LEVELS OF PYRIDINE NUCLEOTIDE TRANSHYDROGENASE DURING ENCYSTMENT IN AZOTOBACTER VINELANDII

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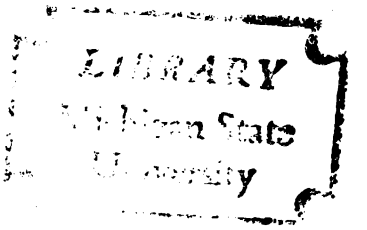
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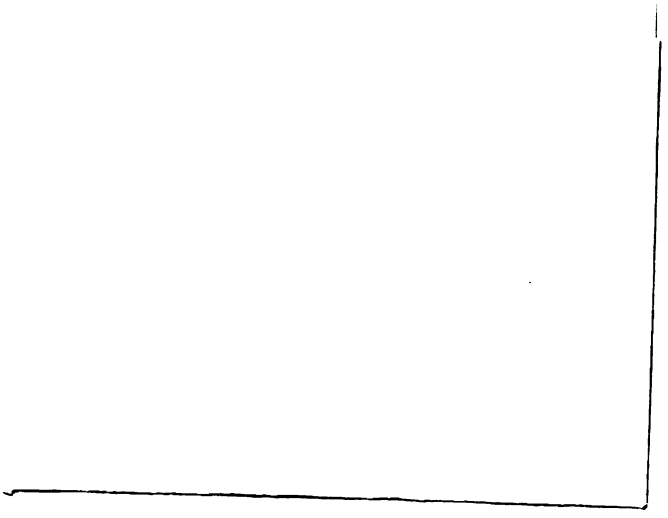


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ABSTRACT

LEVELS OF PYRIDINE NUCLEOTIDE TRANSHYDROGENASE DURING ENCYSTMENT IN AZOTOBACTER VINELANDII

By

Paulanne Chelf

Azotobacter vinelandii, when grown with glucose as a carbon source, contains measurable levels of pyridine nucleotide transhydrogenase activity. The specific activity of the enzyme increases 7-fold within a 4 h period after the glucose in the medium is replaced with B-hydroxybutyrate (BHB) to induce encystment. Vegetative cells of A. vinelandii grown with acetate as a carbon source have no measurable pyridine nucleotide transhydrogenase activity. When acetate is replaced with BHB, very little increase in specific activity of the transhydrogenase occurs. Microscopic observation and plate counting indicate that less than 0.2% of the cells grown in acetate and transferred to BHB form desiccation-resistant cysts. In contrast, more than 70% of the cells grown in glucose form cysts when transferred to BHB. We propose that pyridine nucleotide transhydrogenase is responsible for a shift in the levels of reduced

pyridine nucleotides, and that this shift is a critical step in the encystment process.

LEVELS OF PYRIDINE NUCLEOTIDE TRANSHYDROGENASE DURING ENCYSTMENT IN *AZOTOBACTER VINELANDII*

By

Paulanne Chelf

A THESIS

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1975

DEDICATION

To my mother

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I wish to extend my sincere appreciation to Dr. Harold Sadoff for his never-ending patience and infinite wisdom. Thanks are also due to Dr. James Tiedje and Dr. Clarence Suelter for their interest in this work as members of my guidance committee and to Dr. Ralph Costilow for his excellent review of the manuscript.

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INTRODUCTION

Bacterial pyridine nucleotide transhydrogenase was first described by Colowick et al. in 1952 (8), and subsequently it also has been found in plant and animal tissues (11, 12). The transhydrogenase of Azotobacter vinelandii has been purified to homogeneity and its kinetic properties evaluated (6, 19, 21). The bacterial enzyme catalyzes the transfer of electrons between the reduced and oxidized forms of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) as illustrated in the following equation:

$$NADPH + NAD^+ \rightleftharpoons NADP^+ + NADH$$

The reaction in vitro occurs rapidly from left to right as indicated, but the reverse reaction is relatively slow (6, 19, 20). The enzyme is capable of carrying out this reaction using nicotinamide hypoxanthine dinucleotide or -dinucleotide phosphate (deamino-NAD^+(P)) or thiol-NAD^+(P), and also catalyzes the interconversion of the oxidized and reduced forms of these pyridine nucleotides:

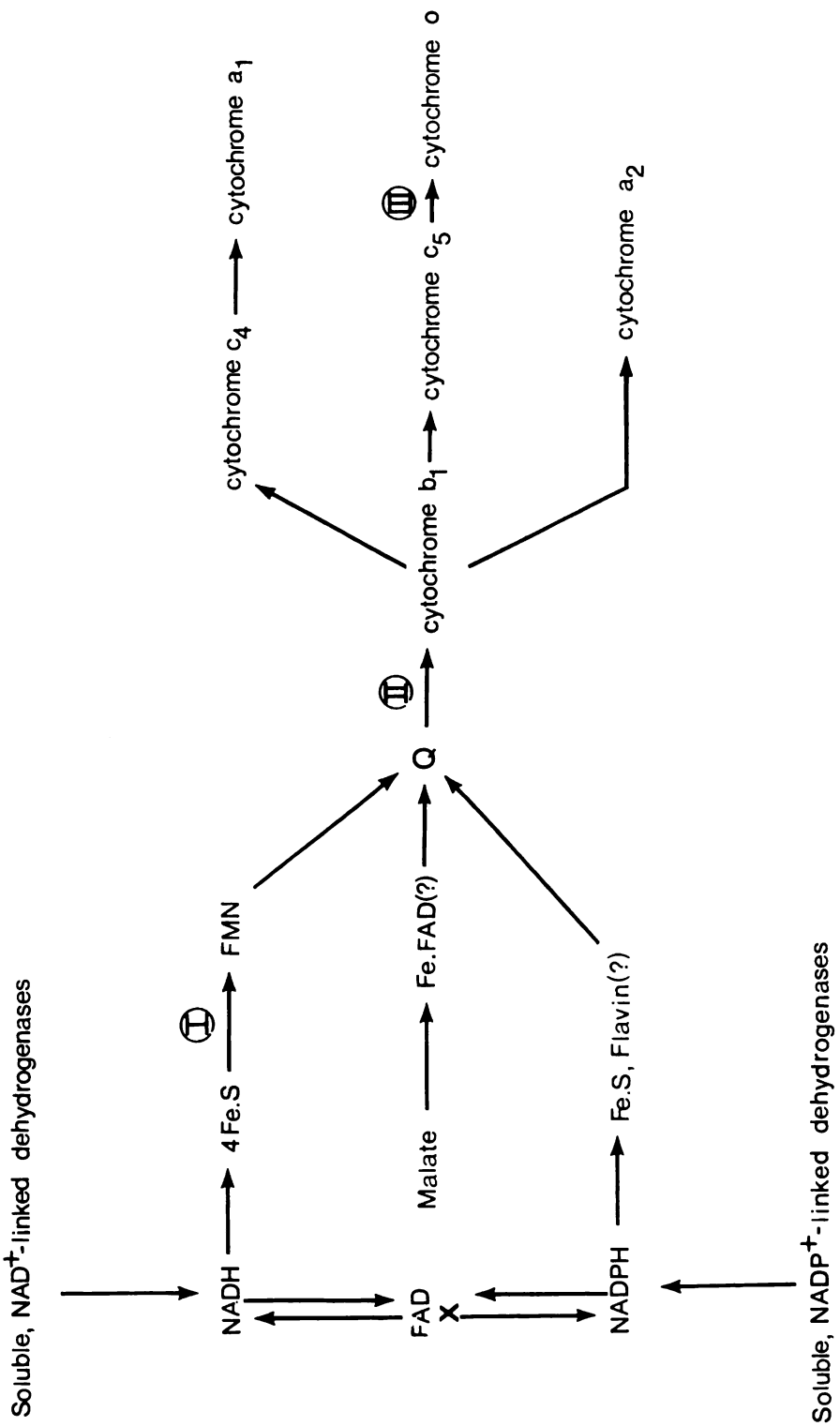
$$NADH + \text{deamino-}NAD^+ \rightleftharpoons NAD^+ + \text{deamino-}NADH;$$

$$NADPH + \text{deamino-}NADP^+ \rightleftharpoons NADP^+ + \text{deamino-}NADPH.$$

The physiological function of the transhydrogenase enzyme with respect to the overall metabolic activity of pyridine nucleotide coenzymes is not well defined. When this enzyme was first discovered, Colowick et al. (8) thought that it might play an important role in regulating the pathway of electron transport. They further suggested that if NADH and NADPH systems were found to differ in their phosphorylation ability, transhydrogenase might serve to regulate the conversion of oxidative energy into phosphate bond energy. Work with the respiratory system of A. vinelandii in more recent years has indeed demonstrated a difference in the phosphorylation efficiency of the NADPH and the NADH respiratory chains (1, 10, 22). The respiratory system of Azotobacter is shown in Figure 1 (Yates & Jones, 22). Thus entry into the respiratory chain at the level of NADH results in greater phosphorylation than entry at the level of malate or NADPH. Transhydrogenase is therefore in a position to exert control over both the intracellular $NAD(P)H/NAD(P)^+$ ratio and the extent of oxidative phosphorylation.

A. vinelandii cells are capable of undergoing a cyclic process of differentiation to produce metabolically dormant, thick-walled cells known as cysts. The cell

Figure 1. Schematic diagram of the electron transport system of Azotobacter. Phosphorylation sites are designated by I, II, and III.


Soluble, NAD+-linked dehydrogenases
NADH
FAD
4 Fe.S
FMN
Malate
Fe.FAD(?)
NADPH
Fe.S, Flavin(?)
Soluble, NADP+-linked dehydrogenases
Q
cytochrome b1
cytochrome c4
cytochrome a1
cytochrome c5
cytochrome o
cytochrome a2

undergoes this process in the laboratory in response to specific inducers, which include n-butanol, B-hydroxybutyrate (BHB), and crotonate (13). Vegetative cells of this organism possess "a very high level of pyridine nucleotide transhydrogenase" (6) ranging from 0.3 - 1.0 unit/mg of soluble protein. Vegetative cells of A. vinelandii are capable of using atmospheric nitrogen, but cessation of nitrogen fixation occurs rapidly upon the induction of encystment with BHB, suggesting that pool levels of NADPH, the primary reductant (4), may be depleted. We have therefore measured the levels of transhydrogenase in A. vinelandii during the course of encystment with the thought that this enzyme may promote shifts in energetic and synthetic pathways.

MATERIALS AND METHODS

Strain and Cultivation

A. vinelandii ATCC 12837, which was used throughout the course of these experiments, was cultivated in Burk's nitrogen-free medium (5) at 30 C. Vegetative cells were grown to mid-exponential phase (0.6 O.D.) with 1% glucose or 0.2% sodium acetate as the carbon source. To prepare cysts, cells were harvested aseptically, washed twice with sterile Burk's buffer (Burk's medium without carbon source), resuspended in sterile Burk's buffer with half the normal phosphate concentration and 0.2% BHB as the carbon source, and incubated with aeration at 30 C. All carbon sources were prepared as aqueous solutions and were autoclaved separately from the Burk's buffer.

Abortive encystment (13) was initiated by cultivating cells in the presence of 1% glucose, washing twice with sterile Burk's buffer, and resuspending the cells in fresh medium containing 0.2% BHB and 0.2% glucose.

Preparation of Cell Extracts

Vegetative and encysting cells of A. vinelandii from 50 ml of culture were harvested by centrifugation

and resuspended in 5 ml of 0.05 M tris(hydroxymethyl) amino methane (Tris) buffer, pH 8.0, containing 10^{-3} M ethylenediamine tetraacetic acid (EDTA). The cells were subjected to sonic oscillation (Measuring & Scientific Equipment, Ltd.) for 15 sec periods followed by 45 sec cooling until 2.5 min treatment was achieved. Cellular debris was removed by centrifugation at 10,000 x g at 4 C in a Sorvall RC2-B refrigerated centrifuge. This supernatant solution was spun again at 35,000 x g for 30 min, and the clarified extract served as the source of the enzyme.

Proteolytic activity was measured by combining 1.0 ml of 0.5% azoalbumin with 0.5 ml of cell free extract. Distilled water was added to the blank, and all tubes were incubated at 30° C for 20 min. At the end of incubation, 2.0 ml of 10% trichloroacetic acid was added to each tube. Tubes were spun at high speed on a clinical centrifuge for 15 min, and the supernatant was read at 340 nm.

Protein was determined by the method of Lowry et al. (14). Desiccation resistant cysts were enumerated according to the method of Socolofsky and Wyss (17), but using Whatman #4 filter paper in place of membrane filters. The Whatman paper disintegrates upon vigorous agitation in solution and therefore allows more accurate quantitation of cysts.

Materials

Deamino-NAD^+ and glucose-6-phosphate dehydrogenase ($NADP^+$-specific) were obtained from Sigma Chemical Co. All other reagents were analytical grade and were obtained from various commercial sources.

Assay of the Enzyme

The enzyme activity was measured at 30 C by modifications of existing assays (8, 19). The conditions were: 0.05 M Tris buffer, pH 8.0, with 10^{-2} M $MgCl_2$, 10^{-4} M $NADP^+$, 6 x 10^{-3} M glucose-6-phosphate (final concentrations in the assay volume), and 10 to 20 ul of a one unit/ml stock solution of glucose-6-phosphate dehydrogenase (one unit oxidizes one umole of glucose-6-phosphate per min at 25 C, pH 7.4) in a total volume of 2.0 ml. This reaction was monitored at 340 nm until no further absorbance change occurred, and then deamino-NAD^+ (final concentration of 10^{-4} M) and 100 ul of transhydrogenase enzyme preparation were added to initiate the reaction. One enzyme unit is defined as the amount which catalyzes the reduction of one umole of deamino-NAD^+ in one min under the above assay conditions. The specific activity is expressed in enzyme units per milligram of protein.

RESULTS

Optimal Method of Cell Breakage

Preliminary experiments were carried out to select a method of cell and cyst breakage which would permit reproducible enzyme recovery. Vegetative cells were easily ruptured using lysozyme, sonic treatment, or extrusion through a French pressure cell. Cysts were very resistant to all of these methods of cell breakage unless the cyst coats were initially disrupted by use of a chelating agent such as EDTA prior to breakage (17).

Enzyme assays of lysate fractions of the vegetative cells indicated that sonication resulted in the greatest yield of enzyme activity. This activity was found in the supernatant fraction resulting from centrifugation of extracts at 78,000 x g for 4.5 h in a Beckman Model L3-50 ultracentrifuge. Cell extracts clarified at 35,000 x g for 30 min had equivalent activities and thus the lower speed was used throughout this work. Cyst lysates prepared by sonication and by extrusion through a French pressure cell possessed comparable levels of enzyme activity. However, lysates

prepared by extrusion through the French pressure cell contained transhydrogenase in both the high speed supernatant and pellet, whereas activity in the sonicated lysates was found only in the supernatant fraction. Sonication seemed to be more effective in solubilizing the transhydrogenase and was therefore used for preparation of all cell and cyst lysates.

Transhydrogenase Levels During Encystment and Abortive Encystment

Cultures which had been grown to mid-exponential phase with glucose or acetate were washed free of carbon source and resuspended in 0.2% BHB. Samples for assay of transhydrogenase activity were taken at this time and again after 1, 2, 3, and 4 h of incubation (Fig. 2). Transhydrogenase specific activity in cultures grown in glucose and undergoing normal encystment underwent a progressive 7-fold increase by 4 h while those cells grown initially in acetate were almost devoid of the enzyme. In abortive encystment the transhydrogenase levels remained at approximately the same as those in glucose-grown vegetative cells. In longer term experiments, Figure 3, it was repeatedly observed that the initial peak of transhydrogenase activity occurred and then fell to zero at 6 h. A smaller peak of activity was detected at 9 h into the encystment process which fell to zero by 18 h and then rose to pre-induction

Figure 2. Comparison of transhydrogenase activity in cultures grown in glucose or acetate containing medium and induced to encyst with BHB. Time -2 h represents vegetative growth. At time 0 h cells were washed and transferred to Burk's buffer plus 0.2% BHB. The blackened areas represent activity in cells grown in acetate, gray areas show activity during abortive encystment; white areas represent cells grown in glucose.

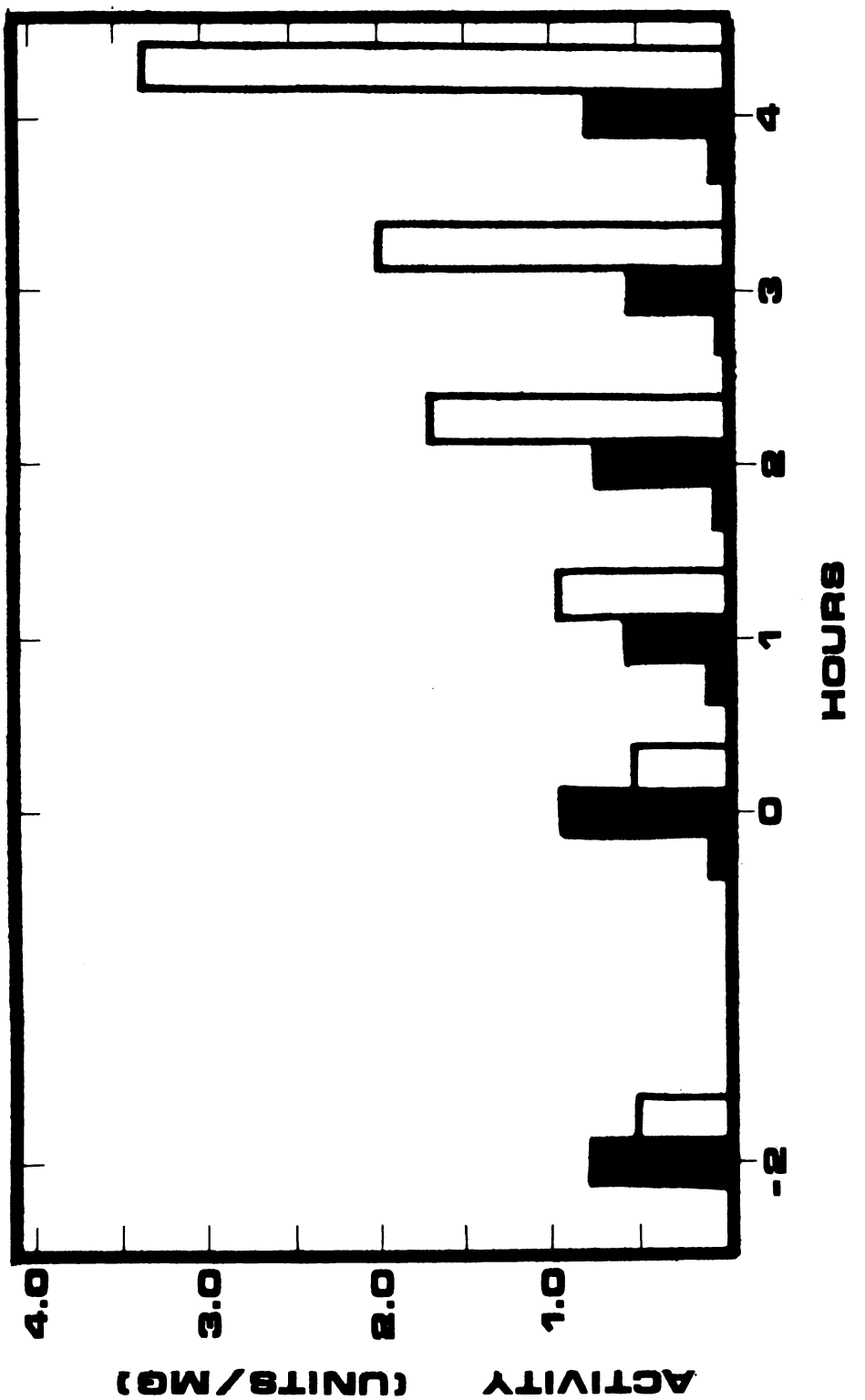
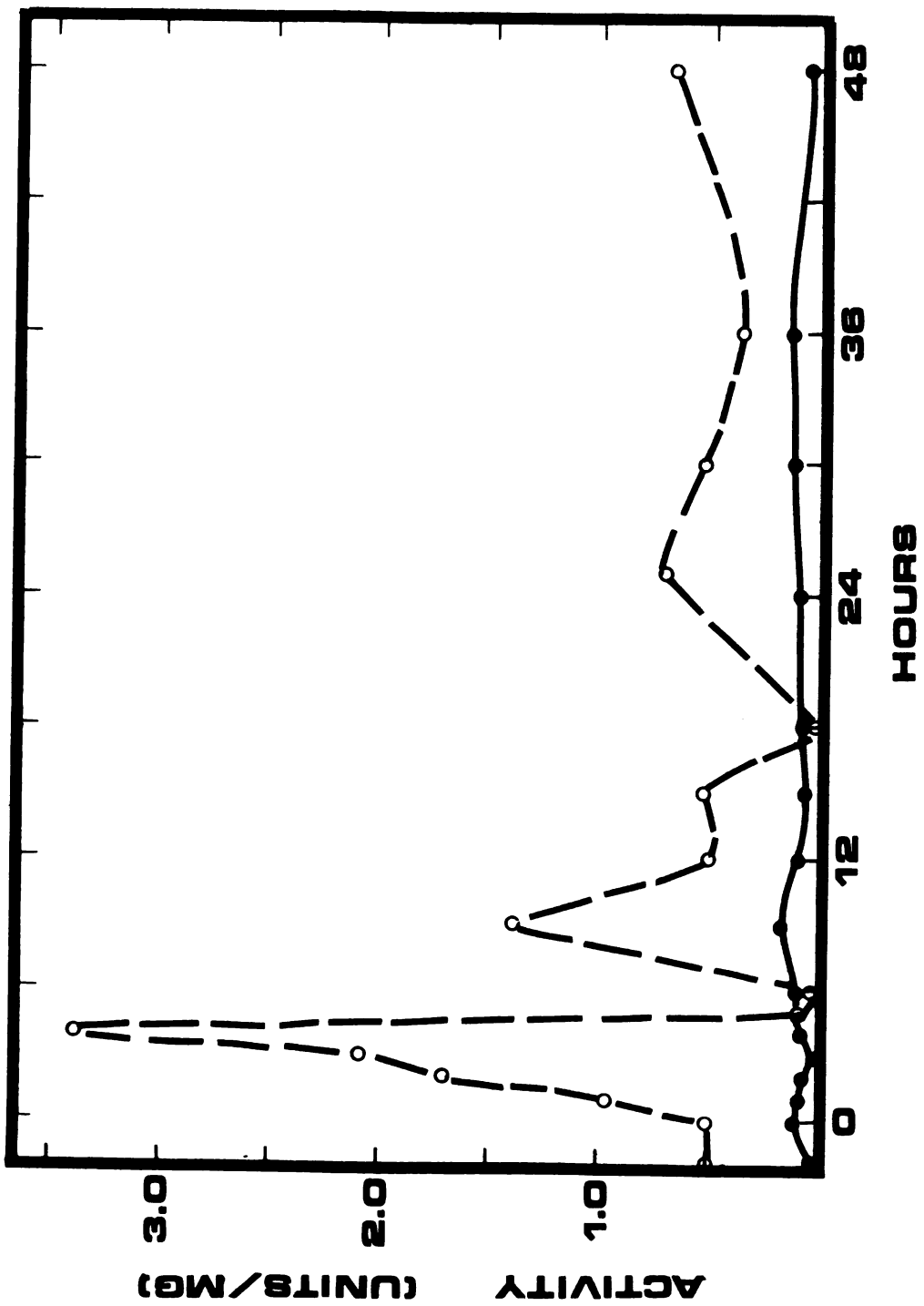

ACTIVITY (UNITS/MG)
4.0
3.0
2.0
1.0
HOURS
-2
0
1
2
3
4

Figure 3. Transhydrogenase activity in cells grown in glucose or acetate and transferred at time 0 h to Burk's buffer plus 0.2% BHB. Symbols: O, glucose-grown cells; ●, acetate-grown cells.


ACTIVITY (UNITS/MG)
3.0
2.0
1.0
0
12
24
36
48
HOURS

levels until the completion of encystment. Intracellular protease could not be detected in extracts at any time during encystment. The specific activity of transhydrogenase in the acetate-grown cells was very low, and additional activity was not induced in cells upon their resuspension in BHB. Levels remained very low throughout the 48 h period after BHB addition.

Microscopic observations and plate counting indicated that less than 0.2% of the acetate-grown culture formed desiccation resistant cysts after resuspension in BHB. In contrast, 48 h after the induction of encystment the glucose-grown culture which had been resuspended in BHB had undergone greater than 70% encystment.

The addition of BHB to the transhydrogenase assay mixture produced no measurable effect and thus the fatty acid is probably not an allosteric effector of the enzyme. NAD^+-linked BHB dehydrogenase which is also induced during encystment (9) was present in these cell-free extracts, but was inactive with deamino-NAD^+. Glucose-6-phosphate dehydrogenase from A. vinelandii was also inactive with this dinucleotide.

In a separate set of experiments, cells were grown to mid-exponential phase with glucose as the carbon source, washed free of glucose, and transferred to an acetate containing medium. Ten hours after this

transfer had occurred, the specific activity of transhydrogenase in these cells had fallen to one-third that in the initial glucose-grown culture. A. vinelandii has a generation time under these conditions of approximately 3 h, so if acetate was responsible for the repression of transhydrogenase synthesis one would expect approximately one-tenth the original activity to remain after 10 h. The results suggest that the synthesis of the enzyme proceeds more slowly in the presence of acetate or that synthesis continues until the concentration of some metabolic product of acetate represses further transhydrogenase synthesis.

DISCUSSION

Pyridine nucleotide transhydrogenase is in a unique position in the cell to regulate both the intracellular NAD(P)H/NAD(P)$^+$ ratio and the extent of oxidative phosphorylation. Since reduced nucleotide levels may have a profound effect on the metabolic processes which are operative during encystment, consideration must be taken of the metabolic processes which contribute to the levels of these molecules during encystment.

Differentiation in Azotobacter involves the functioning of the tricarboxylic acid (TCA) cycle, the glyoxylate cycle, and gluconeogenesis (9). An important TCA cycle enzyme in A. vinelandii is the highly active NADP$^+$-linked isocitrate dehydrogenase. This enzyme constitutes about 2% of the total cellular protein (7) and is probably the principal generator of NADPH in the cell. Barrera and Jurtshuk (3) have proposed that isocitrate oxidation is the primary source of electrons (NADPH) for nitrogen fixation in this organism because the specific activity of the isocitrate dehydrogenase is 10- to 100-fold greater than any other dehydrogenase

in the cell. Nitrogen fixation, however, ceases within one hour after the induction of encystment with BHB (9), and this could result in an accumulation of NADPH. High reduced pyridine nucleotide levels in Azotobacter specifically inhibit citrate synthase, isocitrate dehydrogenase, and enzymes involved in glucose catabolism (16). Inhibition of the TCA cycle would reduce available levels of intermediates and thus affect the glyoxylate cycle and gluconeogenesis as well. It is apparent that reduced nucleotide levels must be closely regulated during the encystment process. Excess NADPH may be oxidized via the NADPH dehydrogenase or may be transhydrogenated to NADH and oxidized via the NADH oxidase system. Ackrell et al. (2) feel that a major fate of NADPH in the cell is transhydrogenation, and that NADPH dehydrogenase functions only at very high substrate concentrations.

The results presented here are consistent with the hypothesis that pyridine nucleotide transhydrogenase is an important regulatory enzyme during encystment. Comparison of transhydrogenase activity in an encysting culture and a nonencysting culture gives support to this argument. In cells grown in glucose containing medium and transferred to BHB, the rise in transhydrogenase activity is accompanied by encystment. In cells grown in acetate and transferred to BHB,

the failure to induce transhydrogenase activity is accompanied by failure to encyst. It appears that the physiological signal which induces transhydrogenase is lacking in acetate-grown cells. This signal may be the level of NADPH. Van den Broek and Veeger (20) have demonstrated two binding sites for NADPH on the enzyme: one catalytic and one regulatory. Cells growing in acetate must utilize the anaplerotic glyoxylate cycle in addition to the TCA cycle. Nagai et al. (15) have presented evidence which suggests that the glyoxylate cycle predominates under these conditions. Since cells utilizing the glyoxylate cycle by-pass the NADPH-generating isocitrate dehydrogenase, pool levels of this reduced nucleotide are probably lower in acetate-grown cells. This is supported by the fact that the rate of nitrogen fixation (for which NADPH is the primary reductant [4]) during vegetative growth on acetate is lower than that of glucose-grown cells (9).

The precipitous drop in transhydrogenase activity early in encystment was repeatedly observed and presumably represents some mode of cellular control of this activity. It is unlikely that this enzyme is degraded extensively and resynthesized since no proteolytic activity could be detected in encysting cells. This does not exclude the possibility of other types

of covalent modification. Alternatively the enzyme may undergo association-dissociation reactions with a tightly bound molecule.

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