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Sequential Metabolic Events During Encystment of *Azobacter vinelandii*[1]

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Cells of *Azotobacter vinelandii* are specifically induced to encyst by β-hydroxybutyrate (BHB). The process of differentiation, which occurs over a period of 36 h, was characterized by an ordered sequence of biochemical events. Upon initiation of encystment, nitrogen fixation and glucose-6-phosphate dehydrogenase activities decreased immediately to very low levels. This was followed by an increase in the specific activities of BHB dehydrogenase, isocitrate dehydrogenase, isocitrate lyase, and malate synthase first at 3 h and then again at 21 h. The peak activity of fructose 1,6-diphosphate aldolase occurred at 6 h, and the enzyme activity then decreased gradually. Fructose 1,6-diphosphatase had peak activities at 9 and 27 h. Deoxyribonucleic acid synthesis ceased just prior to the final cell division at 4 to 6 h, but ribonucleic acid synthesis continued until the 12th h. From labeling studies and the appearance of new enzyme activities, it appeared that protein synthesis continued throughout encystment.

Azotobacter vinelandii cells are prokaryotic organisms which are capable of undergoing encystment, a form of cellular differentiation whose morphogenetic aspects have been demonstrated by phase-contrast and electron microscopy (8, 24, 31, 32). We have studied this process to compare it with sporulation and other systems of morphogenesis, and to discern the principles underlying differentiation in the prokaryotes. Cyst formation can be induced in large quantity and relatively synchronously in vegetative cells by the replacement of glucose with *n*-butanol (31), crotonate, or β-hydroxybutyrate (BHB; 12) as the carbon source. The mechanism by which these compounds induce encystment is unknown. Although a metabolic shift-down occurs when BHB replaces glucose, a shift-down per se (such as occurs when glucose is depleted from the medium) produces few cysts (21). The addition of both glucose and BHB to the growth medium gives rise to abortive encystment which is characterized by a block in the morphological development of cysts (12).

Because of the specificity of BHB as an inducer of encystment and its antagonism by glucose, it appeared that unique metabolic sequences must occur during encystment. A knowledge of the pattern of morphogenesis (8) and the chemistry (13) of *Azotobacter* cysts prompted us to look for the key enzymes in the biochemical pathways from lipids to carbohydrates which would be required for the conversion of BHB to major cyst components, and also to report further (cf., V. M. Hitchins and H. L. Sadoff, Bacteriol. Proc., p. 39, 1971) on the time course for the induction of enzymes of the glyoxylate shunt and gluconeogenesis during encystment.

MATERIALS AND METHODS

Strain and cultivation. *A. vinelandii* 12837 was used throughout these experiments. Vegetative cells or cysts were produced at 30 C in Burk's nitrogen-free medium (30) as described previously (8). Cells grown in the presence of 0.2% glucose and 0.2% BHB gave rise to abortive encystment.

Chemicals. Acetylene (purified grade), ethylene, and a gas mixture of O_2 (22%), CO_2 (0.04%), and argon (78%, high purity) were all obtained from Matheson Co., Inc. (East Rutherford, N.J.). Sodium DL-3-hydroxybutyrate-*3*-^{14}C was purchased from Amersham/Searle (Arlington Heights, Ill.). L-Leucine-*U*-^{14}C was a product of Cal Atomic (Los Angeles, Calif.), and uracil-2-^{14}C was from New England Nuclear Corp. (Boston, Mass.). Coenzyme A (CoA) nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) were obtained from Calbiochem (San Diego, Calif.). Chloramphenicol was purchased from Sigma Chemical Co. (St. Louis, Mo.).

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Incorporation of radioactive-labeled BHB into cysts. BHB-*3*-^{14}C (0.5 μCi, 11.8 Ci/mol) was added to cells in the presence of 0.2% unlabeled BHB to initiate encystment. Radioactive CO_2 was trapped on Whatman no. 1 filter paper saturated with 10% KOH. Cells were collected on membrane filters (pore size, 0.45 μm; Gelman Instrument Co., Ann Arbor, Mich.) and washed three times with fresh Burk's buffer containing 0.2% BHB (26 C). The cell-free medium and the washes were combined and counted for radioactivity. Bray's scintillation fluid (2) was added to the cell-free medium, and a toluene-based scintillation fluid (3) was used for the nonaqueous samples. Radioactivity of the three fractions was measured with a Packard 3000 series Tri-Carb liquid scintillation spectrometer, and the results were corrected for quenching and expressed as radioactivity per milliliter of sample.

Distribution of radioactive-labeled BHB into cysts. BHB-*3*-^{14}C (10 μCi, 4.52 Ci/mol) was added to cells in the presence of 0.2% unlabeled BHB. The distribution of the radioactivity into protein, ribonucleic acid (RNA), deoxyribonucleic acid (DNA), lipid, and acid-soluble fractions was determined by the membrane fractionation method of Roodyn and Mandel (19).

Assay for poly-β-hydroxybutryate granules. The method of Stockdale et al. (27) was used to assay poly-β-hydroxybutyrate (PHB) which accumulates in encysting cells.

Assay for nitrogen fixation. The acetylene reduction technique of Stewart et al. (25) was used as an index of the rate of nitrogen fixation. At regular time intervals, triplicate 2-ml samples of vegetative or encysting cultures were added to 5.0-ml capacity glass serum bottles. The samples were flushed with an O_2-CO_2-A gas mixture prior to fitting the bottles with rubber serum bottle stoppers. Acetylene (0.5 ml/bottle) was then injected into each bottle, and the samples were incubated for 1 h at 26 C. One bottle immediately received 0.3 ml of 2% $HgCl_2$ and served to establish the background of ethylene in acetylene. Acetylene reduction was terminated by the addition of $HgCl_2$ to the remaining bottles. Ethylene formation was detected by gas chromatography with a Varian-Aerograph model 600D gas chromatograph equipped with a flame detector and a 1-m column containing Porapak N, and run at 45 C. The carrier gas was N_2 at a flow rate of 25 ml/min. Duplicate 0.5-ml gas samples were taken from the head space of each serum bottle and injected into the column. The relative rate of nitrogen fixation was expressed as nanomoles of ethylene produced per microgram of cell nitrogen per hour. The nitrogen content of vegetative and encysting cells was determined by the micro-Kjeldahl method (28).

Preparation of cell-free extracts. Packed cells or cysts (0.5 to 1.0 g) were resuspended in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) and were disrupted for 2.5 min in a sonic oscillator (Measuring and Scientific Equipment, Ltd.); the suspensions were then clarified by two cycles of sedimentation at 49,000 × *g* for 20 and 40 min. The resulting supernatant solution (0.25 to 3.0 mg of protein/ml) was assayed directly for enzyme activity. It was assumed that all of the enzymes studied had approximately the same stability in the cell-free extracts and that the specific activity of any one enzyme at a particular time was a reasonable representation of its intracellular concentration. The results are the composite of four separate experiments. All specific enzyme activities are expressed as units per milligram of protein. Protein determination was by the procedure of Lowry et al. (14), with bovine serum albumin V as standard.

Enzyme assays. Spectrophotometric assays were conducted by use of a Perkin-Elmer double-beam spectrophotometer equipped with a Sargent recorder, model SR. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49) activity was assayed by following the reduction of NADP (4) at 26 C. BHB dehydrogenase (D-3-hydroxybutyrate:NAD oxidoreductase, EC 1.1.1.30; reference 21) and isocitrate dehydrogenase (*threo*-D_s-isocitrate:NAD oxidoreductase, EC 1.1.1.41; reference 10) were measured by recording the reduction of NAD in the presence of the appropriate substrate for each enzyme at 37 C. One unit of enzyme was defined as that amount which reduced 1 μmol of NAD or NADP per min when a molar absorbancy at 340 nm for NADH or NADPH of 6.22 × 10^6 cm^2/mol was used.

Isocitrate lyase (*threo*-D_s-isocitrate glyoxylate lyase, EC 4.1.3.1) was assayed by the method of McFadden (15). One unit of enzyme is that amount which catalyzes the disappearance of 1 μmol of D-isocitrate per min at 30 C.

Malate synthase (L-malate glyoxylate-lyase [CoA-acetylating], EC 4.1.3.2) was measured spectrophotometrically by monitoring the deacylation of acetyl CoA (5) at 26 C. Acetyl CoA was synthesized from CoA by the procedure described by Ochoa (17). The cell-free extracts contained no deacylase activity before glyoxylate was added to start the reaction. The molar absorbance coefficient of acetyl CoA at 232 nm is 4.5 × 10^6 cm^2/mol.

Fructose 1,6-diphosphate aldolase (fructose-1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) was assayed by measuring colorimetrically the formation of 2,4-dinitrophenylhydrazine derivative of the triose phosphates formed in 15 min at 38 C (23). One unit of enzyme produced an absorbancy of 1.0 per cm at 546 nm under the conditions of the experiment. Fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11; FDPase) was assayed according to the procedure of Rosen et al. (20). The amount of inorganic phosphate (P_i) liberated was proportional to the amount of enzyme added and was determined by the Fiske-Subbarow method (6). One unit of FDPase is that amount of enzyme catalyzing the release of 1 μmol of P_i/min at 26 C.

Protein synthesis. The synthesis of protein during vegetative growth and encystment was estimated by measuring the incorporation of L-leucine-*U*-^{14}C (5 to 10 μCi, 216 Ci/mol) into trichloroacetic acid-insoluble precipitates (19). These were collected on 0.45-μm membrane filters. The filters were washed three

times with 5% cold trichloroacetic acid with 5 μM L-leucine, dried, and then placed into counting vials containing toluene-based scintillation fluid (3).

RNA synthesis. RNA was measured by the incorporation of uracil-2-^{14}C (10 μCi, 22 Ci/mol; reference 33) in the presence of 20 μg of cytosine/ml (11) and the appropriate substrate for vegetative growth or encystment. Trichloroacetic acid-insoluble precipitates were collected on 0.45-μm membrane filters which were then washed three times with 5% cold (4 C) trichloroacetic acid containing 5 μmol of uracil/ml.

DNA synthesis. DNA was monitored in cells grown in the presence of uracil-2-^{14}C (21.5 μCi, 45 Ci/mol); 45 μM uracil, and a carbon source for vegetative or encysting cells. Samples of 1 ml were incubated with 0.1 ml of 5.5 N NaOH overnight at 37 C to hydrolyze RNA (19). The samples were then cooled in an ice bath and neutralized with 0.1 ml of 6 N HCl. Cold (4 C) 10% trichloroacetic acid was added to make the final concentration 5%. After 30 min, the samples were filtered, washed, and counted.

RESULTS

BHB was incorporated into encysting cells when it was added to washed vegetative cells at the end of their exponential growth (Fig. 1). BHB was metabolized by the cells with the release of radioactive CO_2. At the completion of encystment (36 to 40 h), approximately 10% of the added BHB-*3*-^{14}C had been released as $^{14}CO_2$ and 25% had been incorporated into cyst materials. Radioactive BHB-*3*-^{14}C was incorporated into the RNA, lipid, and protein fractions of encysting cells of *A. vinelandii* (Fig. 2). Radioactivity in the RNA fraction increased steadily until about the 9th h and then decreased slowly. The other fractions showed a steady increase in the incorporation of BHB-*3*-^{14}C. The PHB content per cell increased 10-fold to 1 g of PHB/g of cell nitrogen at 36 h and then decreased to 0.8 g of PHB/g of cell nitrogen at the completion of encystment.

Prior to the completion of exponential growth, vegetative cells were harvested and resuspended in fresh Burk's buffer containing one of the following carbon sources: 1% glucose, 0.5% acetate, or 0.1% propanol for continued vegetative growth: or 0.2% BHB or 0.03% crotonate for encystment. The rate of acetylene reduction (nitrogen fixation) of cells in glucose, acetate, or propanol (vegetative growth) increased, reflecting both an increase in cell mass and an increase in specific activity of the nitrogenase at certain stages of growth. Encysting cells, which underwent a final cell division within 4 to 6 h, lost their ability to reduce acetylene; within 15 min after the addition of BHB, the rate decreased by 70%, and it was approximately zero by 1 h (Fig. 3).

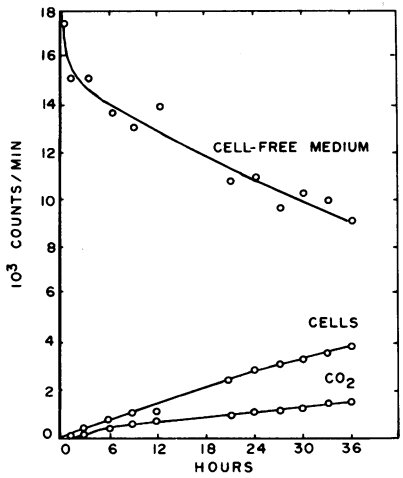

FIG. 1. *Distribution of β-hydroxybutyrate (BHB)-3-^{14}C during encystment. BHB-3-^{14}C (0.5 μCi) and 2% unlabeled BHB were added to 100 ml of Burk's nitrogen-free buffer to initiate encystment.*

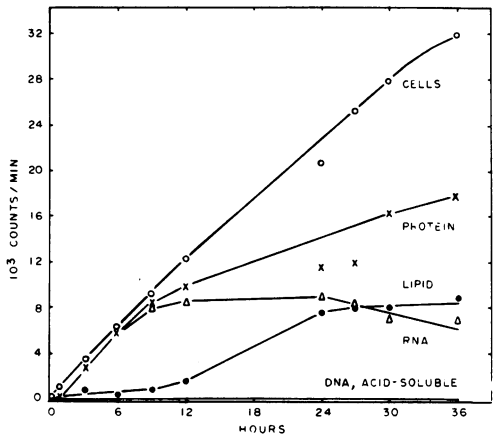

FIG. 2. *Distribution of β-hydroxybutyrate (BHB)-3-^{14}C (10 μCi) and 0.2% unlabeled BHB were added to 100 ml of Burk's nitrogen-free buffer.*

Encystment was also characterized by the steady decrease in specific activity of glucose-6-phosphate dehydrogenase, a key enzyme of the Entner-Doudoroff and the pentose phosphate pathways of glucose catabolism in vegetative cells of *A. vinelandii* (16, 26; Fig. 4). Also shown in Fig. 4 are the activities of BHB dehydrogenase and isocitrate dehydrogenase, each of which exhibited two peaks of activity at 6 and 21 h. The first peak of activity was induced by the addition of BHB to the medium which, in part, was polymerized to PHB. The second peak of BHB dehydrogenase activity was presumably due to the metabolism of PHB. The addition of both glucose and BHB (induction of abortive encystment) to washed,

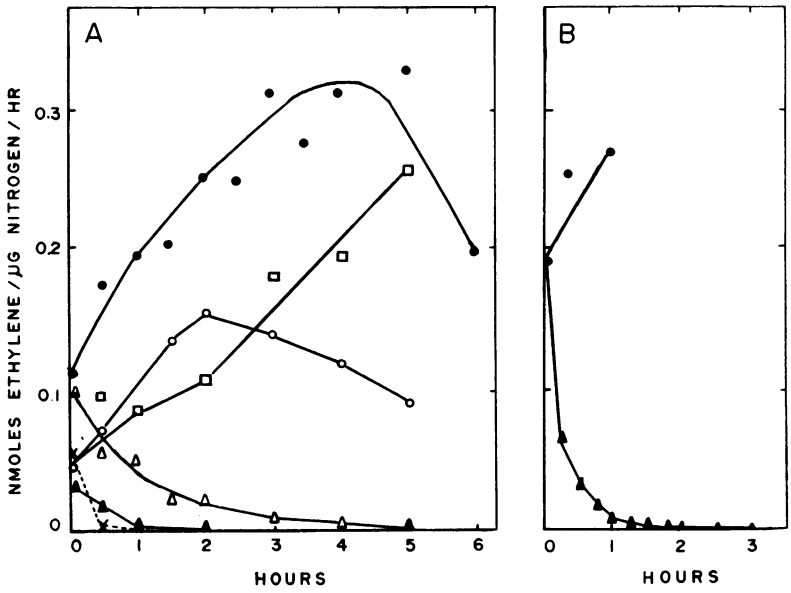

FIG. 3. *(A) Rate of nitrogen fixation expressed as nanomoles of ethylene produced per microgram of cell nitrogen of A. vinelandii grown on different carbon sources. Symbols:* ●, *1% glucose (vegetative growth);* ○, *0.5% acetate (vegetative growth);* ▲, *0.2% BHB (encystment);* △, *0.03% crotonate (encystment);* □, *0.1% propanol; and* × *no substrate. (B) Rate of nitrogen fixation at 15-min intervals during vegetative growth (glucose) and encystment (BHB).*

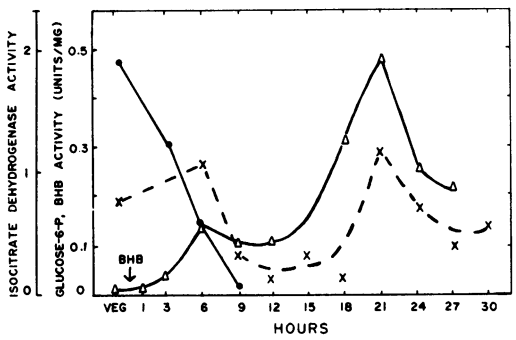

FIG. 4. *Time course for the activities of glucose-6-phosphate dehydrogenase* (●), *BHB dehydrogenase* (△), *and isocitrate dehydrogenase* (×) *upon induction of encystment of A. vinelandii with 0.2% BHB.*

exponentially growing cells resulted in a 3-h time lag in appearance of BHB dehydrogenase and an approximate 25% decrease in its activity.

Isocitrate lyase activity was very low in vegetative cells which had been grown on glucose. However, upon the addition of BHB, an immediate induction of the enzyme occurred, which then decreased to the low initial value by 9 h. The isocitrate lyase was induced once again at 15 h, and the enzyme achieved high levels of activity in cells late in the encystment process. The increase in malate synthase appeared to be coordinated with that of isocitrate lyase (Fig. 5).

Two enzymes of gluconeogenesis, aldolase and FDPase, which appear during encystment have a temporal relationship (Fig. 6). Aldolase activity had a maximal value at 6 h and then decreased to its initial level by the 18th h of encystment. FDPase had two peak activity periods in encysting cells, at 9 h and again at 27 h.

The synthesis of DNA, RNA, and protein during encystment was also examined. Upon the addition of BHB to induce encystment, the rate of DNA synthesis immediately began to decrease and ceased at about 4 h, just prior to a final cell division which resulted in the formation of the "precyst" (9; Fig. 7). On the other hand, net RNA synthesis continued until the 12th h of encystment, albeit at a lower rate than in exponentially growing cells. Protein synthesis, measured by incorporation of ^{14}C-leucine, continued throughout the 36-h encystment period but at approximately one-third the rate of that occurring in vegetative cells (Fig. 8). Immediately upon the induction of encystment, cells of *A. vinelandii* became resistant to chloramphenicol at concentrations of 100 µg/ml. The rate of protein synthesis by these cells was not radically affected by the

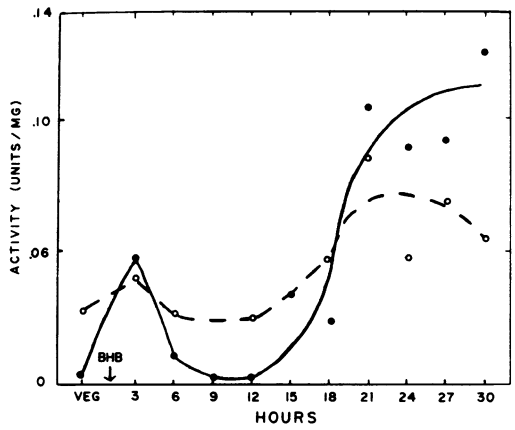

FIG. 5. *Time course for the activities of the two glyoxylate shunt enzymes during encystment.* (●) *Isocitrate lyase;* (○) *malate synthase.*

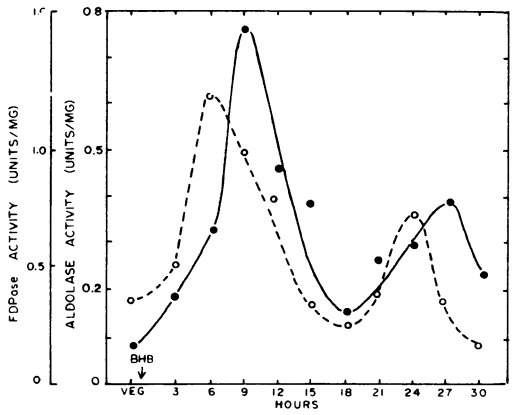

FIG. 6. *Time course for the activities of the two enzymes of gluconeogenesis which appear during encystment:* (○) *aldolase and* (●) *fructose 1,6-diphosphatase. The aldolase data are the average of four experiments. The peak at 6 h has an activity of 0.602 units/mg ± 0.215 (1 standard deviation).*

presence of chloramphenicol nor was the process of morphogenesis interrupted. The usual sequence of events which were discernible by phase microscopy was identical in encysting cells with or without chloramphenicol. In sharp contrast, protein synthesis and growth of exponential cultures of *A. vinelandii* became progressively more inhibited by increasing concentrations of chloramphenicol. Concentrations of 75 and 100 μg of the antibiotic/ml reduced ^{14}C-leucine uptake in cells by 85 and 90%, respectively. The rate of growth of cells grown in the presence of 0.1% propanol or 0.5% acetate was inhibited 60 and 67%, respectively, by the addition 50 μg of chloramphenicol.

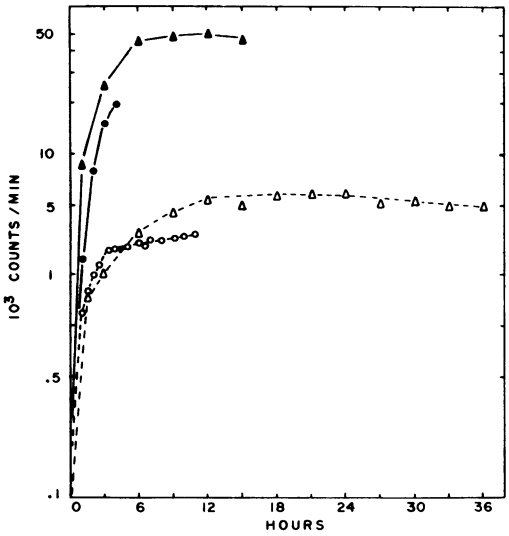

FIG. 7. *DNA (circles) and RNA (triangles) synthesis in cells of A. vinelandii during vegetative growth (closed) and encystment (open).*

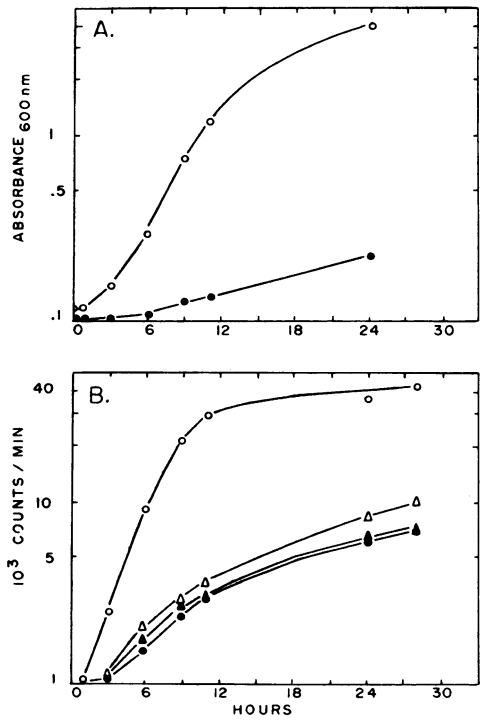

FIG. 8. *Growth of A. vinelandii and extent of protein synthesis during vegetative growth with* (●) *and without* (○) *100 μg of chloramphenicol per ml and during encystment with* (▲) *and without* (△) *100 μg of chloramphenicol per ml.*

DISCUSSION

When BHB was added to cells of *A. vinelandii* to initiate encystment, they lost certain biochemical properties characteristic of vegetative cells and acquired a variety of new enzymes. Of particular interest was the striking decrease in the rate of acetylene reduction (nitrogen fixation) and in the activity of glucose-6-phosphate dehydrogenase. Concomitant with these events was the induction of enzymes necessary for BHB metabolism and gluconeogenesis. Nitrogen fixation is a membrane-associated property in *A. vinelandii* and requires pools of both adenosine triphosphate (ATP) and reduced nucleotides (7, 22), which are produced by the oxidation of substrates such as glucose or acetate. Even in the absence of these compounds, low levels of nitrogenase activity were observed which then decreased to zero within 30 min. The cessation of N_2 fixation in the presence of either BHB or crotonate as substrates therefore constitutes a paradox since either compound can be oxidized to the equivalent of two acetate molecules and ultimately to CO_2. Furthermore, the oxidative enzyme complement which is induced in these cells by BHB is similar to that induced by growth on acetate (9, 21), and thus it would be expected that similar ATP and reduced nucleotide yields would occur.

The effect of BHB on the membrane-bound N_2 fixation system could be quite direct and due to its incorporation into the lipid fraction, in particular, the phospholipids of the cell membrane. In preliminary studies, we have observed that BHB-*3*-^{14}C is rapidly incorporated into the phospholipid fraction of encysting cells (Hitchins and Sadoff, *unpublished data*). Either BHB-CoA or crotonyl-CoA incorporation into fatty acids could generate positional or geometric isomers of the normally occurring unsaturated fatty acids of the membrane. These could then have a profound effect on certain membrane properties or the activities of certain membrane-bound enzymes. The very rapid acquisition of chloramphenicol resistance seen in cells which have been resuspended in BHB may be due to such a membrane modification, which then makes the cell impermeable to the antibiotic. An alternative explanation, the rapid induction of a chloramphenicol acetylating enzyme (18) mediated by BHB or its metabolites, seems less likely, because we have not seen this effect in chloramphenicol-sensitive vegetative cells with other substrates (glucose, propanol, or acetate).

The cyst coats, the exine and intine, contain glucose, mannose, xylose, and rhamnose (13). The polymers containing these sugars must be synthesized during encystment and are derived either from cell constituents present at the time encystment was initiated or from the lipoidal inducers of encystment. The latter alternative would require the functioning of enzymes of the glyoxylate shunt and gluconeogenesis during encystment to synthesize carbohydrates. It is precisely these enzymes which are induced during encystment. A similar induction occurs during microcyst formation in *Myxococcus xanthus* in which there is a marked increase in the activities of the two glyoxylate shunt enzymes (1). During the sporulation of *Bacillaceae*, the enzymes of the citric acid cycle, glyoxylate shunt, and PHB metabolism all have essential functions (29).

Previous studies using time-lapse phase-contrast microscopy and determination of DNA content (21) have shown that encysting cells undergo a final round of cell division and each precyst contains 3.4×10^{-14} g of DNA per cell. Using tracer techniques, we have confirmed that cells finish the synthesis of new DNA at about the 4th h of encystment and there is no incorporation of BHB-*3*-^{14}C into the DNA. RNA synthesis continues until about the 12th h and then reaches a plateau, indicating no further net synthesis of NRA. With BHB-*3*-^{14}C, the distribution of radioactivity in the RNA fraction increased until about the 12th h and then began to decrease, indicating turnover. Protein synthesis continues throughout encystment, as is evident by leucine uptake and by the occurrence of peaks of certain enzyme activities which appear late in the differentiation process.

In the absence of N_2 fixation, macromolecular synthesis during the encystment of *A. vinelandii* must depend on the turnover of both RNA and protein. In this sense, encystment resembles sporulation, but it occurs over a much broader time span. A further fundamental similarity in the differentiation processes is the early completion and cessation of DNA synthesis followed by the segregation of sister nucleoids into separate cells. This leads to the formation of one spore in *Bacillaceae* but two cysts in *Azotobacteraceae*. The metabolic pathways common to sporulation and encystment, both processes of differentiation, result in the formation of dormant cells. The parallel which is seen in these biochemical events and the time course for their appearance suggests that there may exist a general pattern of control of differentiation in the prokaryotes.

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