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Metabolite Concentrations in *Alcaligenes eutrophus* H 16 and a Mutant Defective in Poly-β-Hydroxybutyrate Synthesis

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Abstract. Intracellular concentrations of hexose phosphates, phosphoenolpyruvate, pyruvate, NAD(H) and NADP(H) as well as the protein and poly-β-hydroxybutyrate (PHB) content were measured in suspensions of autotrophically grown cells of *Alcaligenes eutrophus* H 16 and compared with those in a mutant unable to synthesize poly-β-hydroxybutyrate. The parent strain was subjected to successive changes in conditions, and new steady states were rapidly (≃20 min) attained. When the parent strain was provided with carbon and energy but no nitrogen source, it fixed CO_2 and accumulated large amounts of PHB. When the mutant PHB^-4 was exposed to identical conditions, no accumulation of PHB occurred, but pyruvate, malate and citrate were excreted, and a 6-fold accumulation of hexose monophosphate (over the levels in the parent) was observed: in contrast, cofactors in intermediates between fructose-1,6-phosphate and phospho*enol*pyruvate reached steady state as in the parent strain. When ammonium ion was then supplied, growth started and the metabolite concentrations in the mutant returned to the levels observed in the parent strain.

Key words: Metabolite concentrations in bacteria – *Alcaligenes eutrophus* H 16 – Control of intermediary metabolism – Poly-β-hydroxybutyrate deficient mutants.

The measurement of metabolite concentrations is an essential but relatively little used technique to study control of metabolism in vivo. Much elegant work has been done with the very complex, compartmented eukaryotic tissues and cells (e. g. Krebs, 1975). Bacteria can offer a simpler system with a single cell type, a minimum of compartition, and ease of reproducible manipulations, and methods have become available to measure metabolite levels in bacteria (Lowry et al., 1971; Decker and Pfitzer, 1972; Cook et al., 1976).

Alcaligenes eutrophus H 16 is a facultative autotroph capable of rapid growth in simple mineral medium gassed with $H_2 + O_2 + CO_2$. Autotrophically grown cells of this robust organism will oxidize hydrogen in the absence of carbon and nitrogen sources, synthesize the lipid storage polymer poly-β-hydroxybutyrate (PHB) when supplied with CO_2, and then grow when supplied with a nitrogen source (Schlegel and Bartha, 1961; Schlegel et al., 1961). These manipulations are easily done in a defined system (Cook et al., 1976).

In this paper, we present a quantitative description of the steady states of hydrogen oxidation, PHB synthesis and growth in *Alcaligenes eutrophus* H 16 and compare them to a mutant strain (PHB^-4) unable to synthesize PHB. Anomalous behaviour of certain metabolites in the mutant illustrated two loci where enzymes were responding to the metabolic imbalance caused by the mutation.

Materials and Methods

Organisms. The strictly aerobic, facultatively chemolithoautotrophic bacterium *Alcaligenes eutrophus* H 16 (ATCC 17699) and a mutant, PHB^-4 (DSM 541), unable to synthesize the lipid storage material poly-β-hydroxybutyrate (PHB), were used.

Autotrophic Growth Conditions, Harvesting, Experimental Conditions and Methods to Measure Metabolite Concentrations and Bacterial Dry Weight. These were all described by Cook et al. (1976) with the following minor modifications. The assays for ATP in the presence of large amounts of glucose-6-phosphate and fructose-6-phosphate were unusual, in that an oxidation of NADH set in with the addition of hexokinase. The reaction with the standard solution of glucose-6-phosphate was unaltered under these conditions, and the amount of ATP present was calculated as usual by the extrapolation of the recorder-trace, after the normal reaction period, to the time of

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hexokinase addition. No attempt was made to destroy pyruvate during sample-taking, so in the assays for phosphoenolpyruvate or ADP and AMP which contained excess pyruvate, pyruvate was converted to lactate using lactate dehydrogenase and NADH, and the subsequent assay was corrected for the additional fluorescence-quench with the usual internal standard (Cook et al., 1976; Williamson and Corkey, 1969). As the storage of poly-β-hydroxybutyrate produces no additional cell water (Bowien et al., 1974), turbidity increases due to the storage of this polymer were ignored in the calculation of results, as in the data shown in Cook et al. (1976). Intermediates excreted from the cell were measured photometrically in samples of clarified suspension medium with the assays also used to calibrate standard solutions for the fluorimetric assays (see Cook et al., 1976).

The Assay of Poly-β-Hydroxybutyrate. The infrared spectrophotometric assay (Jüttner et al., 1975) was used.

Protein. The method of Kennedy and Fewson (1968) was used.

Materials. All reagents were of the highest grade that could be obtained commercially. With the exception of the following list, all materials were obtained from the sources indicated in Cook et al. (1976). Chloroform, spectroscopically pure, was purchased from Merck, Darmstadt, FRG; bovine serum albumin (fraction 5) was supplied by Serva, Heidelberg, FRG, and membrane filters (11307047) were obtained from Sartorius, Göttingen, FRG.

Results

Alcaligenes eutrophus H 16, the parent strain, was grown autotrophically in mineral medium under an atmosphere of $H_3 + O_2 + CO_2$, harvested, and resuspended in ammonium-free mineral medium under an atmosphere of $O_2 + H_2$. The turbidity remained constant under these conditions (Fig. 1A). The addition of CO_2, the carbon source, to the gas phase led to an increase in turbidity caused by the synthesis of the storage product poly-β-hydroxybutyrate (Fig. 1A), which we found to account for 96–100% of the increase in dry weight confirming previous data (Schlegel et al., 1961). On the subsequent addition of ammonium chloride as nitrogen source, the synthesis of poly-β-hydroxybutyrate ceased and growth, indicated by protein synthesis, started after a lag of about 10 min (Fig. 1A); a low rate of poly-β-hydroxybutyrate synthesis was then observed (Fig. 1A).

In the absence of carbon and nitrogen sources, the intracellular concentrations of metabolic intermediates were comparatively low (<0.1 mM, Fig. 1B, C) though concentrations of cofactors e. g. NAD (Table 1) and ATP (4 mM; from Cook et al., 1976) were high. This condition may be maintained for at least 3 h (not shown) and illustrates non-specifically the ability of the cell to isolate metabolism of the presumably more important cofactors from that of intermediates (Cook et al., 1976). When cells were supplied with CO_2, there was a flow of intermediates into the metabolic pools within 30 s and a steady state was observed within 20 min. There was a flux through at least some pools, as poly-β-hydroxybutyrate was

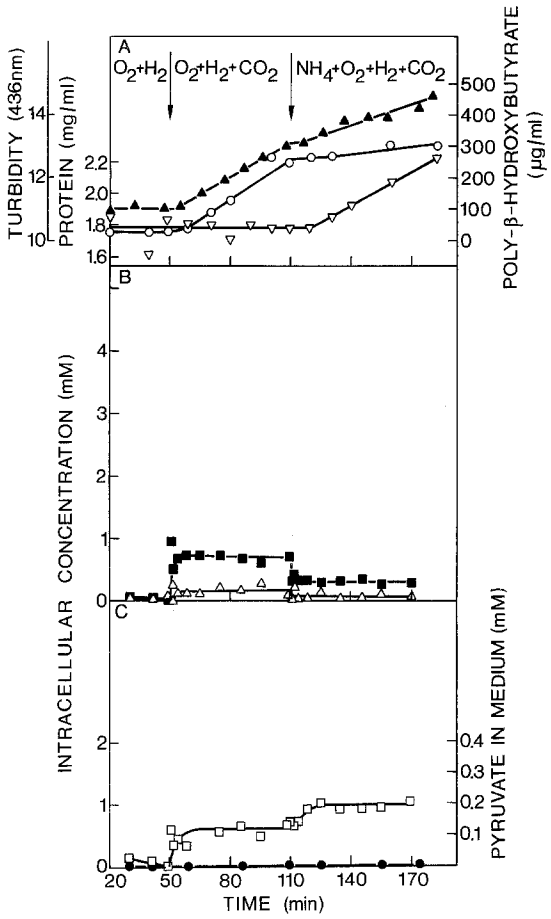

Fig. 1A—C. The effect of successive changes in conditions on intracellular metabolite concentrations and other parameters in *Alcaligenes eutrophus* H 16. Cells grown under autotrophic conditions were harvested, washed, suspended in ammonium-free mineral medium and incubated at 30° C under an atmosphere of $O_2 + H_2$ (2:8 by vol.) as described by Cook et al. (1976). At intervals, samples were taken for turbidity, poly-β-hydroxybutyrate, protein, for acid-stable intracellular metabolites or for excreted metabolites. At the times indicated (↓) CO_2 was added ($O_2 : CO_2 : H_2 = 2:1:7$) and subsequently NH_4Cl. Symbols: ▲——▲ turbidity; ○——○ poly-β-hydroxybutyrate; ▽——▽ protein; ■——■ fructose-6-phosphate; △——△ fructose-1,6-bisphosphate; □——□ phospho*enol*pyruvate; ●——● pyruvate. The data in **A** come frome two experiments; the turbidities correspond to the experiment shown in **B** and **C**, while the other data refer to a parallel experiment at higher celi density (initial turbidity 12.2)

synthesized. The change in concentration of cofactors (Table 1 and data of Cook et al., 1976) was relatively small compared to that of the intermediates (Fig. 1 B, C and data of Cook et al., 1976) presumably further reflecting the separate control of the metabolism of cofactors and of intermediates. The addition of the ammonium chloride led to a new steady state in about 30 min.

The relevant metabolite concentrations shown in Fig. 1 B, C are corrected for material outside the cells. For fructose bisphosphate and fructose-6-phosphate,

Table 1. Levels of pyridine nucleotides in different steady states in *Alcaligenes eutrophus* H 16 and the mutant PHB$^-$4. The parent strain or the mutant was grown under autotrophic conditions harvested, washed, suspended in ammonium-free mineral medium and incubated at 30° C under an atmosphere of $O_2 + H_2$ (2 : 8 by vol.) as described by Cook et al. (1976). At intervals after the 20 min equilibration time, samples were taken for acid-stable and alkali-stable metabolites. The gas mixture was then changed to $O_2 + CO_2 + H_2$ (2 : 1 : 7 by vol.) and the cells started to fix CO_2. In steady state, several samples were taken at intervals. Thereafter, NH_4Cl was added and growth started. In the steady state (after about 30 min) further samples were taken at intervals. The results are given as a function of dry wt (see Williamson, 1969) and corrected for material outside the cell. The standard deviation of the values shown (3 – 5 samples) was usually 3 – 10 %; the values for NADH during "starvation for N" were exceptions where it was about 30 %

Conditions	Starvation for C + N		Starvation for N		Growth	
Supply[b]	$O_2 + H_2$		$O_2 + CO_2 + H_2$		$O_2 + CO_2 + H_2 + NH_4^+$	
Bacterium	Parent	Mutant	Parent	Mutant	Parent	Mutant
Nucleotide	Concentration of nucleotide nmol/mg dry wt.[a]					
NAD	1.7	1.3	2.0	2.2	2.8	[b]
NADH	0.73	0.88	0.36	0.74	0.83	[b]
NADP	0.01	0.03	0.15	0.18	0.13	0.29
NADPH	0.69	0.73	0.38	0.41	0.18	0.38

[a] The dry weight does not include poly-β-hydroxybutyrate; see "Materials and Methods"
[b] No steady state observed in the time course studied (60 min)

extracellular levels reached about 10 % of the total, while for phospho*enol*pyruvate it was about 20 % of the total. Even with 20 % of a substance outside the cell, the concentration gradient across the membrane was $> 1 \times 10^3$, as 80 % of the material was dissolved in the 3 µl intracellular water per ml cell suspension and 20 % was dissolved in the remaining 997 µl. Swedes et al. (1975) observed similar leakage from bacteria.

The experiment shown in Fig. 1 was repeated with the mutant strain, PHB$^-$4, which cannot synthesize poly-β-hydroxybutyrate. As shown in Fig. 2A, a constant turbidity was observed in autotrophically grown cells resuspended in ammonium-free salts-medium under an atmosphere of $H_2 + O_2$. The addition of CO_2 had little effect on the turbidity (cf. Fig. 1A). An increase in turbidity due to growth, illustrated by protein synthesis, was observed on the addition of ammonium chloride (Fig. 2A). When the mutant was starved of carbon- and nitrogen sources, the pattern of metabolite concentrations observed in the wild type was repeated. There were very low concentrations of intermediates present (Fig. 2 B, C) and moderately high concentrations of cofactors e. g. NAD (Table 1) and adenine nucleotides (ATP 3.7 mM, ADP 0.6 mM, AMP 0.6 mM; data from Cook et al., 1976).

The addition of CO_2 led to a flow of material into the pools of intermediates (Fig. 2 B, C). This partially resembles the behaviour of the parent strain in as much as fructose bisphosphate (Fig. 2 B), triose phosphates, 3-phosphoglycerate (not shown) and phospho*enol*pyruvate (Fig. 2C) all came to steady state in about 20 min similar to the wild type (Fig. 1 B, C); the behaviour of the measured cofactors also resembled the wild type (Table 1). In contrast, however, the concentration of fructose-6-phosphate (and glucose-6-phosphate, not shown) rose some six-fold over the level in the parent; the percentage leakage of fructose-6-phosphate from the cells was no higher than in the parent. Also in contrast to the wild type, pyruvate was excreted arithmetically in large amounts (Fig. 2 C). Malate and citrate were also excreted from the cell, though in lower quantities (about 15 and 5 %, respectively).

The low leakage of fructose-6-phosphate from the mutant confirmed the structural integrity of the cytoplasmic membrane. The excretion of pyruvate, malate and citrate from the mutant was thus a controlled response of the cell to the fixation of CO_2 without the normal "sink" in the osmotically inert poly-β-hydroxybutyrate.

When ammonium chloride was added to the cell suspension, the intermediates, whose concentrations already resembled the control values (e. g. fructose bisphosphate and phospho*enol*pyruvate, Fig. 2 B, C), continued to behave as in the parent strain (Fig. 1B, C); the energy charge rose to 0.86 (ATP 4.6 mM, ADP 1.2 mM, AMP 0.2 mM) as observed in the wild type (Cook et al., 1976). The concentration of fructose-6-phosphate (and of glucose-6-phosphate) rapidly fell to about that in the parent strain. The excreted pyruvate (Fig. 2 C; also citrate and malate) was taken up from the medium.

Discussion

The intracellular metabolite concentrations shown here (Figs. 1 B, C; 2 B, C) demonstrate steady states and simple transition patterns especially in the wild type organism. There are no obvious, continued oscillations,

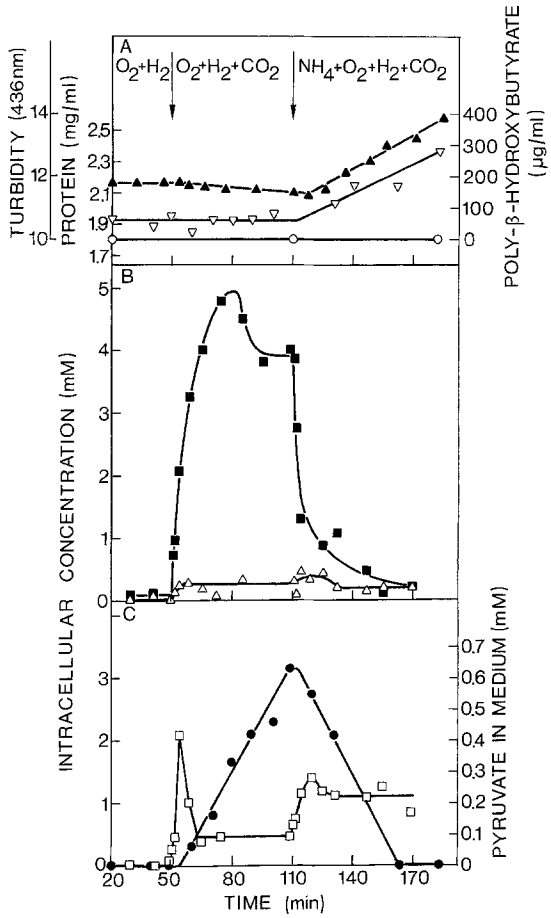

Fig. 2A—C. The effect of successive changes in conditions on metabolite concentrations and other parameters in *A. eutrophus* PHB⁻4. Cells grown under autotrophic conditions were harvested, washed, suspended in ammonium-free mineral medium and incubated at 30° C under an atmosphere of $O_2 + H_2$ (2 : 8 by vol.) as described by Cook et al. (1976). At intervals, samples were taken for turbidity, poly-β-hydroxybutyrate, protein, for acid-stable intracellular metabolites or for excreted metabolites. At the times indicated (↓) CO_2 was added ($O_2 : CO_2 : H_2 = 2 : 1 : 7$) and subsequently NH_4Cl. Symbols: ▲——▲ turbidity; ○——○ poly-β-hydroxybutyrate; ▽——▽ protein; ■——■ fructose-6-phosphate, △——△ fructose-1,6-bisphosphate; □——□ phospho*enol*pyruvate; ●——● pyruvate. The data in **A** come from two experiments; the turbidities correspond to the experiment shown in **B** and **C** while the other data refer to a parallel experiment at higher cell density (initial turbidity 12.1)

though the time scale here is large compared with the well-characterized, yeast-extract system, in which not all substrates produce oscillations (Hess and Bioteaux, 1971; see also Harrison, 1973). The mutually reversed redox balance in NAD and NADP, with NAD^+ having the highest concentration (Table 1, especially left-hand column) qualitatively resembles the patterns in anaerobic bacteria (e. g. Decker and Pfitzer, 1972) and in animal tissues (e. g. Williamson and Brosnan, 1974). The energy charge in *Alcaligenes* oxidizing hydrogen and starved for carbon and nitrogen is as high as during growth (about 0.8; Cook et al., 1976). This supports the idea that the cell maintains a high energy charge when possible.

The mutant PHB⁻ 4 differs from the wild-type in its inability to synthesize and accumulate poly-β-hydroxybutyrate; the metabolic does not concern the enzymes involved in the synthesis of β-hydroxybutyryl-CoA (Oeding, 1972). As shown here, the mutant differs from the wild-type in excreting or accumulating metabolites under conditions which in the wild-type result in PHB synthesis. These metabolites are pyruvate, malate and citrate, which are excreted (see also Vollbrecht and Schlegel, 1978; Vollbrecht et al., 1978), and fructose- and glucose-6-phosphate, which are intracellularly accumulated. The excretion of pyruvate at controlled intracellular phospho*enol*pyruvate concentrations stems presumably from the normal activity of pyruvate kinase in conjunction with a lowered activity of pyruvate dehydrogenase whose product acetyl CoA (i) cannot be directed to poly-β-hydroxybutyrate and, (ii) would be a source of unusable NADH and ATP if oxidized in the tricarboxylic acid cycle. The intracellular accumulation of fructose-6-phosphate and glucose-6-phosphate contrasts with the fructose bisphosphate which behaves as in the parent strain. The present information on the regulation of regeneration of ribulose bisphosphate in CO_2 fixation does not allow precise interpretation of this accumulation of sugar phosphates in the mutant.

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