ●, membrane-bound hydrogenase; ■, RuBPC.

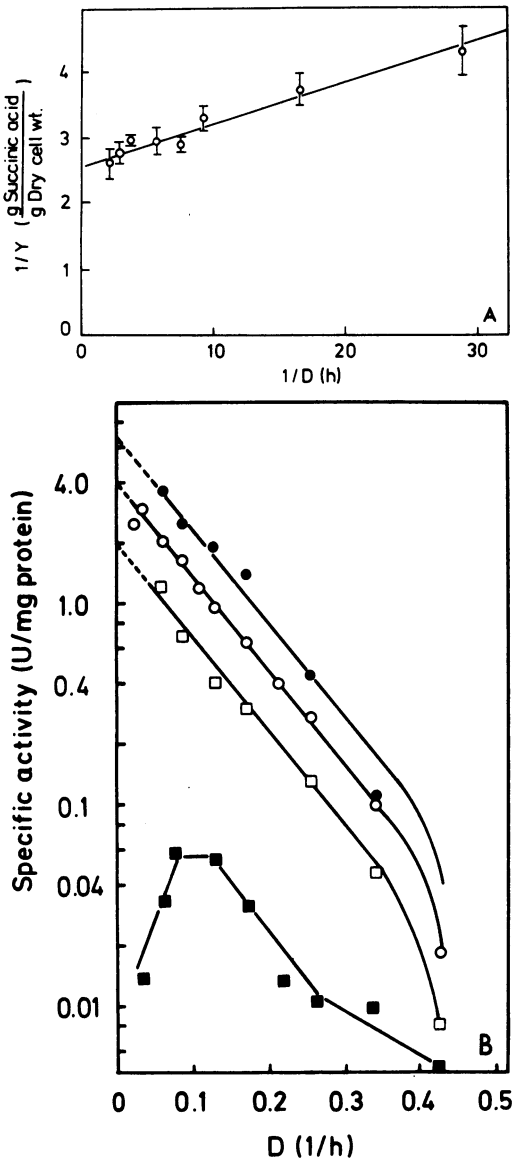

FIG. 2. Growth and enzyme activities in a succinate-limited chemostat. The conditions of succinate limitation were as described in the text. (A) Maximum yield and maintenance requirements were determined from a double-reciprocal plot of $1/Y$ versus $1/D$. (B) Steady-state enzyme activities of whole cells or cell-free extracts were plotted against the dilution rate. Symbols: □ and ●, soluble hydrogenase and particulate hydrogenase, respectively, from cell-free extracts; ○ and ■, soluble hydrogenase and RuBPC, respectively, from whole cells.

The maximum specific activities of the soluble hydrogenase were extrapolated to a dilution rate of 0 h^{-1} and amounted to 4 and 2 U/mg of protein when assays were performed with whole cells and the cytoplasmic fraction, respectively.

The maximum specific activity of the membrane-bound hydrogenase was about 6 U/mg of protein. Although the maximum activities of the soluble and particulate hydrogenases differed, the kinetics of their steady-state activities were identical to the kinetics given by equation 5 (Fig. 2). No hysteresis of the steady-state enzyme activities was observed when changing from low to high dilution rates or vice versa. It should be noted that the specific activities of the hydrogenases obtained at low dilution rates during succinate limitation exceeded those obtained under autotrophic growth conditions in batch cultures by a factor of 5 to 10. RuBPC activity was also found in cells grown under succinate limitation, but at a much lower specific activity than during autotrophic growth. The peak activity of this enzyme appeared at a relatively low dilution rate, and the kinetics of this appearance differed from those of the hydrogenases and were not defined by a simple mathematical model (Fig. 2).

Effect of growth rate on enzyme activities. To determine whether the enzyme activities were growth rate dependent or substrate specific, *A. eutrophus* was grown under nitrogen limitation with ammonium chloride as the nitrogen source and succinate as the heterotrophic substrate. Under these conditions RuBPC was not formed at a dilution rate of 0.060 to 0.40 h^{-1} (Table 2). During ammonia limitation succinate was used to build up intracellular poly-β-hydroxybutyric acid concentrations. Below a dilution rate of 0.21 h^{-1} the rapid rate of succinate consumption exhausted the carbon source in the medium completely, and the activity of the soluble hydrogenase increased drastically with decreasing dilution rate (Table 2). The kinetics of the steady-state activities were identical to those given in equation 5 when the correlation factor was 25.

During succinate-limited growth, the cells could suffer from either carbon or energy limitation. Energy limitation can be achieved either by

TABLE 2. Specific activities of soluble hydrogenase and RuBPC during heterotrophic limitations

Limitation[a]	Dilution rate (h^{-1})	Sp act (U/mg of protein)	
		Soluble hydrogenase	RuBPC
Succinate	0.061	2.25	0.031
	0.300	0.27	0.010
Oxygen	0.060	0.04	<0.001
	0.340	0.01	<0.001
Ammonia	0.060	1.93	<0.001
	0.108	0.95	<0.001
	0.173	0.28	<0.001
	0.21	0.01	<0.001
	0.40	0.01	<0.001

[a] The experimental conditions are described in the text.

restricting the supply of reductant or by restricting the supply of the electron acceptor, molecular oxygen. However, during steady-state oxygen-limited growth with excess succinate in the medium at a dilution rate of 0.060 or 0.34 h^{-1}, the hydrogenase and RuBPC were barely detectable (Table 2). Therefore, limitation of reductant or of carbon for cellular synthesis could account for the derepression of these enzymes under heterotrophic conditions.

Growth limitation by molecular hydrogen. During autotrophic growth of *A. eutrophus* under limitation by molecular hydrogen, the productivity was about 0.13 g of dry cell weight per liter per h, and this value was constant from a dilution rate of 0.032 h^{-1} to a dilution rate of 0.194 h^{-1}. In this range the gas consumption rate was 6.4 to 5.9 liters/liter of medium per h; this rate declined slightly with increasing dilution rate and was typical for a gas-limited chemostat (Fig. 3A).

Both the soluble NAD^+-linked hydrogenase and the membrane-bound hydrogenase were formed in a hydrogen-limited chemostat according to equation 5 with a correlation factor of 9.6 from a dilution rate of 0.08 h^{-1} to a dilution rate of 0.19 h^{-1}. At dilution rates lower than 0.08 h^{-1}, the activities leveled off slightly. The specific activities of these two enzymes, as determined from the soluble and particulate fractions of cell-free extracts, were almost identical at different dilution rates (Fig. 3B). The RuBPC specific activity in cells grown under hydrogen limitation was constant at different dilution rates, with fluctuations around 0.1 U/mg of protein (Fig. 3B).

Carbon dioxide-limited autotrophic growth. Carbon dioxide dissolves well in aqueous media (0.29 mmol/liter of water at 30°C and 1 kPa, compared with 0.008 mmol of H_2 per liter and 0.011 mmol of O_2 per liter under identical conditions). Because of the high solubility of carbon dioxide, the mass transfer rate from the gas to the liquid phase increased, and consequently the availability of CO_2 to the cells increased. Therefore, the productivity increased and was almost twofold higher during carbon dioxide limitation (0.24 g of dry cell weight per liter per h) than during hydrogen limitation. Consequently, the gas uptake rate increased (9.9 liters of gas per liter of medium per h). During carbon dioxide-limited growth, the activity of the soluble hydrogenase increased slightly with increasing dilution rate, although it was generally low (0.1 to 0.25 U/mg of protein) compared with the activity during autotrophic growth in batch cultures (Fig. 4). However, RuBPC activity was highest at a dilution rate of 0.08 to 0.15 h^{-1} (about 0.6 U/mg of protein), and this activity decreased to about 0.2 U/mg of protein at higher and lower dilution rates. It should be noted that the specific activity of this enzyme at these dilution rates was more than fivefold higher than the specific activity during autotrophic growth in batch cultures.

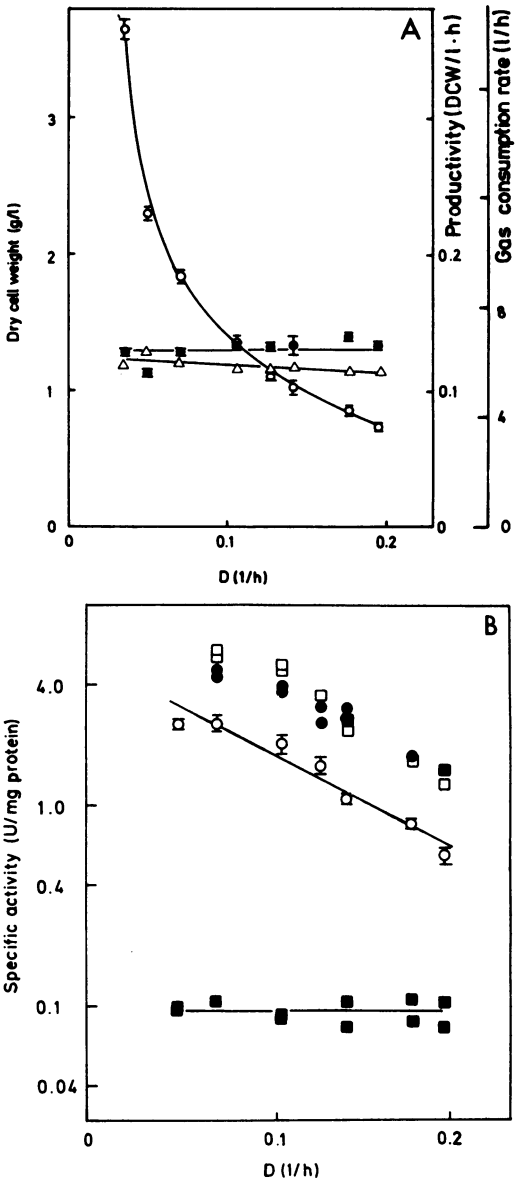

FIG. 3. Growth, cellular productivity, and ezyme activities in a chemostat limited by molecular hydrogen. The composition of the autotrophic gas mixture and the other culture conditions were as described in the text. (A) Symbols: ○, dry cell weight (DCW); ●, productivity; △, gas consumption rate. (B) Enzyme activities were determined from whole cells and cell-free extracts. Symbols: □ and ●, soluble hydrogenase and particulate hydrogenase, respectively, from cell-free extracts; ○ and ■, soluble hydrogenase and RuBPC, respectively, from whole cells.

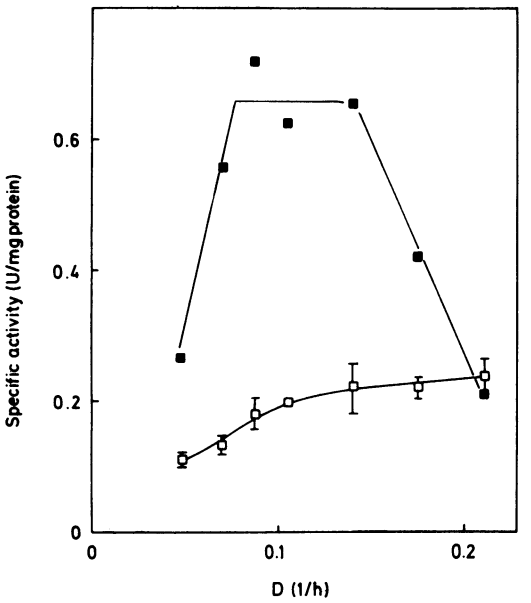

FIG. 4. Specific activities of the key enzymes of autotrophic metabolism during autotrophic growth in a carbon dioxide-limited chemostat. The gas mixture and the culture conditions were as described in the text, and the culture characteristics are also given in the text. Enzyme specific activities were determined with whole cells. Symbols: □, soluble hydrogenase; ■, RuBPC.

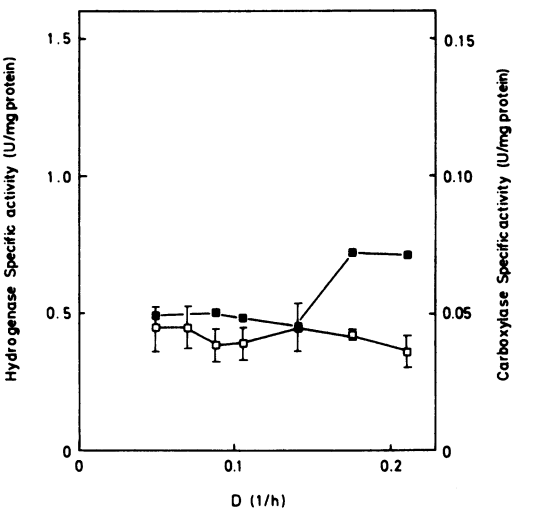

FIG. 5. Specific activities of the key enzymes of autotrophic metabolism during autotrophic growth in an oxygen-limited chemostat. The gas mixture and the culture conditions were as described in the text, and the culture characteristics are also given in the text. Enzyme specific activities were determined with whole cells. Symbols: □, soluble hydrogenase; ■, RuBPC.

Oxygen-limited autotrophic growth. During oxygen limitation productivity increased slightly with increasing dilution rate, perhaps because of decreased maintenance requirements. At a dilution rate of 0.049 h^{-1}, the productivity was 0.116 g of dry cell weight per liter per h, and at a dilution rate of 0.210 h^{-1} this value was 0.169 g of dry cell weight per liter per h. However, the gas consumption rate was constant regardless of the dilution rate and amounted to 8.7 liters of gas per liter of medium per h.

The specific activity of the soluble hydrogenase was about 0.4 to 0.5 U/mg of protein at dilution rates from 0.049 to 0.210 h^{-1}, corresponding to the average level found during autotrophic growth in batch cultures. However, RuBPC was present at a low activity (0.05 to 0.07 U/mg of protein) at the different dilution rates tested (Fig. 5). A carbon dioxide-fixing activity (C_f) of 0.053 U/mg of protein was theoretically necessary for the amount and rate of growth, as determined by the following equation:

$$C_f = \frac{\mu/60 \cdot \mu\text{mol of C/liter}}{\text{mg of protein/liter}} \quad (7)$$

DISCUSSION

These data described above identify the conditions under which the key enzymes of autotrophic metabolism are formed and allow conclusions with respect to the regulation of the formation of these enzymes. First, soluble and membrane-bound hydrogenase activities appeared during growth limitation by the electron donor, whether it was inorganic (molecular hydrogen) or organic (succinate). Under these different growth conditions both hydrogenase activities could be predicted by the same model (equation 5) when different degrees of limitation were given by different growth rates in a chemostat (Fig. 2 and 3B). Limitation of other substrates, such as oxygen and ammonia, did not lead to hydrogenase activities under heterotrophic conditions. Thus, the growth rate per se could not account for the observed activities. Second, RuBPC was not formed coordinately with the hydrogenases; RuBPC activity was derepressed during carbon dioxide limitation, provided that sufficient energy for CO_2 fixation was available (Fig. 4). No RuBPC activity appeared when both carbon and energy were growth limiting (Fig. 1).

The soluble hydrogenase is inactivated in vivo during heterotrophic substrate shifts (20) and in vitro in the presence of NADH and oxygen due to the formation of O_2^- radicals (22). Therefore, a question arose as to whether the hydrogenase

activities reflected the rate of hydrogenase formation or a differential rate of enzyme formation and inactivation. However, immunological studies with cells from batch cultures indicated a perfect correlation between the presence of hydrogenase activity and immunologically measurable hydrogenase protein. Active hydrogenase and hydrogenase inactivated by superoxide radicals reacted with antibodies against the active and inactivated hydrogenases (K. Schneider, personal communication). Therefore, I concluded that the steady-state hydrogenase activities actually reflected their rates of formation.

Generally, the kinetics of enzyme formation that depend on the dilution rate cannot be used as evidence for a certain type of regulation of enzyme formation but must be determined from the individual experimental conditions. *Pseudomonas* sp. or *Spirillum* sp. grown in a carbon-limited chemostat formed several enzymes of central carbon metabolism in an inverse relationship to the dilution rate. This was considered to be due to the release of catabolite repression (i.e., involving the promoter region of the operon) rather than induction (i.e., involving the regulator and the operator genes of the operon) (14).

On the other hand, the concomitant increase in histidase activity with increasing dilution rate of sulfur- or arginine-limited *Aerobacter aerogenes* growth was interpreted in the same way (i.e., release of catabolite repression of histidase synthesis) (11).

In *A. eutrophus* both the soluble hydrogenase and the particulate hydrogenase were formed in a defined first-order fashion with decreasing dilution rate only when the electron donor was limited (Fig. 3B). Derepression of these hydrogenases was apparently linked to the redox level of the cell, either directly or indirectly.

In *Pseudomonas* sp. the total concentration of the pyridine nucleotides increases in a first-order fashion with decreasing dilution rate in a succinate-limited chemostat (15); this resembles the kinetics of hydrogenase formation in *A. eutrophus*. Current studies with *A. eutrophus* are examining the correlation among the intermediate metabolite concentrations, the NAD/NADH ratios, and the rate of hydrogenase formation. Cyclic AMP, which is involved in catabolite repression in *Escherichia coli* and *Aerobacter aerogenes* (10), apparently does not play a role in the repression and derepression of the hydrogenases in *A. eutrophus* (Friedrich, unpublished data). The hydrogenases of *A. eutrophus* were formed both in the presence and in the absence of molecular hydrogen. Therefore, the mechanism leading to hydrogenase formation was distinctly different than that of *Rhizobium japonicum*, in which H_2 is required for the synthesis of some essential component of the H_2 uptake system (13).

Carbon dioxide-limited autotrophic growth of *A. eutrophus* caused a five- to sevenfold increase in RuBPC specific activity compared with the usual level in cells grown either lithoautotrophically with hydrogen and carbon dioxide (Fig. 3B and 5) or autotrophically on formate (8). Also, during heterotrophic succinate-limited growth, RuBPC activity appeared to be at optimum levels (Fig. 2). In carbon-limited chemostats such maxima were considered to be diagnostic for systems that were under multiple control of enzyme synthesis, like induction and catabolite repression, as in the case of *Pseudomonas aeruginosa* acetamidase (2, 3).

Dual control was also considered for the formation of the RuBPC of *A. eutrophus*. First, repression and derepression: this may be determined by the intracellular concentration of carbon metabolites, which would be expected to decrease with decreasing dilution rate. This hypothesis is supported by the finding that the pool concentrations of free amino acids decrease under nitrogen limitation with decreasing dilution rate (23). During carbon dioxide limitation RuPBC was derepressed, but this enzyme was repressed during nitrogen or heterotrophic oxygen limitation, when a surplus of carbon source was present in the medium. Therefore, a compound of central carbon metabolism may act as a signal in the regulation of RuBPC formation. With respect to derepression of RuBPC from *Pseudomonas oxalaticus* OX1, a similar conclusion was drawn when this bacterium was grown with substrate mixtures containing formate and oxalate in a chemostat (4). Second, RuBPC was formed only under conditions of carbon limitation, when sufficient reducing equivalents were available for energy generation. The exhaustion of a heterotrophic carbon source did not lead to RuBPC formation but did lead to a high rate of hydrogenase formation, indicating a lack of reducing equivalents (Fig. 1). Furthermore, under conditions of carbon and energy depletion, RuBPC is not formed, and it is also inactivated (12). Both mechanisms are beneficial to the cells since in this situation energy-consuming carbon dioxide fixation is avoided.

The different mechanisms that regulate hydrogenase or RuBPC formation allow an organism to gain either reducing power or carbon for cellular synthesis under suboptimal growth conditions. In this respect autotrophic conditions could be regarded as suboptimal growth conditions, allowing only relatively slow growth (9). Under these conditions the expression of the key enzymes of autotrophic metabolism results from the balanced limitations of intracellular carbon and reducing power.

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