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Selective Inactivation of Nitrogenase in *Azotobacter vinelandii* Batch Cultures

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When the exhaustion of sucrose or sulfate or the induction of encystment (by incubation in 0.2% β-hydroxybutyrate) leads to termination of growth in *Azotobacter vinelandii* batch cultures, the nitrogenase levels in the organisms decreased rapidly, whereas glutamate synthase and glutamine synthetase levels remained unaltered. Glutamate dehydrogenase activities were low during the whole culture cycle, indicating that ammonia assimilation proceeds via glutamine. Toward depletion of sucrose or during induction of encystment, slight secretion of ammonia with subsequent reabsorption was occasionally observed, whereas massive ammonia excretion occurred when the sulfate became exhausted. The extracellular ammonia levels were paralleled by changes in the glutamine synthetase activity. The inactivation of the nitrogenase is explained as a result of rising oxygen tension, a consequence of a metabolic shift-down (reduced respiration) that occurs in organisms entering the stationary phase.

During the growth cycle of bacterial batch cultures, changes occur in the external medium that often cause switches in metabolic pathways and eventually lead to the establishment of a stationary phase. Termination of the culture cycle can also be induced by the addition of specific reagents that cuase the organisms to form cysts. *A. vinelandii* cultures undergo encystment upon addition of β-hydroxybutyrate (18). The changes in carbon metabolism induced by this addition are under intensive investigation (for reviews see, e.g., references 24 and 26). Further, Hitchins and Sadoff (8) reported a decline in the nitrogenase activity when encystment was induced.

We present data which demonstrate that this decline is a general phenomenon for *A. vinelandii* cultures approaching the stationary phase and that it is not associated with marked changes in the first steps of ammonia metabolism.

MATERIALS AND METHODS

Growth of the organism. *A. vinelandii* OP was grown aerobically in batches of 0.5 or 1.5 liters as outlined previously (12) at 30°C and pH 7.0 in a nitrogen-free medium, similar to that described by Strandberg and Wilson (30), with 2% sucrose as the carbon source. For sulfur-limited cultures, all sulfates in the medium were replaced by chlorides, and Na_2SO_4 was added to a final concentration of 0.12 mM. Induction of encystment was studied after the cells were centrifuged (5 min at 10,000 × *g*), washed with phopshate buffer (10 mM at pH 7.0), and suspended in a culture medium, which now contained 0.2% β-hydroxybutyrate instead of sucrose. Bacterial growth was followed turbidimetrically at 546 nm. Extinctions (epxressed in optical density units) were adjusted to 0.1 to 0.3 by dilution with water. An extinction of 1 corresponds to 0.32 mg of protein per ml.

Preparation of cell-free extracts. Samples were centrifuged for 5 min at 2,500 × *g*, the pellet was suspended in an eightfold smaller volume of imidazole buffer (10 mM, pH 7.0), and the cells were broken in a French press and centrifuged (5 min at 2,500 × *g*) to remove unbroken cells and debris.

Analytical methods. The supernatant fluid of the culture samples was checked for ammonia with an ammonia electrode (Orion Research Inc., Cambridge, Mass.) and for sucrose and sulfate as follows. For the determination of sucrose, the pH of the medium was lowered to 4.8 by addition of 1.0 M acetic acid. To 50 μl of this medium, 50 μl of a solution containing 50 U of invertase was added, and the mixture was incubated for 15 min at room temperature and assayed for glucose and fructose by conventional methods (1). Sulfate concentrations were estimated by isotope dilution analysis (16). Protein concentrations were measured by the microbiuret method with bovine serum albumin solutions as standards (6).

Enzyme assays. Nitrogenase (EC 1.7.99.2) activities in the cell-free extracts (in vitro) were assayed by the acetylene reduction test as described by Shah et al. (27) with the gas phase consisting of 12.5% C_2H_2 and 87.5% N_2. Nitrogenase activity in vivo was measured as described before (12). To samples of encysting cultures, 0.5% sucrose was added before the in vivo measurements to stop encystment during the assay. Glutamine synthetase (GS; EC 6.3.1.2)

was determined by the Mn^{2+}-dependent transferase (GS-T) test at pH 7.1 (this is not the isoactivity pH but that of maximal activity) as outlined by Stadtman et al. (29) and by the Mg^{2+}-dependent synthesis (GS-S) of γ-glutamylhydroxamate from glutamate and hydroxylamine (17). Glutaminase (EC 3.5.1.2) activities were estimated using the GS test without adenosine diphosphate (ADP) and arsenate. Glutamate dehydrogenase (Glu-DH; EC 1.4.1.4) and glutamate synthase (GOGAT, glutamine:2-oxoglutarate amidotransferase oxidoreductase, EC 2.6.1.53) were assayed as described by Meers et al. (20), with a final concentration of 0.01 M KCN in the test mixture to minimize blank reduced nicotinamide adenine dinucleotide phosphate oxidation by oxygen. Attempts to detect asparagine synthetase (EC 6.3.1.1) by two different assay methods (2, 22) were unsuccessful. All assays were performed at 30°C. The activities determined in vitro were expressed as milliunits per milligram of protein. Nitrogenase activities measured in vivo were expressed in terms of milliunits per milliliter of culture. One unit is defined as 1 μmol of product (ethylene, γ-glutamylhydroxamate, glutamate) formed per min. Respiration was measured at 30°C with a Warburg apparatus.

Materials. β-Hydroxybutyrate, hexokinase, glucose-6-phosphate dehydrogenase, and phosphoglucoisomerase were purchased from Boehringer (Mannheim, German Federal Republic); invertase was obtained from Sigma Chemical Co. (St. Louis, Mo.). All other reagents were of analytical grade.

RESULTS

As a reference for the comparison with the events in an encysting culture, *A. vinelandii* was grown in batch cultures in which the transition to the stationary phase was initiated by exhaustion of either sucrose (Fig. 1) or sulfate (Fig. 2). The changes of the enzyme levels of the primary steps of nitrogen assimilation during these growth cycles and some external parameters can be summarized as follows. During both growth cycles, only background activities of Glu-DH and glutaminase (well below 10 mU/mg, not shown) were detected. Attempts to find asparagine synthetase activities during these experiments were unsuccessful. GOGAT activities (not shown in Fig. 2) slowly declined in both cases during logarithmic growth and remained constant during the stationary phase. The growth curves in the final stages, as well as excretion of ammonia and the activities of nitrogenase and GS, were markedly different for both cultures. The transition from exponential to linear growth in Fig. 1 (at a bacterial density of about 4) is probably due to growth limitation by oxygen diffusion, a well-known characteristic of *A. vinelandii* batch cultures grown to high cell densities. However, after reaching the stationary phase, growth stopped completely when the sucrose was exhausted (Fig. 1), whereas a slow increase in the turbidity was observed after the depletion of sulfate (Fig. 2). Ammonia excretion is encountered only occasionally and with poor reproducibility during the transition to the stationary phase when sucrose became exhausted. It was always followed by reabsorption and was never observed in the stationary phase. Otherwise, ammonia levels always remained below 5 μM. When the sulfur source was exhausted, however, massive excretion of ammonia always occurred. Part of this ammonia was often reabsorbed later (Fig. 2). The secretion and reabsorption of ammonia in both cultures were always correlated with considerable changes in the GS activities: as soon as ammonia appeared in the medium, the GS-T activity rose and the GS-S activity dropped steeply. Nitrogenase activities measured both in vivo and in vitro finally dropped to 0 in both cultures. However, upon sucrose exhaustion, both activities show a parallel decrease (Fig. 1; the slow decline of the activity measured in vivo between the end of the logarithmic and the start of the stationary phase is most likely due to oxygen limitation under assay conditions, which in turn should lead to an enhanced intracellular ADP level, which is known to slow down nitrogen fixation; see, e.g., references 13 and 32 for reviews), whereas upon sulfur exhaustion the loss of the in vitro activity always lags behind (up to 5 h) the one determined in vivo.

Respiratory rates [$Q(O_2)$] were measured in parallel experiments. They remained constant at about 3,300 μl of O_2 consumed per h per mg (dry weight) until sucrose or sulfate became exhausted and then declined to less than 300 or 1,500 μl, respectively, within 3 h.

When washed, glucose-grown organisms are suspended in a medium containing β-hydroxybutyrate as the sole carbon source, encystment is induced (18). This induction results in marked changes in the levels of some enzymes associated with carbon metabolism and in the decrease of nitrogenase activity measured in vivo (8). Our investigations show that upon induction of encystment, by changing the carbon source to β-hydroxybutyrate, the levels of the enzymes catalyzing the first steps of nitrogen assimilation do not change significantly, except for nitrogenase (Fig. 3). The activities of Glu-DH remain below 10 mU/mg, whereas GOGAT levels (not shown) reveal the same trend as in Fig. 1. Sporadic excretion and reabsorption of ammonia (an example is shown in Fig. 3) occur and lead to the same reversible changes in the GS activities as described above. Apart from these changes, the GS levels seem

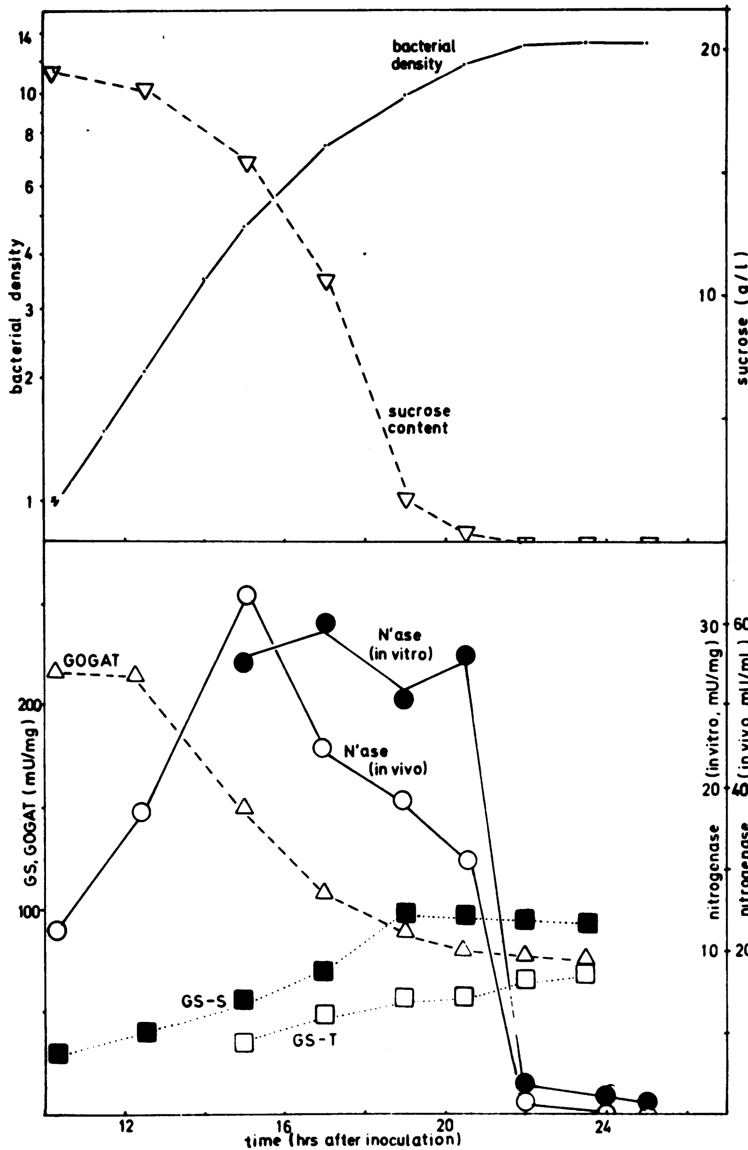

FIG. 1. *Batch culture of A. vinelandii, Growth termination by exhaustion of sucrose. Symbols: (——) bacterial density, (▽) sucrose content, (△) GOGAT, (■) GS-S, (□) GS-T, (○) nitrogenase (determined in vivo), (●) nitrogenase (determined in vitro).*

unaltered. In contrast to these enzymes, the nitrogenase activities determined in vivo as well as in vitro declined rapidly after induction of encystment. However, this decline is not observed if the encysting culture is kept anaerobic (under N_2).

The respiratory rate (determined in a separate run) decreased from 3,050 to 750 μl of O_2 consumed per mg (dry weight) per h within 1 h after the carbon source had been changed to 0.2% β-hydroxybutyrate.

DISCUSSION

The excretion of ammonia by *A. vinelandii* has been the subject of controversial reports during the early phases of research on this organism (for a discussion, see reference 10). Our results might suggest that high levels of ammonia excretion occur when depletion of any nutrient required for protein synthesis other than the carbon source leads to the stationary phase. Under these circumstances nitrogenase

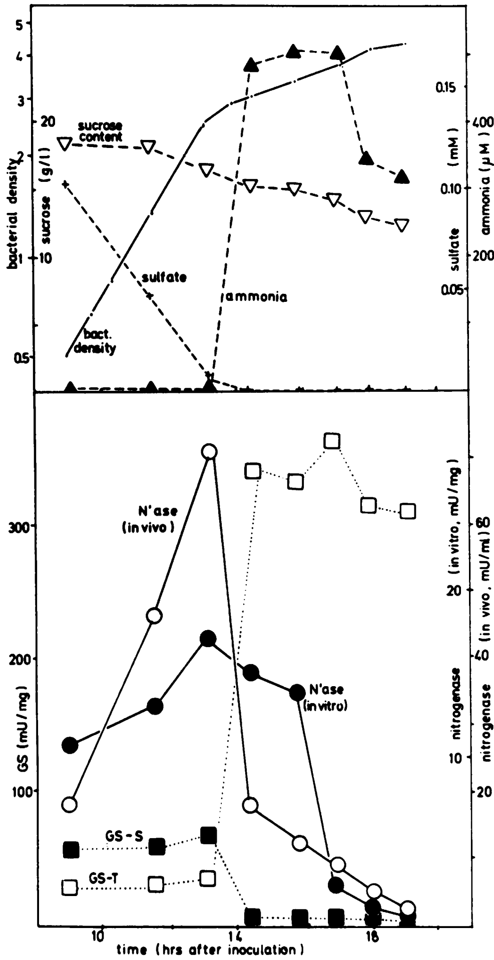

FIG. 2. *Batch culture of A. vinelandii, growth ter- termination by exhaustion of sulfate. Symbols:* (▲) *ammonia in the culture medium,* (×) *sulfate in the culture medium. All other symbols as in Fig. 1.*

will continue to produce ammonia that cannot be completely assimilated. The reason for the sporadic low levels of ammonia excretion and reabsorption during the transition to the stationary phase when sucrose is used up or when encystment is induced remains obscure.

The absence of glutaminase and asparagine synthetase might be due to a lack of genetic information for their synthesis, but it is also possible that special requirements for their induction or derepression have not been met under the culture conditions employed. Glu-DH, however, is known to be synthesized by *A. vinelandii* (19), and our failure to detect this enzyme points toward a repression of its synthesis. In contrast, GS and GOGAT were detected during the whole growth cycle. During the logarithmic phase, GOGAT synthesis appears to be repressed to some degree (Fig. 1), and a plateau is reached during the stationary phase. In contrast, GS activities tend to rise slightly during logarithmic growth (Fig. 1), with a plateau in the stationary phase. An abrupt increase in the transferase activity is encountered whenever ammonia appears in the medium in concentrations higher than 10 μM (Fig. 2 and 3). This regulation of the GS-T activity by extracellular ammonia has been first observed in continuous cultures of *A. vinelandii* (14). It is always accompanied by a rapid decline in the GS-S activity. The nature of these changes is not known; they might reflect regulation by adenylation-deadenylation reac-

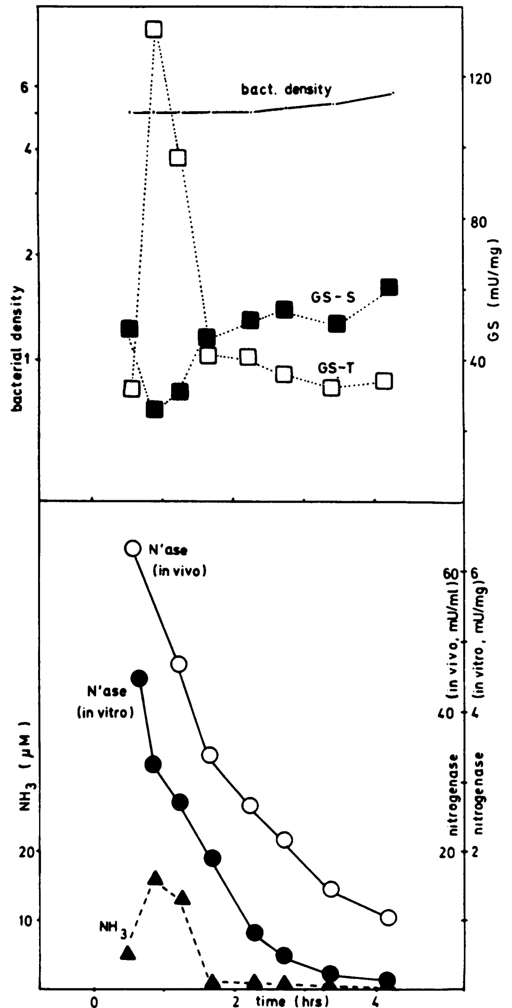

FIG. 3. *Growth of washed, sucrose-grown A. vinelandii cells after transfer to a medium with 0.2% β-hydroxybutyrate as sole carbon source (induction of encystment). All symbols as in Fig. 1 or 2.*

tions, as described for the *Escherichia coli* GS (9, 29).

The presence of GS and GOGAT, as well as the absence of Glu-DH, during the complete growth cycle indicates that ammonia assimilation occurs via glutamine, as has been reported for several bacteria under N_2-fixing conditions (21).

After reaching the stationary phase, nitrogenase activity declines rather fast under all conditions tested, indicating that this loss of activity is a general phenomenon and not only associated with induction of encystment. The decline of the enzyme level in cell-free extracts suggests an inactivation and not an allosteric regulation (e.g., by ADP). The lag between the decline of in vivo and in vitro activity, upon exhaustion of sulfate, can be explained by the rise in the GS-T activity, which has been shown to be correlated with a decrease of the nitrogenase activity determined in vivo but not with the one determined in vitro (14). How can this inactivation be explained? Hitchins and Sadoff (8) tentatively suggested that incorporation of β-hydroxybutyrate into the membrane may affect nitrogenase activity directly, as it is known that the *Azotobacter* nitrogenase is membrane associated (23). This hypothesis, however, does not explain the declining nitrogenase contents in cell-free extracts. Moreover, this inactivation is not only induced by β-hydroxybutyrate, but seems to occur whenever *A. vinelandii* cultures reach a stationary phase. A common characteristic is the metabolic shift-down that occurs upon transition into the stationary phase (11, 25), which is connected with a reduced respiratory activity, which in turn should cause a rising oxygen tension in the culture.

Purified nitrogenases are extremely oxygen sensitive. Postgate and co-workers (4, 5, 7) postulated two mechanisms for their protection in obligate aerobes: (i) a fast respiration to reduce the intracellular O_2 tension (respiratory protection) and (ii) – if that is not sufficient – a conformational change that renders the nitrogenase catalytically inactive and not susceptible towards damage by oxygen (termed "switch-off"). The oxygen-insensitive crude nitrogenase preparations obtained by passing cells through a French press are supposed to contain the enzyme in the switched-off state. To switch off nitrogenase, however, seems to be possible only when respiration is unrestricted. It fails to operate when respiration is slowed down by carbon limitation in continuous cultures (4) and by a high supply of adenosine 5'-triphosphate in batch cultures (31). In accordance with these results, Shah et al. failed to detect synthesis of active nitrogenase when respiration was blocked by azide (28). Thus, an unrestricted respiration is probably an essential requirement to enable the nitrogenase to be protected against damage by O_2 by undergoing a conformatonal change. However, in all cases tested here the entrance into the stationary phase is associated with a decreased respiration. Therefore, the most likely explanation for the decline in nitrogenase activity is an inactivation by O_2. This is corroborated by the fact that no inactivation occurs when induction of encystment is studied under anaerobic conditions.

The subsequent fate of the nitrogenase proteins is uncertain. A partial or total destruction might be indicated by the results of Fig. 2. After the decline in nitrogenase activity in vitro, a small increase in sucrose utilization, together with a significant absorption of previously excreted ammonia and a correlated small increase in growth, could indicate a turnover of the nitrogenase proteins that contain a relatively high amount of acid-labile sulfur (3, 15).

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