

Physiological Studies of Encystment in *Azotobacter vinelandii*¹

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Azotobacter vinelandii, in late exponential growth phase, encysts when the glucose in the medium is replaced with β -hydroxybutyrate. A final cell division then occurs without apparent deoxyribonucleic acid (DNA) synthesis, resulting in a reduction from two to one nucleoids per cell and a final DNA content of 3.2×10^{-14} g per cell. This is also the DNA content per cyst. A β -hydroxybutyrate dehydrogenase is derepressed by the addition of the inducer and is identical to the enzyme in acetate-grown cells in its pH optimum, Michaelis constant for substrate, temperature-activity response, and mobility during electrophoresis in acrylamide gel.

Cysts of *Azotobacter vinelandii* are resting cells which are analogous to endospores of bacilli in that they are considerably more resistant than vegetative cells to deleterious physical and chemical agents (17). Cultures of this organism may be induced to encystment by growth on agar plates of Burk's nitrogen-free medium supplemented with 0.2% *n*-butyl alcohol as the carbon source (18, 23). Alternatively, cultures may be grown to late exponential phase in liquid medium with glucose as the carbon source; the cells are then induced to encystment by the replacement of the glucose with either β -hydroxybutyrate (BHB) or crotonate (9). This two-step process can be carried out in relatively large culture volumes and has been applicable to the study of the time course of this unique cellular differentiation process at the physiological (9) or fine structural level (6). We have been concerned with the biochemical events which occur upon the induction of encystment and have examined the immediate effects of the addition of BHB on cell growth, cell division, and enzyme induction.

MATERIALS AND METHODS

Strain and cultivation. *A. vinelandii* ATCC 12837, which was used throughout these experiments, was cultivated in Burk's nitrogen-free medium (22) at 30 C. In the two-step method for cyst production (9), cultures were grown to late exponential phase (2×10^8 cells/ml) with 1% glucose as the carbon source. The cells were centrifuged from the medium aseptically, suspended in sterile Burk's medium with 0.2% BHB as the carbon

source, and incubated with aeration at 30 C. Aqueous solutions of both glucose and BHB (sodium salt) were sterilized separately by autoclaving.

Preparation of cell extracts. Vegetative and encysting cells of *A. vinelandii* were suspended in 0.05 M tris (hydroxymethyl)aminomethane (Tris) buffer (pH 7.5) containing 10^{-3} M ethylenediaminetetraacetic acid (EDTA), and were then disrupted in a sonic oscillator (Measuring & Scientific Equipment, Ltd.). The chelating agent (EDTA) was necessary to achieve rapid and reproducible cell rupture.

Analytical procedures. The nitrogen content of vegetative and encysting cells was determined by the micro-Kjeldahl analysis (19). Deoxyribonucleic acid (DNA) was determined by the method of Burton (2). Cells were extracted with 10% trichloroacetic acid at 95 C for 15 min, and the deoxynucleotides were assayed by using highly polymerized calf thymus DNA as a standard. Proteins were estimated spectrophotometrically (20). Compounds containing amino sugars were assayed by the Rondle and Morgan (15) procedure with glucosamine as the standard. The D- β -hydroxybutyric acid dehydrogenase of encysting cells was assayed by the procedure of Jurtschuk et al. (7). The rate of oxidation of BHB was equal to the rate of reduction of nicotinamide adenine dinucleotide (NAD), measured at 340 nm. One unit of enzyme oxidized 1 μ mole of substrate per min at 37 C, and specific activity was expressed in units of protein per mg.

Photomicroscopy. Time-lapse phase-contrast photomicroscopy of encysting *A. vinelandii* was carried out with a Zeiss Photomicroscope by using slide cultures of the organism on Burk's nitrogen-free agar medium containing 0.2% BHB. Nuclear staining of *A. vinelandii* at various stages in its life cycle was performed by the procedures of Piekarski (14). Direct cell counts, which were made with a Petroff-Hauser counting chamber, corresponded well with viable counts obtained by standard plating techniques.

Gel electrophoresis. The relative mobilities of BHB

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dehydrogenases in acrylamide gel electrophoresis at pH 8.3 and 4 C were tested by the method of Davis (4). Vertical gels of 5% Cyanogum 41 (10 by 20 by 0.3 cm) were prepared in a vertical gel electrophoresis cell (E-C Apparatus Corp., Philadelphia, Pa.). Tris-glycine buffer (pH 8.3) was employed, and a constant current of 6 ma was maintained. Bromophenol blue, the anionic marker, migrated to within 1 cm of the anionic end of the gel in 1.75 hr. The BHB dehydrogenases were stained specifically by coupling nitroblue tetrazolium reduction to the oxidation of the substrate (16).

RESULTS

Growth and encystment. The two-step encystment procedure (9) was scaled-up to a 3-liter volume in the following manner. Cultures of *A. vinelandii* were grown in 100-ml volumes of Burk's medium (1% glucose) per 500-ml Erlenmeyer flask. When a cell concentration of 2×10^8 per ml was achieved (approximately 18 hr at 30 C with shaking), 200 ml of culture was introduced into 2,800 ml of the same medium at 30 C in a 3-liter New Brunswick fermentor. The culture was stirred at 400 rev/min and aerated at 3 liters/min. Samples of this culture were taken at intervals for analyses of turbidity, cell count, total nitrogen, DNA, and BHB dehydrogenase. When the cell concentration reached 2×10^8 per ml, the cells were harvested by centrifugation and suspended in fresh Burk's nitrogen-free medium containing 0.2% BHB. Stirring, aeration, and sampling were continued through the initial stages of encystment.

Figure 1 presents growth data for *A. vinelandii* under the conditions described. After the induction of encystment by BHB, the culture appeared to undergo one cell doubling. This was apparent from both the turbidity and total nitrogen data and was substantiated by time-lapse phase-contrast micrography (Fig. 2). A cluster of six vegetative cells on a slide culture was observed over a period of 8 hr. These cells had been grown in liquid Burk's nitrogen-free medium containing glucose. They were washed once in buffer and spread over the BHB-containing medium. The initiation of cell division was readily apparent at 2.5 and 3.5 hr. At 8 hr, five of the cells had divided, and their progeny appeared to be in the early stages of encystment. At that time, the sixth cell was in its final division.

Concomitant with the last cell division, an arrest of nitrogen fixation occurred. The total nitrogen of the culture remained constant with approximately 95% in the cellular material. DNA synthesis stopped at the time of the last cell division. The DNA content per cell dropped from 15×10^{-14} g per cell in exponentially growing cultures to 3.4×10^{-14} g per encysting cell (Fig.

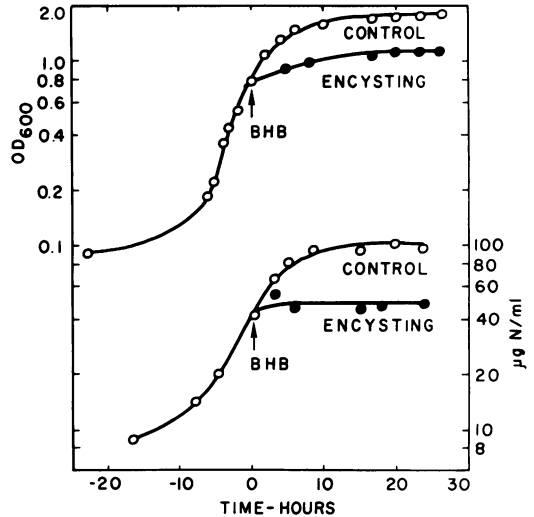


FIG. 1. Growth curve of *A. vinelandii* in Burk's nitrogen-free medium containing 1% glucose, in which turbidity (optical density at 600 nm) or micrograms of nitrogen per milliliter are plotted as functions of time. Cells in late exponential growth were induced to encystment with 0.2% BHB with subsequent inhibition of nitrogen fixation and cell growth.

3). The latter value is precisely the DNA content per cyst of *A. vinelandii*. Nuclear staining showed that cells in early exponential growth had as many as four nucleoids, whereas late exponential-phase cells had two. Cysts contained only one nucleoid.

An alternative procedure for initial extraction (3) and then DNA analysis was performed to validate the observations of DNA content per cell. A 100-ml culture of *A. vinelandii* was grown in Burk's medium (1% glucose) to a concentration of 1.8×10^8 cells/ml. The culture was chilled to 4 C; the cells were harvested, washed in 0.1 M Tris-0.1 M NaCl buffer (pH 9.0), and resuspended in 18 ml of the same buffer. Two milliliters of cold 10% sodium dodecyl sulfate was added, and the suspension was stirred for 20 min in an ice bath. An equal volume (20 ml) of water-saturated phenol was added, and the mixture was slowly stirred for an additional 20 min. The aqueous and phenol layers were separated by centrifugation, and the DNA in the aqueous layer was precipitated by the addition of three volumes of ethanol. The DNA precipitates were collected and suspended in 0.1 M NaCl. The total DNA recovered from 1.8×10^{10} cells was 840 µg, amounting to 4.7×10^{-14} g/cell. The amino sugar content of the DNA preparation was used as a measure of its contamination with cell envelope constituents (21). The DNA preparation contained an equivalent of 25 µg of glu-

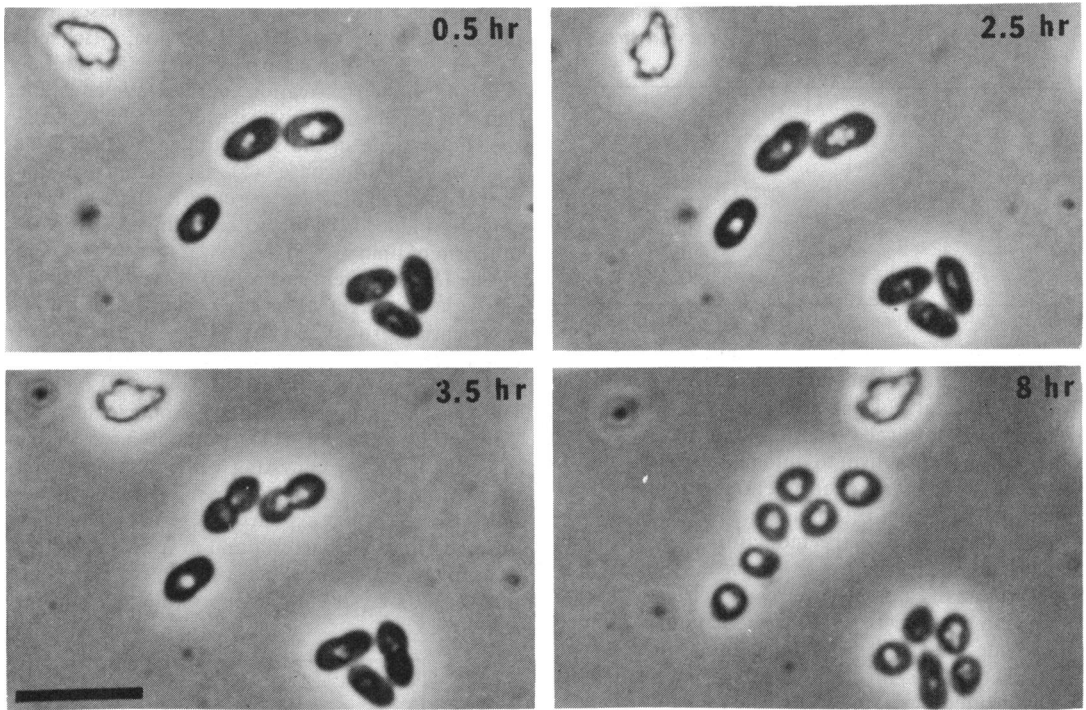


FIG. 2. Time-lapse, bright phase-contrast photomicrographs of encysting *A. vinelandii*. Cells were grown to late exponential growth, and slide cultures were prepared on Burk's agar containing 0.2% BHB. The marker indicates 5 nm.

cosamine whereas the supernatant solution contained 1860 μ g of glucosamine.

The addition of BHB to late exponentially growing cultures of *A. vinelandii* resulted in the induction of a soluble β -hydroxybutyric acid dehydrogenase which was NAD dependent. This enzyme was not found in young cells; growing on glucose however, within 1 hr of the addition of BHB, a specific activity of 0.03 units/mg of protein was noted in cell extracts (Table 1). Because of the relationship of BHB metabolism to encystment, we considered the possibility that the dehydrogenase was a unique "encystment enzyme" analogous to several which occur in cells with the onset of sporulation in *Bacillus* species (1, 5). A soluble BHB dehydrogenase has been studied in cells of *A. vinelandii* grown on acetate as the sole source of carbon and energy (7). We grew 3-liter cultures of the organism in Burk's nitrogen-free medium containing 1% sodium acetate, harvested the cells, and prepared extracts. The specific activity of the BHB dehydrogenase was 0.025 units/mg. This enzyme was compared to that occurring during encystment by observing enzyme activities over the temperature range 26 to 48 C, pH responses, Michaelis constants for substrate, and relative electrophoretic mobilities in acrylamide gels. The results of the

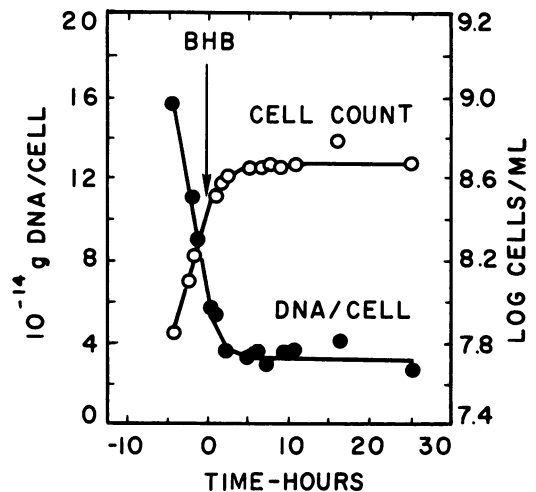


FIG. 3. Cell concentration and DNA content per cell of encysting *A. vinelandii* as a function of time.

comparisons are presented in Fig. 4 and Table 2 and indicate that the two enzymes are very similar.

DISCUSSION

The replacement of glucose with BHB in the two-step induction of encystment is similar in

TABLE 1. Specific activity of β -hydroxybutyrate dehydrogenase upon induction of encystment of *Azotobacter vinelandii*

Time after addition of BHB (hr)	Specific activity (units/mg)
0	0
1	0.030
1.5	0.027
2	0.023
3	0.023
4	0.021

many respects to a metabolic shift-down (8). At the time of its replacement by BHB, about 50% of the original glucose still remained in the medium (9). These cells must have shifted from carbohydrate metabolism to lipid metabolism with the attendant need for gluconeogenesis. Macromolecular synthesis and cell division which was in progress at the time of the induction of encystment appeared to be completed without the initiation of new rounds. Control cultures which were maintained in the glucose-containing medium continued to divide and achieved final population levels of 1.1 ± 10^9 cells/ml. Approximately 1% of these cells formed cysts.

The total nitrogen in the culture increased 1.25-fold after induction of encystment, and the total cell count increased 1.4-fold, a value consistent with the 1.8-fold increase in optical density. A reduction in the DNA content per cell was achieved in this process, the final level equaling the DNA content of mature cysts. A derepression of BHB dehydrogenase synthesis occurred, and relatively high levels of the enzyme were present in cells within 1 hr of induction of encystment. The specific activity of this enzyme during encystment was equal to that in cells grown on acetate. A complete cessation of nitrogen fixation took place approximately 3 hr after the addition of BHB.

The shift-down per se did not induce encystment. This metabolic state occurs after glucose exhaustion from the medium; however, under these conditions, relatively few cysts are produced (9). The specificity of the inducers of encystment, (*n*-butanol, crotonate, or BHB) suggest that unique metabolic sequences may be involved in cyst formation. In some unknown manner, the metabolites of *n*-butanol, which stop normal growth, control the formation of cyst components. Even though key intermediates may exist, they must be transitory because ultimately 90% of all exogenously added BHB is oxidized to CO₂ (9).

The BHB dehydrogenase which was elaborated during encystment was identical to the enzyme which occurred in *A. vinelandii* during vegetative growth on acetate on the basis of the following

parameters. The two soluble dehydrogenases were indistinguishable in their pH optima, Michaelis constants, electrophoretic mobilities in acrylamide gels, and in their activation energies. We conclude that BHB dehydrogenase is not an encystment-specific enzyme in the sense that protease (1) or glucose dehydrogenase (5) is related to the sporulation of *Bacillus cereus*. Rather, the enzyme is induced when the cells shift to the metabolism of lipid materials.

The DNA content per cell of *A. vinelandii* varied with the stages of its life cycle. Cells in mid-exponential growth contained 15×10^{-14} g of DNA/cell and were multinucleate. During encystment a minimal value of 3.4×10^{-14} g of DNA/cell was observed for mononucleate cells. This latter value amounts to about 2.5% of the dry weight of the encysting cells, assuming that they contain 8% nitrogen. The reduction in nucleoids per cell after exponential growth has been observed in other bacteria and in metabolic shift-down experiments in which cell growth rate is altered (8, 10). Cysts contain approximately 10 times

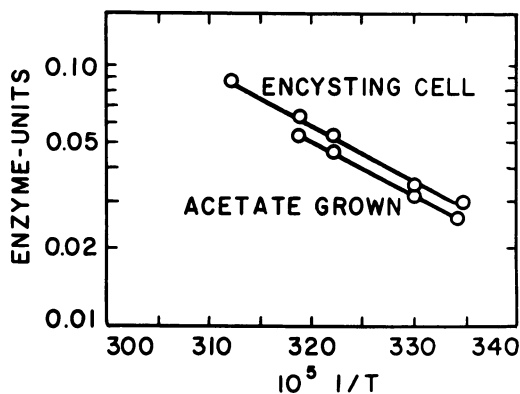


FIG. 4. Arrhenius plot of the effect of temperature over the range 25 to 48 C on the activities of 0.045 units of BHB dehydrogenase from acetate grown cells and 0.055 units from encysting *A. vinelandii*. The activities are expressed as equivalent units (37 C).

TABLE 2. Properties of β -hydroxybutyrate (BHB) dehydrogenase of *Azotobacter vinelandii*

Source	pH optimum ^a	K _m (mM) ^b	R _m ^c
Encysting cells	9.0	4.0	0.38
Acetate-grown cells . .	9.0	5.5	0.38

^a The enzyme (0.05 units) was assayed over a pH range of 7.5 to 11.0 in 0.05 M Tris-glycine buffer.

^b Michaelis constants for either enzyme were determined with 0.02 to 0.05 units of enzyme over a BHB dehydrogenase concentration range of 0.001 to 0.03 M.

^c Mobility relative to bromophenol blue in electrophoresis on 5% acrylamide gel at pH 8.3.

as much DNA per nucleoid as does *Escherichia coli* (10). Similar DNA contents for synchronous cultures of *A. vinelandii* have been reported by Zaitseva et al. (24). Müller and Kern (13) observed that the DNA contents of various radiation resistant mutants of *A. chroococcum* ranged from 10^{-14} to 19×10^{-14} g/cell. Despite these corroborative findings, we considered the possibility that the high values of DNA content per cell were artifacts of the assay procedure. If the hot trichloroacetic acid used to hydrolyze the cell DNA also hydrolyzed a part of the cell envelope, the values for the DNA assay might have been inordinately high. Therefore, we extracted DNA from 100 ml of a culture in late exponential growth phase and, after a standard DNA assay, calculated the DNA per cell. To detect contamination of the DNA preparation by the cells' lipopolysaccharide, we monitored hexosamine content of our precipitated DNA and the aqueous phase from which it was precipitated. Only 1.3% of the hexosamine-containing material originally present in the aqueous phase was precipitated with the DNA. Assuming a 70% yield of DNA on extraction, we calculated that late exponential cells of *A. vinelandii* contain 6.7×10^{-14} g of DNA/cell. Since these cells contained an average of two nucleoids per cell, the individual nucleoids must have contained 3.4×10^{-14} g of DNA. This value is identical to that which we have obtained for encysting cells and cysts of *A. vinelandii* by our DNA assay. The relatively large mass of the *A. vinelandii* nucleoid suggests that there may be considerable redundancy in its genome and may account for the difficulty in obtaining mutants of this organism (11, 12).

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