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Diauxic Growth in *Azotobacter vinelandii*†

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Azotobacter vinelandii **exhibited diauxie when grown in a medium containing both acetate and glucose as carbon sources. Acetate was used as the primary carbon source during the acetate-glucose diauxie. Uptake of acetate was constitutively expressed during both diauxic phases of growth. Induction of the glucose uptake system was inhibited in the presence of acetate. Acetate was also the preferred growth substrate for *A. vinelandii* grown in a medium containing either fructose, maltose, xylitol, or mannitol. The tricarboxylic acid cycle intermediates citrate, isocitrate, and 2-oxoglutarate inhibited glucose utilization in cells grown in glucose medium containing these substrates, and diauxic growth was observed under these growth conditions. Temporal expression of isocitrate-lyase, ATPase, and nitrogenase was exhibited during acetate-glucose diauxie.**

Diauxic growth is observed when an organism is grown in a medium containing two carbon sources, and there is a preferential utilization of one carbon source before the metabolism of the other. A biphasic growth curve results. A classical example is observed when *Escherichia coli* is grown in a medium containing both glucose and lactose as carbon sources. Under this condition glucose is metabolized preferentially; after it is depleted from the medium, lactose catabolism commences. The inability of cells to ferment lactose in the presence of glucose has been attributed to the inhibitory effects of glucose or its metabolic products on the synthesis and activity of certain enzymes involved in lactose utilization.

Catabolite repression or catabolite repression-like phenomena have been described in bacteria other than *E. coli*. Recently such a phenomenon has been reported for *Rhizobium meliloti*, a symbiotic nitrogen-fixing bacterium. Diauxic growth is observed when this organism is grown on succinate and lactose (17). The addition of cyclic AMP to this system does not cause reversal of the diauxie. Diauxie has also been described in *Propionibacterium shermanii* when it is grown anaerobically in glucose-lactate medium (12). The yeast *Candida tropicalis* exhibits glucose repression of cellobiose utilization, also producing diauxie (1). Dijkhuizen et al. (8) have described a diauxie phenomenon for *Pseudomonas oxalaticus* growing on mixtures of oxaloacetate and fumarate or acetate. Oxaloacetate transport is not inhibited by fumarate or acetate, suggesting that the pathway intermediate oxalyl coenzyme A may be the target for formate and acetate inhibition.

We report here diauxic growth which occurs when *Azotobacter vinelandii*, a nonsymbiotic nitrogen-fixing bacterium, is grown on medium containing both acetate and glucose. The effects of acetate on the glucose uptake system as well as other carbohydrates in *A. vinelandii* are reported. These studies reveal some aspects of possible regulatory processes in *A. vinelandii* not yet studied with regard to substrate uptake and biological nitrogen fixation.

MATERIALS AND METHODS

Chemicals and enzymes. The following chemicals used in this study were reagent grade and obtained commercially. Chloramphenicol, D-glucose, D-fructose, xylitol, D-mannitol, glyoxylic acid, trisodium DL-isocitrate, sodium citrate, sodium pyruvate, *cis*-oxalacetic acid, hydroxylamine, and *o*-dianisidine dihydrochloride were obtained from Sigma Chemical Co., St. Louis, Mo.; potassium acetate, sodium citrate, 2-oxoglutarate, trichloroacetic acid, malate, and sodium succinate were obtained from Fisher Scientific Co., Pittsburgh, Pa.; fumarate was from National Biochemicals Corp., Cleveland, Ohio; and triethanolamine hydrochloride was obtained from Eastman Kodak Co., Rochester, N.Y.

D-[U-^{14}C]glucose (13.9 mCi/mmol) and sodium salt–[1,2-^{14}C]acetate (56.5 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass.; D-[U-^{14}C]fructose (241 mCi/mmol), D-[U-^{14}C]mannitol (58 mCi/mmol), and [U-^{14}C]xylitol (87.5 mCi/mmol) were obtained from Amersham Corp., Arlington Heights, Ill.

Acetate kinase (EC 2.7.2.1; 638 U/mg) was from Millipore Corp., Bedford, Mass.; glucose oxidase (EC 1.1.3.4; 183 U/mg) was from Worthington Diagnostics, Freehold, N.J.

Media and buffers. Burk minimal medium (BM) contained the following (per liter of distilled water): 0.2 g of KH_2PO_4; 0.2 g of $MgSO_4 \cdot 7H_2O$; 0.09 g of $CaCl_2 \cdot 2H_2O$; 0.25 mg of $NaMoO_4 \cdot 2H_2O$; 5.0 mg of $FeSO_4$; and 10 g of a given carbon source. Either ammonium acetate, ammonium chloride, or ammonium sulfate (each at a final concentration of 30 mM) was used as a nitrogen source in the above medium. Burk buffer (BB) at pH 7.0 was used for the uptake assays and contained all of the components in BM except the carbon source.

Bacterial strains and culture conditions. *A. vinelandii* OP was maintained on nitrogen-free BM slants at 4°C. Cultures (20 ml) were routinely obtained from stock and grown for up to 24 h, sedimented, and suspended in 1 ml of BB before experimentation. All cultures were incubated at 28°C on a rotary shaker. Experimental cultures were inoculated to 20 to 30 Klett units of cells, and growth was monitored turbidimetrically with a Klett-Summerson photoelectric colorimeter (Klett filter no. 66). Diauxie cultures were pregrown in medium containing sucrose (2%, wt/vol) and ammonium acetate (30 mM). These cells were used as inoculum for the diauxie medium, which contained 2% glucose and 30 mM ammonium acetate. Cultures for some of the substrate uptake studies were precultured for 36 h on BM–2% sucrose, and then a 0.5% inoculum was transferred to a Fernbach flask containing 500 ml of BM with 1% carbon source. The cultures were grown for 18 to 20 h at 28°C on a

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rotary shaker. Cells were harvested by centrifugation (8,000 × *g*) at room temperature, washed in 250 ml of BB, and then suspended in 5 ml of BB at 20 Klett units.

Uptake studies. Uptake analyses were conducted in 10-ml glass vials (25 by 55 mm) placed on a magnetic stir plate. The assay mixtures (3.5-ml final volume) contained 1.2 ml of BB, 0.3 ml of chloramphenicol (1 mg/ml), and 2 ml of the cell suspension. A continuous flow of air was supplied to the transport mix by using a 15-cm Pasteur pipette attached to an air pump. A microspin bar (2 by 7 mm) was placed in the vial to allow continuous mixing of cells during the assay. The uptake mixtures, containing approximately 10^9 cells per ml, were incubated at 25°C. The experiment was initiated by the addition of a 200-μl sample of labeled substrate to the uptake mixture. Uptake was terminated by removing a 200-μl sample from the transport mix and diluting into 10 ml of BB at room temperature. The sample was mixed on a Vortex mixer and immediately filtered through a Gelman membrane filter (0.45-μm pore size) mounted on a Millipore sampling manifold attached to a vacuum pump. All membrane filters were washed before the experiment with 5 ml of BB. After filtration the filters were placed into scintillation vials, to which 5 ml of scintillation fluid [666 ml of toluene, 333 ml of Triton X-100, 5.5 g of (2,5-diphenyloxazole), and 0.15 g of dimethyl 1,4-bis(5-phenyloxazolyl)benzene] was added, and vials were counted in a Packard liquid scintillation spectrometer. Uptake was expressed as nanomoles of labeled substrate taken up per milligram (dry weight) of cells.

Biochemical assays. A glucose oxidase assay (20) was used to determine concentrations of glucose in media during diauxic growth. Samples (0.7 ml) of cells were removed from the culture at appropriate time intervals and filtered with Gelman membrane filters (0.45-μm pore size). A 1:5 dilution was made of the filtrate, and 0.3 ml of this diluted filtrate was added to a 3-ml cuvette already positioned in a Guilford spectrophotometer set at 460 nm and 25°C. Before the addition of the sample this cuvette contained 0.1 ml of oxygenated 1% *o*-dianisidine hydrochloride in 2.5 ml of 0.1 M potassium phosphate buffer at pH 6.0 and 0.1 ml of a stock solution of peroxidase (200 mg/ml). The reaction was initiated by the addition of 0.1 ml of glucose oxidase (1 mg/ml) and the addition of the filtered sample to the cuvette. The reaction velocity was determined by the increase in absorbancy at 460 nm resulting from the oxidation of *o*-dianisidine by means of the peroxidase-coupled system. Glucose concentrations were read from a standard curve of known glucose concentrations versus reaction velocity.

Acetate concentration in the diauxic culture medium was determined by the method of Bergmeyer and Mollering (3). Samples (2 ml) were removed from a 100-ml culture at appropriate times and centrifuged at 10,000 × *g* for 10 min. The supernatant solution was diluted 1:6 in reaction buffer, and a 0.1-ml sample of the dilution was used in the assay. The concentration of acetate in samples was interpolated from an acetate standard curve and reported as the percentage of acetate remaining in the culture medium.

Isocitrate lyase activity was measured by the procedure of McFadden (13) by using glyoxylate to prepare a standard curve. Samples (0.5 ml) were withdrawn at appropriate time intervals from a 75-ml growth culture. These samples were centrifuged at 7,000 × *g* for 15 min at 4°C and suspended in 2.5 ml of BB, and the cells were disrupted by sonication. The disrupted cells were centrifuged at 10,000 × *g* at 4°C for 30 min, and the crude extract was assayed for isocitrate lyase activity. A 0.1-ml sample of the crude extract was used per assay in 1.5 ml of Tris-Mg^{2+} buffer (1.0 M Tris [pH 7.7] containing 3 mM $MgCl_2$) and 0.2 ml of 0.125 M glutathione (freshly prepared in Tris-Mg^{2+} buffer). This mixture was preincubated at 30°C for 10 min. The reaction was initiated by the addition of 0.2 ml of 40 mM trisodium DL-isocitrate (prepared in Tris-Mg^{2+} buffer) and incubated for 10 min at 30°C. The reaction was terminated upon the addition of 10 ml of 10% trichloroacetic acid. A 0.1-ml sample was removed from this mixture and placed in a 30-ml beaker. A 5:1 mixture of 10 mM oxalic acid and 1% phenylhydrazine hydrochloride (6.0 ml) was added, and the mixture was heated until just boiling. The solution was immediately removed and cooled at room temperature for 5 min and then chilled on ice for 2 min. Upon removal from the ice, 4.0 ml of concentrated HCl and 1.0 ml of 5% potassium ferricyanide (freshly prepared) were added. The contents of the beaker were then transferred to a spectrophotometric tube; after exactly 7 min of incubation at room temperature, the absorbance at 520 nm was read against a buffer blank. Total protein was determined by the biuret assay of Chaykin (6).

Acetylene reduction assays were performed by the procedure of Bishop et al. (4). Cells (1-ml samples) were removed at appropriate times from the diauxic culture, and N_2-fixing ability was measured via acetylene reduction. Cell dry weight was determined by weighing 1 ml of cells (washed and suspended in 1 ml of BB) dried at 80°C for 2 h.

The luciferin-luciferase technique of Cole et al. (7) with the modifications of Wilson et al. (18) was used to determine intracellular ATP concentrations during diauxie. Samples of cells (2.0 ml) were removed from 100-ml diauxic cultures at various times, and 0.4 ml of each was used in the assay.

RESULTS

Diauxic growth of *A. vinelandii* on medium containing glucose and acetate. Diauxic growth of *A. vinelandii* occurred in medium containing 2% glucose and 30 mM potassium acetate (Fig. 1). However, diauxie was not observed when cells were grown on BM-glucose containing ammonium chloride or ammonium sulfate as the sole nitrogen source. The acetate-glucose diauxic growth phases were separated by a lag phase of about 4 h, after which the second growth phase of diauxie began. Cells grown on medium containing only 30 mM ammonium acetate (i.e., the concentration used in the diauxic medium) as the sole carbon and nitrogen source ceased growing after 6 h of incubation, corresponding to the onset of the acetate-glucose diauxie lag. These results suggested that the first phase of diauxie might result from the utilization of acetate as the primary carbon source, since cells continue to grow on media containing only 2% glucose and either ammonium sulfate or chloride as the source of nitrogen. The duration of growth during the first phase of diauxie was dependent upon the concentration of ammonium acetate in the diauxie medium (data not shown). Increasing the ammonium acetate concentration to 60 mM extended the onset of the diauxic lag to 12 h compared with 6 h obtained with 30 mM ammonium acetate.

Measurements of the rate of acetate and glucose utilization during diauxie clearly substantiated the preferential use of acetate as the primary carbon source (Fig. 2). The concentration of acetate in the medium decreased during the first phase of diauxic growth and was depleted after 6 h of incubation, corresponding to the onset of the diauxic lag. Once acetate was depleted from the diauxie medium, *A. vinelandii* began to metabolize glucose (Fig. 2). There was no immediate switch to glucose utilization since there was no appreciable decrease in the concentration of glucose until

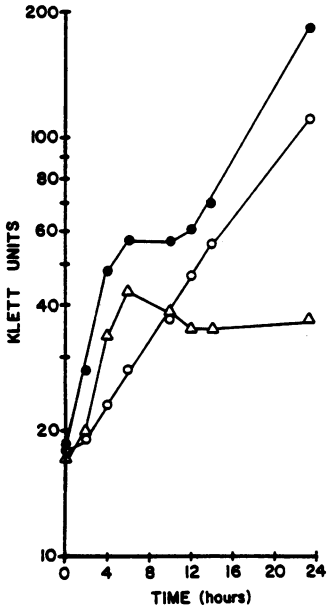

FIG. 1. Growth of *A. vinelandii* in glucose (○), glucose and acetate (●), and acetate (△). Cultures were pregrown in BM–1% sucrose, 30 mM potassium acetate, and 30 mM ammonium chloride at 28°C with vigorous shaking for 16 h before inoculation into experimental media containing BM, 30 mM ammonium chloride, 1% glucose, or 30 mM potassium acetate (or a combination) where applicable and were incubated with vigorous shaking at 28°C.

after about 10 h of incubation, some 4 h after the onset of diauxie.

Uptake of glucose and acetate during diauxie. Two mechanisms might be suggested to explain the occurrence of diauxie, namely, catabolite repression and inducer exclusion. Catabolite repression would involve an acetate-mediated repression of the synthesis of proteins needed for utilization of glucose, whereas inducer exclusion would involve an exclusion of the uptake of glucose by acetate. To investigate these possibilities, we studied glucose and acetate uptake during acetate-glucose diauxie (Fig. 3 and 4).

Cells harvested at various times during the first stage of diauxic growth incorporated labeled acetate and retained this ability throughout the second phase of acetate-glucose diauxie (Fig. 3). On the other hand, cells harvested during the initial diauxic growth phase did not significantly take up radiolabeled glucose (Fig. 4). The capacity to take up glu-

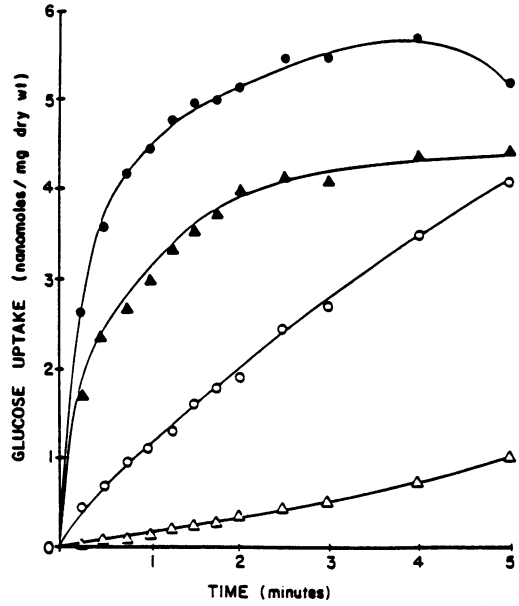

FIG. 3. Acetate uptake during *A. vinelandii* diauxie. Cultures were pregrown and incubated as in Fig. 1. Samples for transport studies were taken at 2 h (●), 7 h (●), 14 h (■), and 23 h (▲).

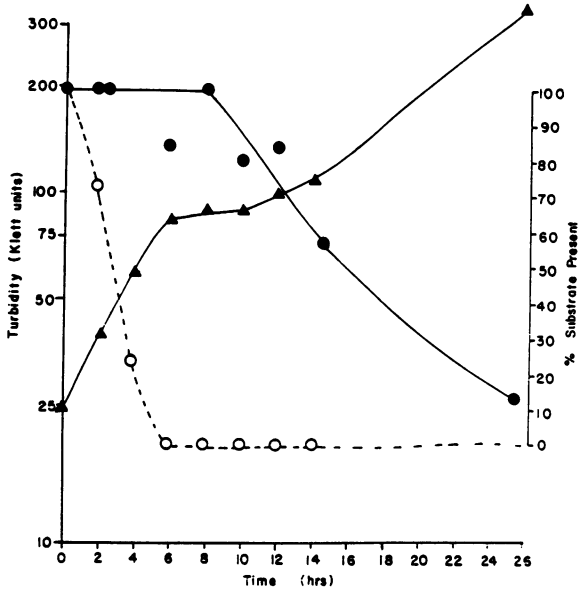

FIG. 2. Utilization of acetate (○) and glucose (●) during *A. vinelandii* diauxic growth (△). Cultures were pregrown and incubated as in Fig. 1. Acetate concentration was measured with acetate kinase, and glucose concentration was determined with glucose oxidase.

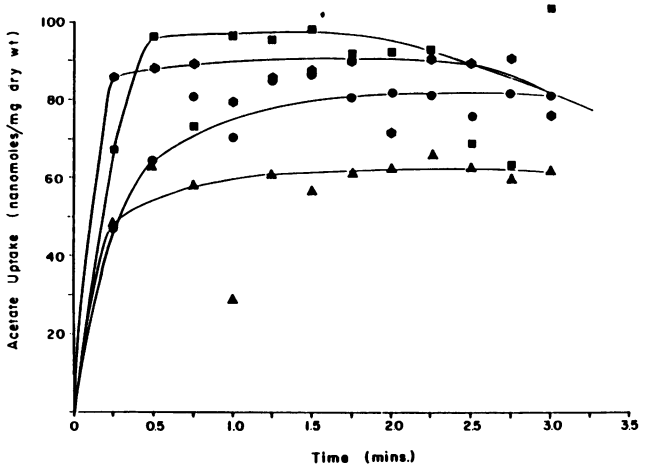

FIG. 4. Glucose uptake during diauxic growth of *A. vinelandii*. Glucose uptake was performed on cultures harvested after 2 h (△), 8 h (○), 14 h (●), and 22 h (▲) of growth on BM–1% glucose plus 30 mM ammonium acetate. The diauxic curve is shown in Fig. 1.

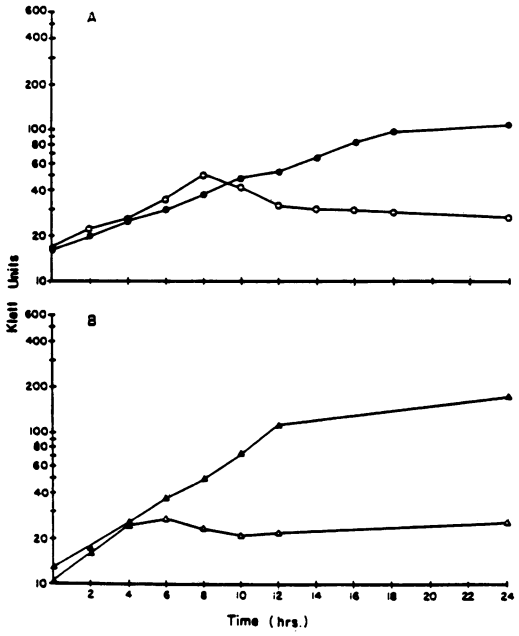

FIG. 5. Growth of *A. vinelandii* in maltose or xylitol medium. Cultures were grown in 20 ml of BM containing either (A) 1% maltose or (B) 1% xylitol. Ammonium acetate (○, △) or ammonium chloride (●, ▲) was added to the media at a final concentration of 30 mM.

cose increased during the subsequent stages of diauxie, indicating that the proteins required were inducible, whereas the acetate system appeared constitutive, under the conditions examined. *A. vinelandii* cells grown solely on glucose initially took up labeled glucose in the presence of exogenously added acetate (data not shown). These results suggested that acetate repressed synthesis of the glucose transport system rather than inactivating this transport system. Diauxic growth exhibited by *A. vinelandii* growing on medium containing acetate and glucose resulted from the use of acetate as the primary carbon source. Upon the deletion of acetate, the glucose uptake system was expressed, and *A. vinelandii* utilized glucose.

Effects of acetate on the uptake and utilization of other substrates. Cells grown on BM-sucrose incorporated both glucose and fructose (data not shown), but failed to transport mannitol or xylitol; cells grown on acetate, however, failed to take up any of these substrates (data not shown). *A. vinelandii* grew in BM containing maltose as a sole carbon source and NH_4Cl as a nitrogen source (Fig. 5A). However, when *A. vinelandii* was grown in medium containing acetate and maltose, only growth on acetate was detected. Unlike the acetate-glucose diauxie described above, growth on maltose was inhibited by the prior presence of acetate. This was also observed when cells were grown in medium containing acetate and xylitol (Fig. 5B).

Inhibition of glucose utilization by various TCA intermediates. Acetate is metabolized in many bacteria via the glyoxylate cycle. We became interested in determining what effect, if any, various intermediates of acetate metabolism had on the utilization of glucose by *A. vinelandii*. Such an investigation would allow us to assess those particular intermediates responsible for the inhibition of glucose utilization during acetate-glucose diauxie. For this purpose, cells were pregrown on sucrose and a tricarboxylic acid (TCA) intermediate, harvested, placed in fresh medium containing glucose and the TCA intermediate indicated, and allowed to incubate for 1.5 h. After this time the concentration of glucose remaining in the medium was determined (Table 1). These cultures failed to utilize glucose in the presence of acetate as shown earlier (Fig. 2); citrate, isocitrate, and 2-oxoglutarate were also effective inhibitors of glucose utilization (Table 1). Fumarate and malate inhibited glucose utilization also, but to a lesser extent. Under these conditions neither pyruvate nor oxaloacetate significantly inhibited glucose utilization. Succinate was found to stimulate glucose utilization (Table 1).

Those compounds which inhibited glucose utilization were added to BM-glucose containing *A. vinelandii* cells, and growth was monitored. Diauxic growth occurred in BM-glucose containing either 30 mM citrate or isocitrate (data not shown). The initial phase of growth for both citrate-glucose or isocitrate-glucose diauxie terminated after 6 h of incubation. The duration of the diauxic lag was found to vary, i.e., 9 h for isocitrate-glucose diauxie and 6 h for citrate-glucose diauxie, respectively.

Expression of isocitrate-lyase during diauxic growth. In the acetate-glucose medium, the initial growth phase was associated with a sixfold increase in isocitrate lyase activity (Fig. 6). This activity subsequently decreased during the diauxic lag followed by another burst of synthesis during growth on glucose in the second diauxie phase. It thus would appear that *A. vinelandii* regulates isocitrate lyase activity during diauxic growth.

Nitrogen fixation during acetate-glucose diauxie. *A. vinelandii* can use atmospheric nitrogen due to the presence of the nitrogenase enzyme complex. To investigate the expression of nitrogenase during diauxic growth, cells were grown in nitrogen-free BM containing acetate and glucose as described above. Nitrogenase activity, as measured by acetylene reduction, was found to increase during the initial growth phase of diauxie, reaching a maximum value of about 40 nmol of C_2H_2/h per mg (dry weight) (Fig. 7). This peak activity was reached after 5 h of growth and began to decrease with the onset of the diauxic lag. No nitrogenase activity was detected during the diauxic lag. During the second diauxic phase the nitrogenase activity increased to about 115 nmol of C_2H_2/h per mg (dry weight) (Fig. 7).

TABLE 1. Effects of TCA intermediates on glucose utilization in *A. vinelandii* [a]

Competitor	% of glucose metabolized
None	48
Acetate	0
Citrate	0
Isocitrate	6
2-Oxoglutarate	10
Succinate	95
Fumarate	21
Malate	30
Oxaloacetate	44
Pyruvate	48
Acetate + cyclic AMP	0
Acetate + cyclic GMP	0

[a] *A. vinelandii* cells were pregrown in BM–1% sucrose containing 30 mM ammonium chloride and 30 mM TCA intermediate competitor at 28°C on a rotary shaker at 200 rpm. After 12 h of incubation, cells were harvested and inoculated into cultures containing BM–5 mM glucose with 30 mM ammonium chloride and 30 mM competitor and incubated for 1.5 h, after which the percentage of glucose was determined with glucose oxidase as described in Materials and Methods.

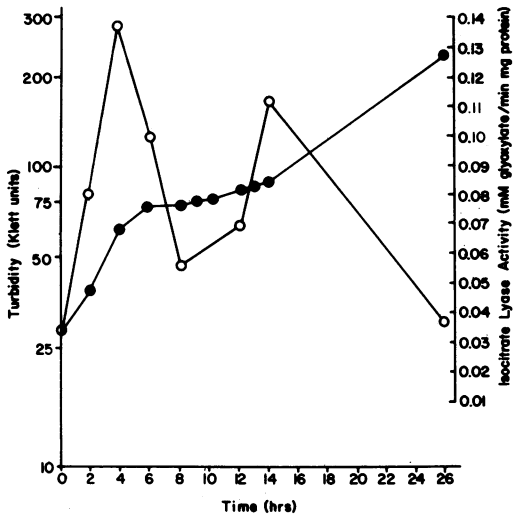

FIG. 6. Isocitrate lyase activity (○) during *A. vinelandii* diauxic growth (●). Cells were withdrawn during various stages of diauxie and assayed for isocitrate lyase activity.

One possible basis for the fluctuations in levels of nitrogen fixation during the various stages of diauxie was investigated. The fixation of atmospheric nitrogen and other cellular activities are dependent upon cellular energy in the form of ATP (7). We therefore sought to determine the extent to which the intracellular concentration of ATP could be correlated with nitrogenase activity during the entire diauxie period (Fig. 8). During the first growth phase a slight increase in intracellular ATP was noted (i.e., from 0.2 to 0.65 nmol of ATP per mg [dry weight]). There was very little increase in the levels of ATP observed during the diauxic lag; however, an increase of approximately fourfold in ATP levels was observed during the glucose phase of diauxie. These results may indicate a correlation between the expression of nitrogenase activity and the intracellular levels of

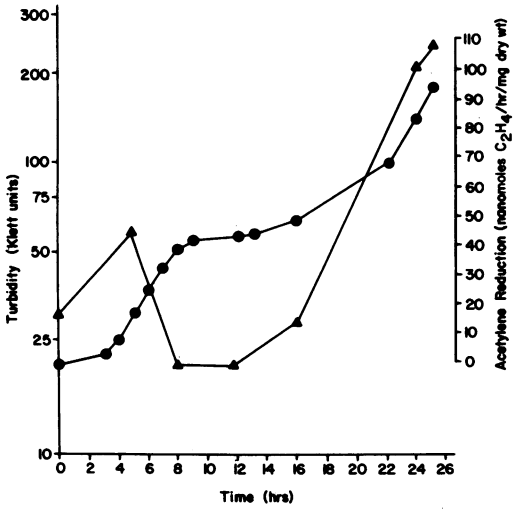

FIG. 7. Acetylene reduction (▲) during *A. vinelandii* diauxic growth (●). Cultures were grown on acetate-glucose medium and incubated at 28°C with vigorous shaking. Samples were withdrawn and assayed for acetylene reduction.

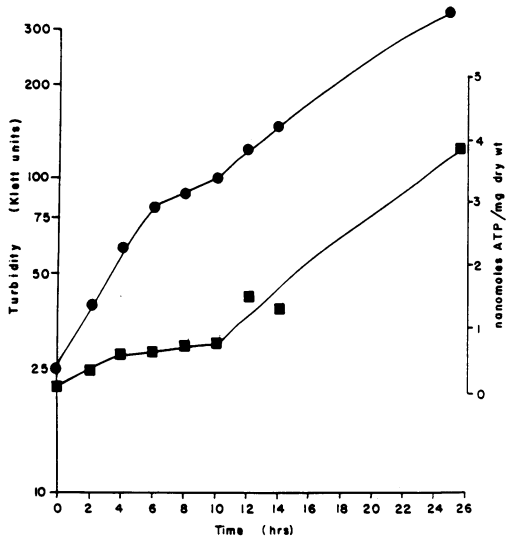

FIG. 8. Intracellular ATP concentration (■) during *A. vinelandii* diauxic growth (●). Cultures were prepared as in Fig. 7. Samples were taken and assayed for intracellular ATP concentration by using firefly lantern extract.

ATP during diauxic growth of *A. vinelandii*. Because other factors (i.e., reducing power) may be involved, it is difficult for us at this time to ascertain the role of ATP in the observed nitrogenase activity during diauxie.

DISCUSSION

Diauxic growth was observed when *A. vinelandii* was grown in medium containing both acetate and glucose as sole carbon sources (Fig. 1). Glucose was not metabolized until acetate was exhausted from the diauxic medium. Our results indicated that acetate transport uptake, like acetate oxidation (15), was constitutively expressed (Fig. 3). Since glucose was not taken up until after the diauxic lag (Fig. 2), it appeared that the synthesis of proteins necessary for glucose uptake was repressed in the presence of acetate.

Barnes (2) notes that in the presence of acetate only 7% of the normal transport of glucose occurs in *A. vinelandii* membrane vesicles. Acetate is also known to prevent the transport of serine in membrane vesicles prepared from *Bacillus subtilis* (16). Acetate has an uncoupling effect on the cytochrome-electron transport-linked carrier protein. In other microorganisms, acetate affects substrate utilization. Dijkhuizen et al. (8) have reported that *P. oxalaticus* uses acetate as the primary carbon source when it is grown in medium containing both acetate and oxalate. The oxalate metabolizing enzymes are repressed by acetate since uptake of oxalate is not inhibited by this substrate.

The inhibitory effect of acetate on glucose uptake in *A. vinelandii* led us to investigate whether TCA intermediates in general might exhibit some effects on glucose metabolism. Among the TCA intermediates tested (Table 1), only citrate, isocitrate, and 2-oxoglutarate strongly inhibited glucose utilization in *A. vinelandii*. Postma and Van Dam (15) have found that citrate and isocitrate use the same inducible translocation system in this organism, whereas succinate, fumarate, malate, and oxaloacetate enter the cell using a different transport system. Pyruvate oxidation in *A. vinelandii* is constitutive and does not inhibit glucose utilization. Burris and colleagues (5) have reported that respiration is increased in *A. vinelandii* grown on sucrose in the

presence of succinate, fumarate, 2-oxoglutarate, malate, or oxaloacetate. Citrate, formate, malate, and succinate have the same effect on mannitol oxidation.

The glyoxylate shunt is the main pathway by which acetate is metabolized in *A. vinelandii*. Growth of *A. vinelandii* on acetate during the first phase of diauxie stimulated isocitrate lyase activity (Fig. 6). This activity also increased during the glucose phase of diauxie. In *E. coli* no induction of the enzyme occurs when acetate is added to glucose medium (11). Pyruvate, on the other hand, represses isocitrate lyase synthesis in *E. coli* (11), *Arthrobacter atrocyanens* and *Arthrobacter pyridinolis* (19), and *Chlorella pyrenoidosa* (18). Oxaloacetate also inhibits isocitrate lyase activity in *C. pyrenoidosa* (10).

The majority of the nitrogen fixation studies in *A. vinelandii* have been conducted with cells grown in sucrose growth medium. Bishop and colleagues (4) report that *A. vinelandii* wild-type strain CA grown on sucrose has a very high rate of N_2 fixation. Interestingly, Haaker and Veeger (9) have determined that cells grown on sucrose and pyruvate, respectively, have different nitrogen-fixing capabillties. We wanted to determine nitrogen-fixing capabilities under the conditions of diauxie.

We observed a temporal expression of nitrogenase during diauxie (Fig. 8). The rate of nitrogen fixation under diauxie conditions for cells growing on acetate was 50% of that during growth on glucose (Fig. 7). The rate of nitrogen fixation was greatly decreased during the transition from acetate to glucose metabolism under diauxie.

Presently we cannot conclude a direct correlation of nitrogen fixation with ATP pool levels since other factors also need to be considered which can affect nitrogenase activity, i.e., reducing power. Milekhina and co-workers (14) have studied intracellular ATP accumulation in *A. vinelandii* cells grown on sucrose under N_2-fixing conditions. Maximum ATP concentrations are obtained after approximately 9 h of growth, remain constant for 24 h, and then decrease corresponding to the onset of stationary growth. Thus, *A. vinelandii* seemingly modulates ATPase and nitrogenase activities respective to carbon and nitrogen sources available during diauxic growth.

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