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GRADUATE COLLEGE

ARTHROBACTER ATROCYANEUS AS A SYSTEM FOR THE STUDY OF PROCARYOTIC MORPHOGENESIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

GARY JAMES STANLAKE

Norman, Oklahoma

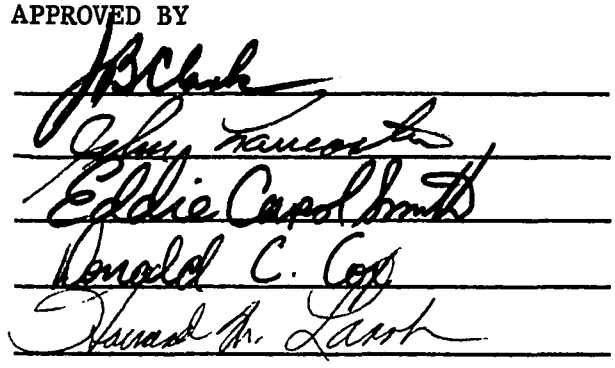
1976

ARTHROBACTER ATROCYANEUS AS A SYSTEM

FOR THE STUDY OF PROCARYOTIC

MORPHOGENESIS

APPROVED BY



DISSERTATION COMMITTEE

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PAPER III

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ARTHROBACTER ATROCYANEUS AS A SYSTEM FOR THE STUDY OF PROCARYOTIC MORPHOGENESIS

INTRODUCTION

Procaryotic cellular differentiation is of interest primarily as a simplified model of eucaryotic cellular differentiation (20). The simple morphogenic cycle (coccus to rod to coccus) exhibited by members of the genus Arthrobacter has been a favorite tool for the study of procaryotic cell differentiation (3).

Two theories have been proposed to explain the morphogenic cycle of the genus Arthrobacter. According to the theory of Ensign and Wolfe (4), morphogenesis, as expressed by sequential changes in cellular morphology, can be separated from growth. Some nutrients support growth without a morphogenic cycle (i.e., A. crystallopoietes grown in a mineral salts glucose medium). Others support growth with a morphogenic cycle (i.e., A. crystallopoietes grown in a mineral salts succinate medium). The term "morphogenesis inducing compounds" is a product of this theory and has greatly influenced the study of Arthrobacter morphogenesis. The second theory was proposed by Luscombe and Gray (22, 23) based upon chemostat experiments. This theory proposes that morphogenesis and growth are inseparable with no "specific inducer" being required for the morphological change of cocci to rods.

Both theories have been applied principally to the explanation of the morphogenic step of "induction". Induction has, by convention, been designated as the first step of the morphogenic cycle. The morphological expression of induction is the elongation of cocci into rods. Although induction has received the greatest attention, two other steps are always associated with a complete morphogenic cycle. The second step of a morphogenic cycle consists of a series of binary rod divisions or a single multiseptate rod division. This stage is designated "fragmentation" and results in an increase in cell numbers accompanied by a decrease in cell length. The last step in the morphogenic cycle is the "reversion" of rods back to cocci and is closely associated with fragmentation.

Fragmentation and reversion are not invariable consequences of induction. Hariri demonstrated that fragmentation of A. crystallopoietes did not result from the exhaustion of the inducing compound (8). Failure of A. crystallopoietes rods to undergo fragmentation and reversion in a buffer solution (5) indicates a need for an exogenous energy source for the terminal morphogenic steps in A. crystallopoietes.

In the present investigation the majority of carbon sources tested supported only a partial morphogenic cycle in A. atrocyaneus CBRI 21001. Induction and various degrees of fragmentation often occurred but complete fragmentation and reversion were supported by only a few carbon sources. Like A. crystallopoietes fragmentation and reversion did not occur in the absence of an exogenous energy source. In addition, the terminal morphogenic steps were inhibited by the inducing carbon source succinate.

The inhibition of fragmentation and reversion of A. atrocyaneus CBRI 21001 rods back to cocci by an inducing carbon source supports Ensign and Wolfe's (4) induction theory for morphogenesis. Neither theory, however, adequately explains fragmentation and reversion in A. atrocyaneus.

The cellular morphological changes (cocci to rods to cocci) traditionally associated with Arthrobacter morphogenesis do not constitute the only markers for the study of morphogenesis. Changes in Arthrobacter cellular morphology are not as pronounced as those which designate the "cell cycle" of Caulobacter crescentus (18). Other markers would therefore be of value in the study of Arthrobacter morphogenesis. Some possible markers are: 1. specific activity of enzymes (6, 11, 19, 25, 28); 2. mRNA population (24); 3. cyclic adenosine 3':5'-monophosphate effects (9); 4. cell wall changes (10, 15, 16); 5. phage sensitivity (1); 6. pigment; and 7. motility (2, 17, 18, 26). A. atrocyaneus CBRI 21001 possesses many of these additional markers.

A. atrocyaneus was initially described by Kuhn and Starr (17). Its carbohydrate catabolism involves both the Entner-Doudoroff and hexose monophosphate pathways but not the Embden-Meyerhof-Parnas pathway (27). A survey of its response to various carbon sources has been performed (21). Reclassification of the Arthrobacter species has led to the inclusion of A. atrocyaneus as a strain of A. globiformis (14). Such a grouping of many of the Arthrobacters under one species is justified when based on nutritional characteristics because of the high degree of sharing of these characteristics (7). Serological criteria,

however, indicate that strains (previous species) can be differentiated. Therefore, in this paper A. atrocyaneus CBRI 21001 designates a specific strain of A. globiformis.

Additional work in our laboratory has shown that A. atrocyaneus CBRI 21001 is phage sensitive (13) and easily mutatable (12). With the advent of these findings it becomes apparent that A. atrocyaneus CBRI 21001 has the possibility of becoming a good system for a study of the genetic controls of Arthrobacter morphogenesis. Such a study would be a departure from the "inducing compounds" approach of the last decade.

The purpose of the present investigation was to determine the suitability of A. atrocyaneus CBRI 21001 as a system for the study of procaryotic morphogenesis. Particular emphasis was given to the study of motility as a morphogenic marker. The recognition of the possible use of this strain in a genetic study of morphogenesis prompted the development of a rapid method for its positive identification. This ability to identify a morphological, auxotrophic or morphogenic mutant as a progeny of A. atrocyaneus CBRI 21001 would be crucial to any genetic study of an organism of such variable characteristics (i.e., Gram stain and morphology). A comparison of rod and coccus antigens was made to demonstrate cell wall changes due to morphogenesis. In addition, a better understanding of the metabolism of this organism was sought. This would facilitate the detection and evaluation of auxotrophic mutants and aid in the comparison of A. atrocyaneus CBRI 21001 to other strains of Arthrobacter.

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PAPER I

MOTILITY AS A CHARACTER OF MORPHOGENESIS IN *ARTHROBACTER*

MOTILITY AS A CHARACTER OF MORPHOGENESIS IN *ARTHROBACTER*

ABSTRACT

Motility in *Arthrobacter atrocyaneus*, *A. citreus* and *A. simplex* was found to correlate with the morphogenic cycle of these organisms. The percentage of the *A. atrocyaneus* and *A. simplex* populations which were flagellated at a given time during the growth cycle differed significantly from that of the non-morphogenic *Pseudomonas aeruginosa* population. Flagellation in *A. atrocyaneus* was shown to be dependent upon the morphogenic cycle rather than growth. The commitment to flagellar synthesis in *A. atrocyaneus* was found to occur only after induction to the rod morphology. Flagellar synthesis in *A. atrocyaneus* was shown to be restricted to only a small segment of the morphogenic cycle.

INTRODUCTION

The genus Arthrobacter is composed of bacteria possessing a coccus to rod to coccus morphogenic cycle (6). Motility in the genus was first noted by Clark and Carr (3) in a review of the soil Corynebacterium which included C. simplex (later classified as A. simplex). Motility has also been observed in A. citreus (14), A. atrocyaneus (7), A. globiformis, and A. ureafaciens (1).

In the initial description of A. citreus, Sacks (14) reported that only rod shaped cells were motile and that this motility occurred for only a short period of time. In a survey of Arthrobacter soil isolates the majority of motile strains were found to possess flagellated but non-motile cocci (1). Kuhn and Starr (7) in their initial description of A. atrocyaneus reported that the cocci were generally non-motile and atrichous.

In his review of the genetics of bacterial flagellation Iino (5) described several non-morphogenic bacteria in which the location or number of flagella per cell changed in relation to changes in cellular morphology or age. For the dimorphic bacterium Caulobacter crescentus, Shapiro and Maizel (15) reported a correlation between cellular morphogenic changes and flagellation. The initial observations of motility in the genus Arthrobacter indicated that such a correlation also existed in this genus.

MATERIALS AND METHODS

Organisms. Arthrobacter atrocyaneus (CBRI 21001), Arthrobacter citreus (CBRI 21003), Arthrobacter simplex (ATCC 6946), and Pseudomonas aeruginosa (OU stock collection) were maintained on tryptone glucose yeast extract slants at 10 C. Cultures labeled CBRI were obtained courtesy of the Cell Biology Research Institute, Canada Department of Agriculture, Ottawa, Canada.

Media and culture conditions. The complex medium used to study the correlation of motility with the morphogenic cycle was ½ strength DIFCO Plate Count Broth (TGY). The mineral salts medium (MS) for Arthrobacter described by Ensign and Wolfe (4) was used with L-lysine (0.5% w/v) as the carbon source for growth without a morphogenic cycle.

One hundred ml of inoculated broth in a 250 ml flask were incubated on a rotary shaker at 200 rev min^{-1} and 25, 30 or 37 C.

Synchronization of cultures. Arthrobacter cultures are generally in synchrony through the first fragmentation division if the inoculum consists of morphologically uniform coccoidal cells of the same age. Some species of Arthrobacter, when grown in complex medium, do not consist of uniform coccoidal cells even after 4 to 5 days of growth. To increase uniformity in the inoculum, Arthrobacter coccoidal cells were separated according to size and collected by the method of Mitchison and Vincent (13).

Motility and the morphogenic cycle. Motility was determined

by direct observation of wet mount preparations with a dark field microscope at 970x magnification.

Cellular morphology was documented by Gram stain or crystal violet stain. Flagella were stained using Baltimore Biological Laboratory flagella stain with a borax-methylene blue counter stain according to the method of Liefson for broth cultures (9). As a control for the staining procedure motile cells were stained concomitantly with non-motile cells when non-motile cells were assayed for the presence of flagella.

The percentage of cells flagellated for the various stages of the morphogenic cycle was determined from stained preparations using a Leitz Ortholux microscope at 1000x magnification. The percentage of cells flagellated for each sample period was compared for significance of difference by the statistical t test (12).

Relative cell densities were determined from absorbance at 480 nm on a Bausch and Lomb Spectronic 20.

Biochemicals. L-lysine, Rifampicin (3[4-methylpiperazinylimino-methyl] Rifamycin S V) and L-Chloramphenicol (Chlormycetin) were obtained from Sigma Chemical Co. Rifampicin was used at a concentration of 100 μg/ml to inhibit messenger ribonucleic acid synthesis. L-Chloramphenicol was used at a concentration of 20 μg/ml to inhibit protein synthesis (11).

RESULTS

Growth of A. atrocyaneus in mineral salts L-lysine medium. A. atrocyaneus exhibited an extremely slow growth rate in MS medium with L-lysine as the sole carbon source when compared to growth in complex medium (Fig. 1). For the entire growth cycle (lag phase, exponential growth phase, and stationary phase) the cells grown in MS lysine medium retained the coccoidal morphology. No morphogenic cycle accompanied the growth cycle. No motility was observed during the growth cycle and flagella could not be detected by staining procedures.

Correlation of motility with the morphogenic cycle. Figure 2 shows the correlation of changes in cellular morphology with the presence of flagella during growth of A. atrocyaneus in complex medium (TGY). As with A. atrocyaneus, A. simplex and A. citreus showed a correlation of motility with the morphogenic cycle. For all three species motility began after induction to rod morphology and ended prior to reversion to cocci.

Variations in percent of cells flagellated vs. growth. For the nonmorphogenic P. aeruginosa population the percent of the cells which were flagellated increased through the log phase and early stationary phase (i.e., 0 to 27th h) then plateaued at 90% flagellation for the remainder of the stationary phase (Fig. 3). In contrast, the A. atrocyaneus population reached a peak of 60% flagellation after induction, yet prior to the first fragmentation division. The percent of the population which was flagellated then rapidly declined from this peak to near zero at reversion. A. simplex followed basically the same

pattern of population flagellated as A. atrocyaneus, reaching a peak of 75% flagellation at about the time of the first fragmentation division. A. citreus showed a very low incidence of flagellation. All organisms were predominantly monotrichous.

Commitment to flagella synthesis in A. atrocyaneus. When A. atrocyaneus was grown at a temperature permissive for flagella synthesis, the culture was motile by the 6th h. During the first 6 hours of incubation the culture completed the morphogenic stages of induction and elongation to long rods, and had commenced fragmentation.

At permissive growth temperatures above 34C A. atrocyaneus grew well but was atrichous. If a culture was shifted to 37C, after 1, 2, 3, or 4 hours of incubation at 25C or 30C, motility was prevented. If the culture grew for 5 hours at 25C or 30 C before being shifted to 37C, motility was expressed at the 6th h and persisted for several hours.

Chloramphenicol or rifampicin, when added to a 30 C culture of A. atrocyaneus in the same time sequence as the temperature shifts (25C to 37C), greatly retarded the expression of motility. If the antibiotics were added to motile 6 hour cultures, motility continued approximately 1 h. Both antibiotics inhibited the synthesis of flagella when added to the culture prior to the sixth h.

When A. atrocyaneus was grown at 37C, it passed through the same morphogenic steps for the first 6 h as it did when grown at 25C or 30 C. Major differences did occur in the cultures when they were compared to cultures grown at 30C. The growth rate was increased, the rods were atrichous and tended to clump while undergoing fragmentation. If a culture was shifted from 37C to 30C at a time before the sixth h

of incubation, motility was subsequently observed. If the culture was maintained at 37C for 6 h, then shifted to 30C, motility did not subsequently ensue. When the shift (37 to 30C) was made at the 5th h, 26% of the cells subsequently were flagellated after 3 h incubation at 30C. When the shift (37 to 30C) was made at the 6th h, less then 6% of the cells subsequently were flagellated after 3 h incubation at 30C (Table 1).

DISCUSSION

For all three Arthrobacter species studied flagellar synthesis commenced after induction and cellular deflagellation occurred during reversion. The percentage of A. atrocyaneus and A. simplex cells flagellated throughout the growth cycle differed significantly when compared to the non-morphogenic P. aeruginosa. The data in figure (3) indicate that the Arthrobacters synthesized flagella for a much shorter duration of their growth cycle than did P. aeruginosa. From this it was hypothesized that A. atrocyaneus and A. simplex synthesized flagella only once during their morphogenic cycle. Subsequent fragmentation divisions resulted in one daughter cell receiving the parental flagellum. The daughter cell which did not receive a parental flagellum did not synthesize a new flagellum. The rapid increase in cell numbers during fragmentation then resulted in a sharp drop in the percentage of the population which was flagellated.

Flagellar synthesis as a function of the morphogenic cycle and not merely the growth cycle was shown by growing A. atrocyaneus in MS lysine medium. Lucas and Clark (10) have shown that A. atrocyaneus grew only as a coccus in this medium, making it possible to separate the growth cycle and its aging effects from the morphogenic cycle. Although the coccoidal cells of some Arthrobacter species are flagellated (1, 2), the A. atrocyaneus cocci were atrichous as exponentially growing cocci and stationary phase cocci. When the A. atrocyaneus cocci were induced to enter the morphogenic cycle by the addition of an inducing carbon source to the medium (10), the resulting rods were flagellated and motile.

The temperature shift and antibiotic experiments gave insight into the temporal aspect of motility in A. atrocyaneus. Shifting the culture from a temperature permissive for flagella synthesis to a non-permissive temperature showed that a commitment to flagellation was made during elongation (5th h), shortly before the appearance of flagella. Shifting the culture from a non-permissive temperature to a permissive temperature showed that the commitment period could extend into the long rod stage but not into the fragmentation stage. Results using rifampicin, an inhibitor of messenger ribonucleic acid synthesis, indicated that the genetic information for flagellin synthesis and motility was not transcribed until the elongation stage (5th h).

Since the genetic information for flagellar synthesis is transcribed sometime after the commencement of the morphogenic cycle we may assume that flagellar synthesis is not obligatory to the morphogenic cycle of A. atrocyaneus. This would correlate with the data indicating that flagellar synthesis is not obligatory to the cell cycle of C. crescentus (8).

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Table 1. Culture motility for A. atrocyaneus as a function of time and temperature.

Growth at 30C

h of incubation	O.D. 480 nm	Motility	% of cells flagellated	Morphological stage
3	0.05	-	1.3	coccus
4	0.05	-	0.8	induction
5	0.07	-	1.2	induction
6	0.10	+	7.0	long rod
7	0.16	+	47	fragmentation

Growth at 37C followed by growth at 30C

Time of incubation						
h @ 37C	h @ 30C	Total h	O.D. 480 nm	Motility	% of cells flagellated	Morphological stage
3	3	6	0.21	+	14	fragmentation
3	4	7	0.34	+	44	fragmentation
4	2	6	0.26	-	1.1	long rod
4	3	7	0.41	+	18	fragmentation
5	1	6	0.36	-	0	long rod
5	2	7	0.56	+	3.8	fragmentation
5	3	8	0.75	+	26	fragmentation
6	1	7	0.68	-	0	fragmentation
6	2	8	0.85	-	0.5	fragmentation
6	3	9	1.20	-	5.8	fragmentation

Fig. 1. Growth of A. atrocyaneus in complex medium and MS lysine medium. Initial inoculum was MS lysine grown coccoidal cells. Symbols: 0, complex medium; □, M.S. lysine medium.

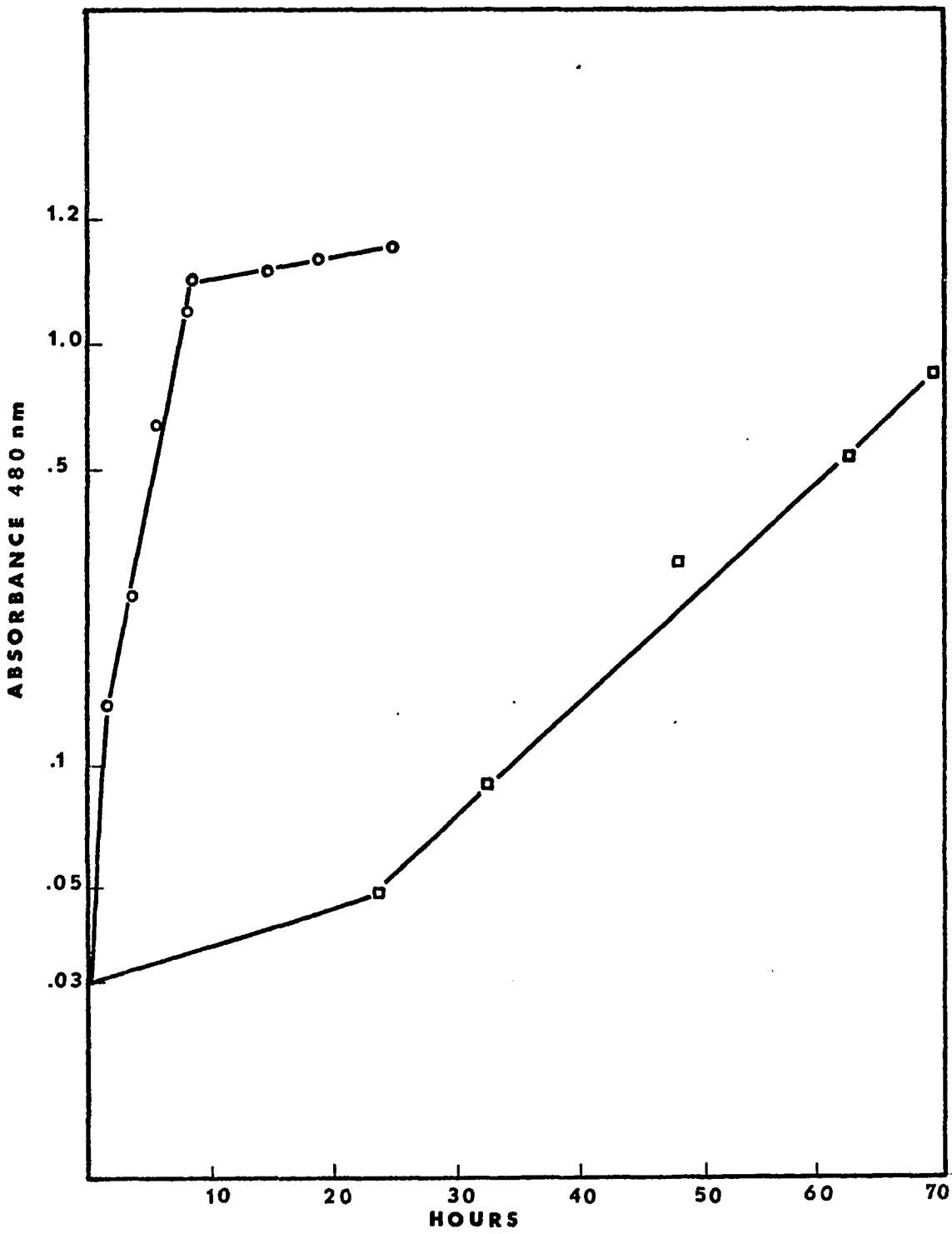

ABSORBANCE 480 nm
1.2
1.0
.5
.1
.05
.03
10
20
30
40
50
60
70
HOURS

Fig. 2. Life cycle of A. atrocyaneus grown in complex liquid medium. (A) atrichous cocci of uniform size; (B) induction, cocci elongating into rods and synthesizing flagella; (C) long rod stage, motile rod undergoing first fragmentation division; (D) and (E) fragmentation, a series of cell divisions resulting in flagellated and atrichous rods of decreasing cell length; (F) reversion, atrichous and non-motile flagellated cocci. Flagellated cocci subsequently lose their flagella restoring stage (A). Motility is observed in stages (C) through (E). Synchrony of the cultural morphogenic cycle degenerates during fragmentation.

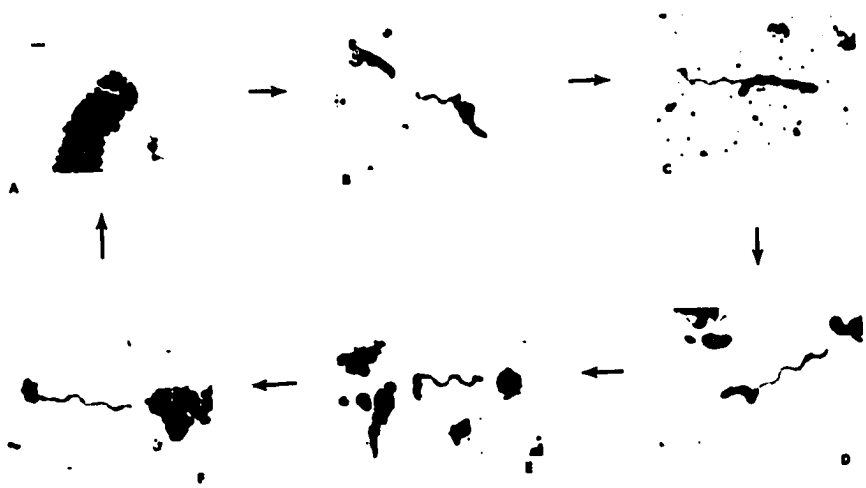
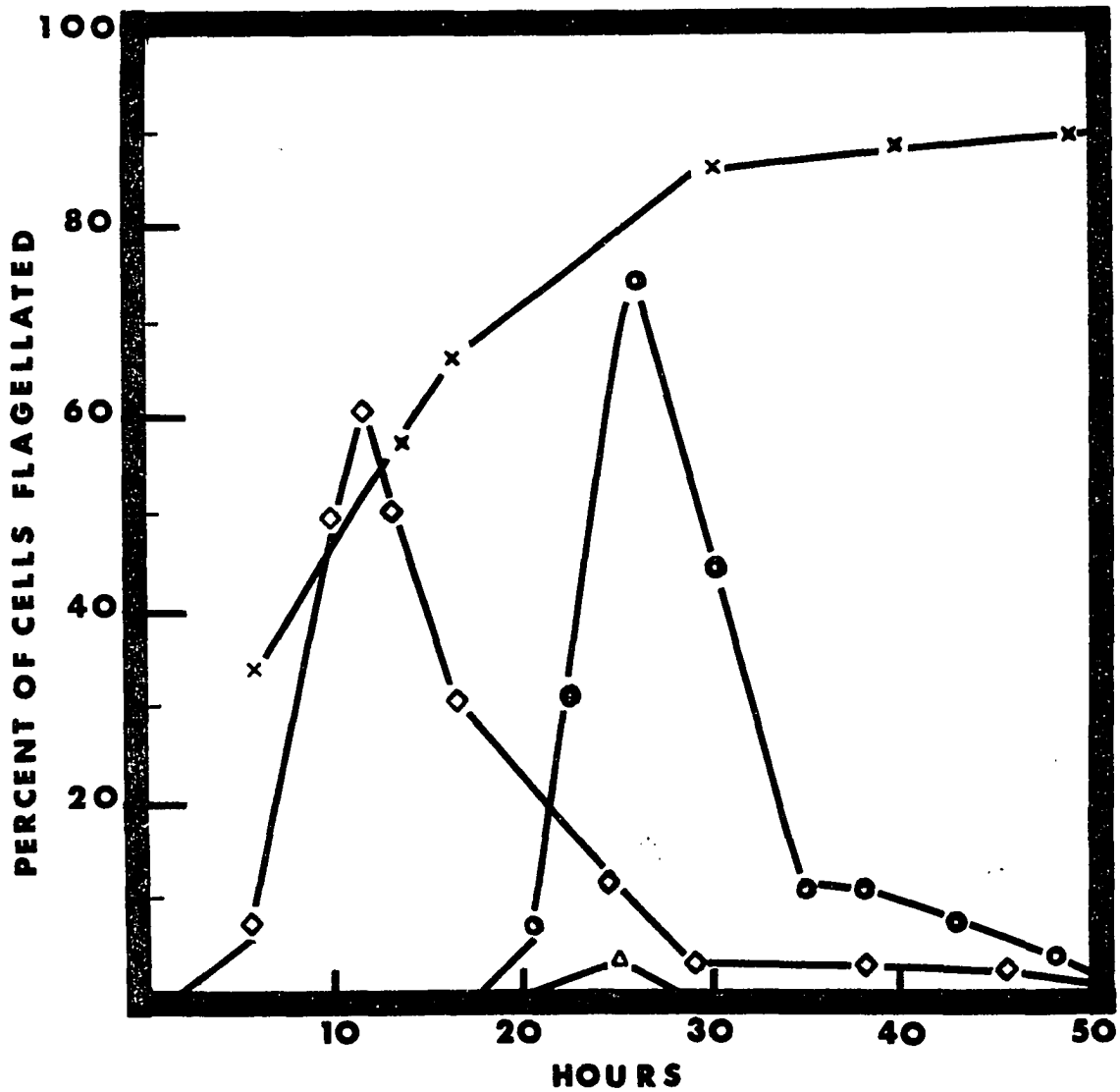

A
B
C
D

Fig. 3. The percent of cells flagellated in a population vs. time for P. aeruginosa and the morphogenic cycle for A. atrocyaneus, A. simplex, and A. citreus. Cultures were grown in complex liquid medium at 25 C and 200 rev min^{-1}, beginning with synchronized cocci. The percent of cells falgellated was determined from prepared material stained for the presence of flagella. Symbols; ◇ , A. atrocyaneus; ▲ , A. citreus; 0, A. simplex; X, P. aeruginosa.



ind. frag. rev. A. atrocyaneus

ind. frag. rev. A. simplex

PAPER II

A SLIDE AGGLUTINATION AND IMMUNODIFFUSION STUDY OF *ARTHROBACTER ATROCYANEUS*

A SLIDE AGGLUTINATION AND IMMUNODIFFUSION STUDY OF ARTHROBACTER ATROCYANEUS

ABSTRACT

Cross reactivity between Arthrobacter atrocyaneus, A. citreus and A. globiformis was demonstrated by slide agglutination. The flagella of A. atrocyaneus and A. simplex were shown not to be antigenically similar. Arthrobacter species agglutination titers differed depending upon the morphogenic (rod or coccus) specificity of the antiserum and the morphogenic form of the antigen. Immunodiffusion studies revealed that the rod and coccoidal forms of A. atrocyaneus were antigenically different but that both morphogenically derived cocci and non-morphogenically derived cocci were basically similar.

INTRODUCTION

Due to the possession of a morphogenic cycle (1), the ability to degrade chemical substances (5) and the challenge of its taxonomy (3) the physiology and morphology of the genus Arthrobacter has received a great amount of study. However, immunological techniques have been applied only to a limited extent in the study of the genus.

The antigenic relationships between species of the genus Arthrobacter were surveyed by Katznelson (6). Ten years later Holland (4) showed that the rod and coccoidal forms of A. globiformis var. polychromogens were antigenically distinct.

The present study was undertaken: (1) to determine if the antigenic distinctness of rod and coccoidal cell walls is true for a second species of Arthrobacter (A. atrocyaneus); (2) to see if antigenic distinctness of the rod and coccoidal forms is of importance in evaluating the antigenic relatedness between members of the genus Arthrobacter; and (3) to question if the antigenicity of the cell walls of cocci, which have undergone growth and division without a morphogenic cycle and those which have developed via a morphogenic cycle, differs.

MATERIALS AND METHODS

Organisms and cultural conditions. The Arthrobacter cultures used and their sources are listed in Table 1. The cultures exhibited a morphogenic cycle when grown in tryptone, glucose, yeast extract (TGY) medium Baltimore Biological Laboratory, Inc.) on a New Brunswick Pyscrotherm rotary shaker (200 rev min^{-1}) at 30C.

In addition to the named cultures, morphological and auxotrophic variants of A. atrocyaneus were used. The variants were naturally occurring or induced mutants isolated in our laboratory. The leuco variant was a naturally occurring mutant which failed to produce a yellow pigment on TGY plates. The rough and capsular variants were isolated in our laboratory by Ralph Jones. They were resistant to lysis by the phage Phi A 21001 upon initial challenge. The rough mutant exhibited much branching during rod growth and produced a rough, convoluted colony. The capsular mutant possessed a thick capsule and produced a large mucoid colony. The variant M-8 was isolated by Randy Jeter using UV irradiation. It produced the blue pigment indigoidine constitutively and was arginine negative. The lysine negative mutant required lysine for growth. When A. atrocyaneus was grown in a minimal salts (MS) medium with 0.5% L-lysine as the sole carbon source (8) it grew as a coccus and did not go through a morphogenic cycle.

Preparation of antigens for antiserum production. For the production of specific antisera to be used in agglutination and immunodiffusion tests A. atrocyaneus cells were grown in either TGY medium to obtain motile rods or in MS-lysine medium to obtain cocci. The

stationary phase coccoidal cell walls, used for immunization, were prepared by sonication according to the method of Holland (4) with the exception that the phosphate-buffered saline (pH 7.3) did not contain sucrose or L-cysteine hydrochloride.

Preparation of antigens for immunodiffusion. A. atrocyaneus cells, to be used as the source for diffusible antigens in the immunodiffusion tests, were grown in either TGY medium to obtain motile rods and morphogenically derived stationary phase cocci or in MS-lysine to obtain dividing cocci and nonmorphogenically derived stationary phase cocci. The cells were harvested by centrifugation, treated with formalin, washed twice, and resuspended to a concentration of 100/mg/ml in buffered saline.

Preparation of antigens for slide agglutination tests. Cells to be used as antigens for slide agglutination were grown in TGY medium to the desired morphology (rod or coccus), collected by centrifugation, treated with formalin, washed twice in 0.01M phosphate buffer (pH 7), and standardized to a final concentration giving an absorbance of 0.5 at 480 nm.

In preliminary studies cell suspensions of A. aurescens, A. citreus, A. crystallopoietes, and A. ramosus as rods and/or cocci spontaneously agglutinated, resulting in false positive antigen controls. This problem was alleviated or reduced by mild sonication. Individual cells were then separated from clumped cells by layering the cell suspension over a 40% sucrose solution and centrifuging for 10 minutes (2500 xg) at 25°C in a Sorval HB-4 rotor. A diffuse band, containing

the individual cells, and a pellet were formed. The cells in the band were collected, washed, and resuspended to the desired concentration in 0.01M phosphate buffer.

Preparation of antisera. A. atrocyaneus flagellated rods or cell wall fragments of atrichous cocci were mixed with an equivalent amount of Freund's complete adjuvant, emulsified, and 1 ml of the antigen emulsion injected intradermally into white New Zealand rabbits at weekly intervals for 3 weeks. This was followed by 3 weekly injections of the cell suspension without adjuvant. The rabbits were then test bled and the titer was maintained by weekly 1 ml intravenous injections prior to final bleeding. The serum was stored at -20C.

Adsorption of immune serum was performed by incubating the serum with a heavy suspension of whole cells at 25C for 2-4 hours and then overnight at 4C. The immune serum was separated from the cells by centrifugation and used the same day or stored at -20C. In some experiments repeated adsorptions were required to remove residual antibodies. Immune serum specific for A. atrocyaneus flagella was prepared by adsorbing the rod immune serum with rods deflagellated by sonication.

Slide agglutination test. A 0.03 ml sample of immune serum, diluted in phosphate buffered saline, was mixed with an equivalent amount of standardized antigen suspension on a microscope slide and then incubated for 5 minutes at 25C. A coverslip was then added and agglutination read at 100x magnification with darkfield illumination. The endpoint was recorded as the highest dilution of antiserum showing agglutination. Antigen controls and normal rabbit serum controls were run with all tests.

Nonspecific agglutination of some Arthrobacter species by normal rabbit serum (10) was also observed. The problem of nonspecific agglutination was overcome by heating the normal serum or immune serum at 56C for 5 minutes and using a serum dilution of 1:40 or greater for assay.

Immunodiffusion studies. The diffusible antigens of morphogenic forms of A. atrocyaneus cells were compared by the micro Ouchterlony method (2). Purified agar was dissolved in physiological saline, containing 0.1% w/v merthiolate, to give a final agar concentration of 1.35% w/v. A 50 mm x 75 mm glass slide, precoated with a thin layer of 2% w/v agar, was covered with enough melted agar to give a layer 1 mm thick. Wells of 4 mm O.D. were cut in the solidified agar so that 6 mm separated all peripheral wells and 3 mm separated each peripheral well from the central well. The wells were then charged with either the antigen suspension or undiluted immune serum and incubated for 48 hrs at 25C.

RESULTS

Characterization of Arthrobacter sp. var. lysine negative. A lysine negative Arthrobacter variant was isolated from a diauxic (succinate and lysine) grown culture of A. atrocyaneus. The lysine negative strain possessed a morphogenic cycle, synthesized a blue pigment, and was motile in TGY medium. Its auxotrophic requirement for lysine was determined by testing the organism for growth in MS base with various carbon sources and growth factors added (Table 2). The only carbon source which supported growth was L-lysine. It was found that various carbon sources supported growth of the lysine neg. strain when the medium was supplemented with 2.73×10^{-4} moles/liter (0.005% w/v) L-lysine. The lysine neg. strain did not grow in MS medium with 0.005% L-lysine as sole carbon source. When the lysine neg. strain, grown in the L-lysine supplemented MS media with various carbon sources, was compared to the morphologically similar A. atrocyaneus or the type species A. globiformis it differed metabolically. Based on the carbon sources tested the lysine neg. strain did not correlate 100% with any species tested by Lucas and Clark (8).

Pyruvate plus aspartate, picolinic acid and diaminopimelic acid, intermediates of lysine biosynthesis in bacteria (9), did not replace the need for lysine. Diaminopimelic acid is the last intermediate in the biosynthetic pathway leading to lysine. Its inability to substitute for lysine would indicate that the decarboxylation of diaminopimelic acid could be the blocked step.

Slide agglutination. The rsults of slide agglutination

reactions between specific antisera produced against A. atrocyaneus rod and coccoidal forms and antigens consisting of rod and coccoidal forms of various Arthrobacter which have been grouped taxonomically as A. globiformis (7) are presented in Table 3. A maximum titer of 1:1600 was obtained with each antiserum and its respective homologous antigens. The rod and coccoidal antisera reacted to different degrees with both morphogenic forms of A. atrocyaneus. The anti-rod serum cross reacted with both morphogenic forms of A. globiformis while the anti-coccoidal serum cross reacted only with the coccoidal form of A. globiformis. No cross agglutination was detected between the A. atrocyaneus anti-rod and anti-coccoidal sera and the rod and coccoidal forms of the other A. globiformis group Arthrobacter species tested (7).

The cross reactivity between the rod and coccoidal antisera of A. atrocyaneus and the rod and coccoidal forms of two other motile, but taxonomically distinct (7), species (i.e. A. citreus and A. simplex) was investigated (Table 4). The anti-coccoidal serum cross reacted with the rod and coccoidal forms of A. citreus. No cross reactivity was detected between the rod forms of A. citreus or A. simplex when they were tested with antisera prepared against the rod form of A. atrocyaneus. No cross reactivity was detected between the rod form of A. simplex and anti-serum rendered specific for A. atrocyaneus flagella (Titer 1:800) by adsorption with atrichous rods.

After 9 months storage at -20C the cocci cell wall specific anti-serum gave lower titers in slide agglutination test with the rod and coccoidal morphologies of A. atrocyaneus. In addition, the anti-serum no longer showed higher titers for cocci as opposed to rods.

Adsorbtion of the anti-serum by whole rods of A. atrocyaneus failed to produce an immune serum specific for cocci. The anti-serum no longer cross agglutinated A. globiformis cocci at a dilution of 1:100 but did at 1:40.

Slide agglutination tests of four morphological variants of A. atrocyaneus gave positive results in 3 cases and a negative result in the case of the capsular variant. The slide agglutination test for the A. sp. var. lysine negative was negative at the 1:40 antiserum dilution (Table 5).

Immunodiffusion. The precipitation reactions between the diffusible antigens of (1) MS lysine grown A. atrocyaneus dividing cocci, (2) TGY grown A. atrocyaneus log phase rods, (3) MS lysine grown A. atrocyaneus stationary phase cocci, and (4) TGY grown A. atrocyaneus stationary phase cocci and specific immune serum prepared against type 3 A. atrocyaneus cell wall fragments are presented in Figs. 1 and 2. An inner line of precipitation developed which was identical for all antigens tested. A second line of identity developed for a diffusible antigen common to the coccoidal forms. A third precipitation line developed opposite coccoidal antigen number 4 only when it was positioned between coccoidal antigens 1 and 3, and was formed by the combination of 2 lines of partial identity of 4 to 1 and 4 to 3. These results suggest that coccoidal antigen 4 has an additional antigenic determinate group which is not shared by the coccoidal antigens 1 or 3.

A minor line of precipitation developed opposite antigen number 3 between the first and second lines of precipitation. When the same

cell wall antiserum was adsorbed by either the whole cells of 1, 3, or 4, no precipitation line developed when the adsorbed serum was retested against the four antigens. Adsorption of the cell wall antiserum with no. 2 whole cells resulted in the development of a precipitation line opposite 1, 3 and 4 which was identical to the second line of precipitation in Fig. 1 (Fig. 3).

DISCUSSION

The discovery of cross reactivity of A. atrocyaneus with A. globiformis and A. citreus indicated a greater degree of antigenic similarity for cell wall antigens of the genus Arthrobacter than previously observed (6). This increased expression of cross reactivity may have been due to a difference in the method of immunization. Although there was reactivity of A. atrocyaneus with A. globiformis, this relatedness did not extend to reactivity with a majority of the other Arthrobacters grouped taxonomically as strains of A. globiformis (7).

Attempts to agglutinate flagellated A. simplex rods with anti-serum specific for A. atrocyaneus flagellated rods or anti-serum rendered specific for A. atrocyaneus flagella were negative, which indicates that the flagella of these two species are antigenically distinct. A similar evaluation of the antigenic flagellar composition of A. citreus could not be made because of the physical manipulation required for preparing self agglutinating species as antigens (Methods).

Slide agglutination of suspected auxotrophic and morphological variants of A. atrocyaneus provided a rapid screening procedure for the identification of true mutants. The suspected auxotrophic mutant, Arthrobacter sp. lysine negative, shared major morphological markers with A. atrocyaneus but was immunologically dissimilar. Subsequent nutritional testing confirmed that the lysine negative strain was not A. atrocyaneus. The routine use of immunology would be of assistance for any type of genetic work involving an organism as morphologically variable as Arthrobacter.

Arthrobacter species agglutination titers differed depending upon the morphological specificity (rod or coccus) of the organisms used to stimulate anti-serum production and the morphological form of the antigen used in the agglutination tests. This was supported by immunodiffusion studies which showed that the rod and coccoidal forms of A. atrocyaneus are antigenically different.

During morphogenic growth of A. atrocyaneus the step of induction, i.e. change from cocci to rods, resulted in the loss of an antigenic component. After a series of divisions as rods, reversion to cocci occurred, resulting in the reappearance of the lost antigenic component. During cultural growth without morphogenesis (MS lysine) the antigenic component was present in both dividing cocci and maximum stationary phase cocci. This suggests that the loss and regain of the antigenic component during morphogenesis is not a result of aging, but instead is directly associated with the changing morphology.

Non-morphogenically derived MS lysine grown cocci and morphogenically derived TGY grown cocci share most antigenic components in common; however, the TGY grown cocci and the MS lysine grown stationary phase cocci to possess unshared antigenic characteristics. Whether these characteristics are due to the morphogenic cycle or the difference in media is not known.

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TABLE 1

Cultures and Sources

A. atrocyaneus	CBRI 21001
A. aurescens	CBRI 21002
A. citreus	CBRI 21003
A. crystallopoietes	ATCC 15481
A. globiformis	ATCC 8010
A. oxydans	CBRI 21010
A. ramosus	CBRI 21013
A. simplex	ATCC 6946
A. ureafaciens	CBRI 21017

CBRI - Cell Biology Research Institute, Canada Department of Agriculture, Ottawa, Canada.

ATCC - American Type Culture Collection, Rockville, Md.

TABLE 2

Arthrobacter sp. var. lysine negative grown in MS media containing 0.5% of carbon source.

Carbon Source	Growth Factor	Growth
AMINO ACIDS		
Alanine	YE[1]	-[2]
Glycine		-
Serine		-
Asparagine		0[3]
Glutamate	YE	-
Arginine	YE	0
Lysine		+[4]
Histidine	YE	-
Proline		0
NON-NITROGEN CARBON SOURCES		
Adipic acid		-
Citrate	YE	-
Gluconate		-
Malate	YE	-
Pyruvate		-
Succinate	Biotin[5]	-
Glycerol		-
Fructose	YE	-
Glucose	Biotin	-

TABLE 2 Continued

Carbon Source	Growth Factor	Growth
Galactose		0
Lactose	YE	0
Mannose	YE	0
Sucrose		0

1. Yeast extract @ 20 μg/liter.
2. No growth; less than 3X increase in OD at 480 nm.
3. Slight growth; increase in OD @ 480 nm of 3X but not 5X.
4. Growth; 5X or greater increase in OD at 480 nm.
5. Final concentration of 4 μg/liter.

TABLE 3

Agglutination reactions between A. atrocyaneus specific antisera and taxonomically related Arthrobacter species[1].

Antigen	Agglutination titers of antisera	
	A. atrocyaneus rod specific	A. atrocyaneus coccus specific
A. atrocyaneus rods	1:1600	1:400
A. atrocyaneus cocci	1:1200	1:1600
A. aurescens rods	0[2]	0
A. aurescens cocci	0	0
A. crystallopoietes rods	0[3]	0
A. crystallopoietes cocci	0	0
A. globiformis rods	1:200	0
A. globiformis cocci	1:200	1:100
A. oxydans rods	0	0
A. oxydans cocci	0	0
A. ramosus rods	0	0
A. ramosus cocci	0	0
A. ureafaciens rods	0	0
A. ureafaciens cocci	0	0

1] Arthrobacter species classified as A. globiformis in Bergey's Manual of Determinative Bacteriology, 8th ed.

2] Negative at 1:40 and 1:100 antiserum dilutions.

3] Nonspecific agglutination with normal and immune rabbit serum at 1:40 serum dilution.

TABLE 4

Agglutination reactions for some motile Arthrobacter species

Antigen	Flagellated A. atrocyaneus rod specific	Atrichous A. atrocyaneus coccus specific	A. atrocyaneus flagella specific
A. atrocyaneus rods	1:1600	1:400	1:800
A. atrocyaneus cocci	1:1200	1:1600	0
A. citreus rods	0	1:200	
A. citreus cocci	0	1:200	
A. simplex rods	0	0	0
A. simplex cocci	0	0	

TABLE 5

Agglutination reactions between A. atrocyaneus coccoidal cell wall antiserum and suspected morphological mutants of A. atrocyaneus[1)]

Antigen	Agglutination titer
A. atrocyaneus CBRI 21001	1:400
A. atrocyaneus capsular	0 @ 1:40
A. atrocyaneus leuco	1:300
A. atrocyaneus rough	1:400
A. atrocyaneus M-8	1:600
A. sp. var lysine negative	0 @ 1:40
A. globiformis ATCC 8010	1:40

1) Antiserum had been stored for 9 mo. at -20C prior to use.

Fig. 1. Diagram of the precipitation reactions between immune serum prepared against cell wall fragments of MS lysine grown stationary phase A. atrocyaneus cocci (S) and the antigens in A. atrocyaneus whole cell (1) MS lysine grown dividing cocci, (2) TGY grown log phase rods, (3) MS lysine grown stationary phase cocci, and (4) TGY grown stationary phase cocci.

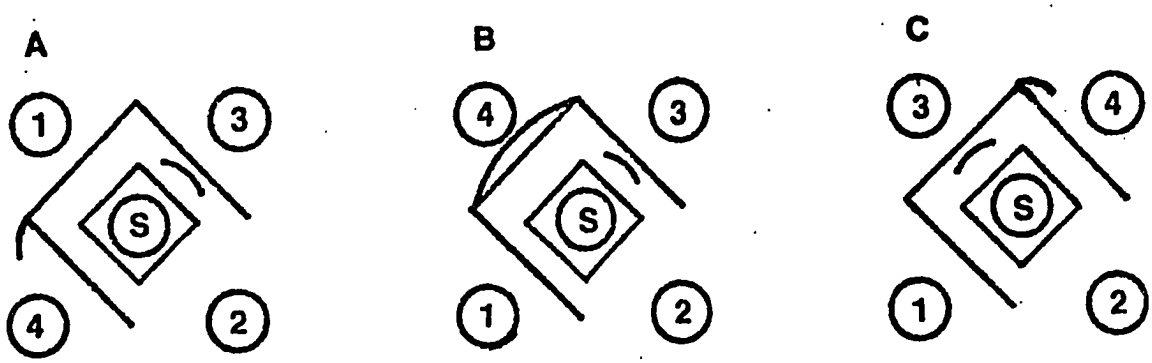

A
1
3
S
4
2
B
4
3
S
1
2
C
3
4
S
1
2

Fig. 2. Precipitation reaction between immune serum prepared against cell wall fragments of MS lysine grown stationary phase A. atrocyaneus cocci and the antigens in A. atrocyaneus whole cell (1) MS lysine grown dividing cocci, (2) TGY grown log phase rods, (3) MS lysine grown stationary phase cocci, and (4) TGY grown stationary phase cocci. This reaction is depicted diagrammatically in Fig. 1-B.

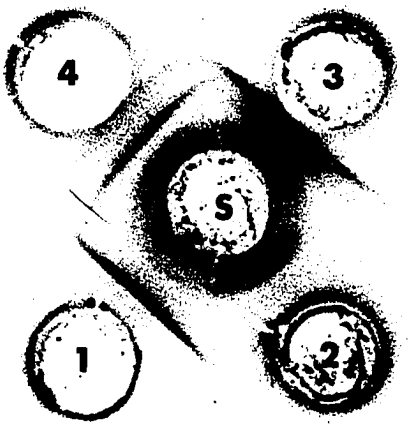
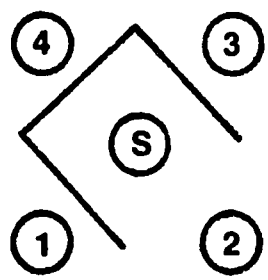

4
3
S
1
2

Fig. 3. Diagram of the precipitation reaction between rod adsorbed immune serum and the antigens in A. atrocyaneus whole cell (1) MS lysine grown dividing cocci, (2) TGY grown log phase rods, (3) MS lysine grown stationary phase cocci, and (4) TGY grown stationary phase cocci. The immune serum prepared against cell wall fragments of MS lysine grown stationary phase A. atrocyaneus cocci was adsorbed by TGY grown log phase rods.


4
3
S
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2

PAPER III

METABOLISM AND ITS RELATIONSHIP TO THE MORPHOGENIC CYCLE IN *A. ATROCYANEUS*

METABOLISM AND ITS RELATIONSHIP TO THE MORPHOGENIC CYCLE IN A. ATROCYANEUS

ABSTRACT

A study of the effects of various carbon sources and vitamins upon growth and morphogenesis of Arthrobacter atrocyaneus (CBRI 21001) was made. Not all carbon sources which supported growth with induction supported reversion. Reversion was shown to require an exogenous energy source in this strain. The TCA cycle was shown to be functional. Biotin stimulated growth of this strain in nearly all carbon sources and had a greater effect than the other vitamins tested. Some carbon sources did not support growth without added biotin while others did. Those which did not support growth were pyruvate or pyruvate precursors. A model is proposed for the control of metabolism by biotin in this strain. Comparisons of specific activities to the morphogenic cycle for NADP specific isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and malic enzyme are made.

INTRODUCTION

Morphology and nutrition are interrelated in the genus Arthrobacter (6, 14, 15, 17). A great emphasis is placed upon the ability or inability of a nutrient to cause cellular morphological changes while supporting growth of an Arthrobacter species. The ability of a nutrient to initiate induction (change in cellular morphology from coccus to rod) is most frequently the only morphogenic criterion noted (6, 14). The effects of nutrients on fragmentation of rods and reversion of rods back to cocci are generally ignored. A strain of A. globiformis (NCIBI 10683) undergoes fragmentation and reversion in phosphate buffer with a complete loss of viability (15). A. crystallopoietes (ATCC 15481), on the other hand, does not undergo reversion in phosphate buffer nor does it exhibit as great a loss of viability (7). Reversion in A. globiformis is apparently powered by endogenous metabolism (15), while reversion in A. crystallopoietes is not. This paper investigates the effects of different nutrients on induction and reversion of A. atrocyaneus (CBRI 20110) and reports the requirement of an exogenous energy source for reversion in this organism.

All named species of Arthrobacter utilize respiration for energy production and are never fermentative (10). Oxidation of pyruvate by the tricarboxylic acid (TCA) cycle has been shown for A.

globiformis (16). The TCA cycle, in whole or part, is probably functioned in all Arthrobacter species. A. atrocyaneus differs from A. globiformis in not utilizing the Embden-Meyerhof-Parnas pathway for glucose metabolism (25). It instead catabolizes glucose via gluconate through the Entner-Doudoroff and the hexose monophosphate pathways (25). The ability of TCA intermediates to support growth of A. atrocyaneus (CBRI 21001) is investigated in this paper and the suppression of L-lysine transport by succinate is shown.

The literature concerning the requirement of A. atrocyaneus for exogenous biotin is contradictory. Wolfson and Krulwich (24) report the growth of A. atrocyaneus (ATCC 13752) in mineral salts glucose medium without the addition of biotin. Keddie (10), however, reports that A. atrocyaneus (M. P. Starr) requires the addition of biotin for growth in a mineral salts glucose medium. This paper reports the vitamin requirement of A. atrocyaneus (CBRI 21001) and presents a mechanism for control of glucose catabolism based on biotin.

The specific activities of A. atrocyaneus enzymes during growth, with and without a morphogenic cycle, were studied. These patterns of enzyme activity were compared to those in A. crystallopoietes (8) and A. globiformis (26).

MATERIALS AND METHODS

Chemicals. The chemicals used were obtained from either Calbiochem, Los Angeles, California; Fischer Scientific Co., Fair Lawn, New Jersey; New England Nuclear, Boston, Massachusetts; Nutrient Biochemicals Corporation, Cleveland, Ohio; or Sigma Chemical Company, St. Louis, Missouri.

Organism. Arthrobacter atrocyaneus CBRI 21001 was obtained courtesy of the Canadian Cell Biology Research Institute, Canada Department of Agriculture, Ottawa, Canada. The culture was maintained on tryptone, glucose, yeast extract slants at 4 C.

Media and culture conditions. The complex medium used was ½ strength DIFCO Plate Count Broth (TGY). The mineral salts (MS) medium for Arthrobacter described by Ensign and Wolfe (6) was used with 0.5% w/v carbon source unless otherwise noted. The chemically defined medium used to support a complete morphogenic cycle (diauxic growth curve) was MS plus 0.006% w/v succinate, 0.4% w/v L-lysine and 2 μg 1 biotin.

Cultures in Erlenmeyer flask, filled no more than ½ volume, were incubated at 30 C and 200 rev. min. $^{-1}$. Growth was initiated by the addition of coccoidal cells to an absorbance of 0.02-0.04 at 480 nm.

Growth studies. Growth in mineral salts media was monitered spectrophotometrically at 480 nm. Morphology was followed by crystal violet, 0.05% w/v, stain or Gram stain. Growth was followed for a minimum of 72 h.

The vitamins used and their final concentrations in μg/l were: biotin, 0.2; folate, 5; niacin, 1; Ca^{++} pantothenate, 1; pyridoxal, 40; pyridoxin, 40; riboflavin, 1; and thiamine, 1.

Cocci used as inoculum for the Avidin experiments were first washed twice in 0.5 M tris (hydroxymethyl)- amino-methane (TRIS)-HCl (pH 7.6). Four ml aliquots of MS culture were incubated in ½ inch colorimeter tubes. The control contained no added Avidin. The test contained 1 unit of Avidin per ml. One unit of Avidin neutralizes 1 μg 1 of biotin.

Reversion. Cells were grown to maximum stationary stage in MS succinate plus biotin medium, then harvested by centrifugation. The cells were resuspended to the same cell density in fresh media and incubated an additional 11 days. Relative viability was determined on TGY streak plates.

Radioactivity studies. Cells were grown to the appropriate morphological stage in MS succinate, lysine, biotin (diauxic growth) medium or the appropriate growth stage in MS lysine medium. They were harvested and washed with cold 0.5M TRIS-HCl buffer (pH 7.6). The cells were inoculated into 18 ml of MS medium with 0.1% w/v L-lysine to give a final absorbance of 0.1 at 480 nm. To this suspension in a 250 ml Erlenmeyer flask, was added 2 ml of ^{14}C L-lysine in distilled water giving a final concentration of 1 μ Ci/ml ^{14}C. The flask was incubated for 20 minutes at 30 C and 200 rev. min^{-1}. The flask was cooled in ice and the suspension filtered on a 0.2 μm Nalge filter and washed with 10 volumes of cold 0.5 M TRIS-HCl (pH 7.6). Each membrane was placed

in a scintillation vial containing 10 ml of fluor (Beckman cocktail D: 100 g naphthalene, 5 g diphenyloxazole, 10 ml H_2O, Q S to 1 l with 1,4- dioxane) and counted in a Beckman Model DPM 100 liquid scintillation system.

Preparation of cell free extracts. Cells were grown to the appropriate stage and harvested by centrifugation for 10 minutes at 8,000 x g at 4 C. The cells were washed with 0.5 M TRIS-HCl (pH 7.6). The cells were resuspended in buffer (4 x w/v) and disrupted by sonication (Blackstone, Model BP-2) for 3-6 min (1 min pulses) at 4 C.

The extract was then centrifuged at 35,000 x g for 30 min at 4 C and the supernatant used for enzymatic assays. Protein was determined by a modified Lowry method (19).

Enzyme assays. All enzyme assays were carried out at 25 C and activity was measured on a Gilford model 2000 recording spectrophotometer. One unit of activity was defined as the amount of enzyme which transformed 1 μM of substrate per minute.

NADP isocitrate dehydrogenase (Threo-D-isocitrate: NADP osicoreductase (decarboxylating); E C 1.1.1.42). Activity was measured by the method of Baldwin and Milligan (1).

Glucose-6-phosphate dehydrogonase (D-glucose-6-phosphate: NADP oxidoreductase; E C 1.1.1.49). The method of Kuby and Noltman (12) was used to measure the reduction of NADP by glucose-6-phosphate.

Malic enzyme. (L-malate: NADP oxidoreductase (decarboxylating); EC 1.1.1.40). Reduction of NADP by malate was measured by the method of Ochoa (18).

RESULTS

Effects of different carbon sources on the morphogenic cycle. The ability of A. atrocyaneus to exhibit a morphogenic cycle in minimal medium differed with the carbon source supplied. L-lysine as the sole carbon source supported growth without induction (14). Most carbon sources supported growth with induction. Only a few amino acids supported growth with both induction and reversion (Table 1). The cell density in MS lysine medium was often higher than that obtained using inducing carbon sources.

Reversion from rod to coccus. Table 1 shows that MS succinate, 0.5% w/v, supported growth with induction but without subsequent reversion. If smaller concentrations of succinate were used, reversion did occur. A concentration of 0.005% w/v succinate supported growth with induction and nearly 100% reversion. A concentration of 0.025% w/v succinate supported growth and induction with no reversion. Concentrations of succinate between 0.005% w/v and 0.025% w/v supported growth with induction and partial reversion. The smaller the concentration of succinate the greater the percentage of rods which reverted to cocci. The increasing concentrations of succinate resulted in higher cell numbers.

Reversion was not observed in a culture incubated for 121 days in MS succinate, 0.5% w/v, supplemented with biotin. A. atrocyaneus rods grown to maximum stationary phase (4 days) in MS succinate, biotin medium failed to revert to cocci when transferred (at the same cell density) to fresh MS base plus biotin or MS succinate plus biotin and

incubated for an additional 11 days. A. atrocyaneus rods did revert to cocci when transferred to fresh MS lysine plus biotin medium. Viability was higher for cells transferred to MS plus biotin or MS lysine plus biotin than for the original culture or cells transferred to fresh MS succinate plus biotin medium (Table 2).

A chemically defined medium which supports an A. atrocyaneus morphogenic cycle. A complete morphogenic cycle, with a high cell density, was obtained in minimal medium containing the carbon sources succinate and L-lysine and the vitamin biotin. A diauxic growth curve resulted. A plateau occured (24th h-30th h Fig. 1), which differentiated rod growth (0 - 24th h) from coccoidal growth (30th h- 75th h). Reversion commenced after the 24th h and was completed by the 40th h.

The uptake of ^{14}C L-lysine was retarded during the rod stage of growth. The level of ^{14}C L-lysine uptake rose sharply during reversion (10th h Fig. 2B) to a level which remained steady during coccoidal growth. The uptake of ^{14}C L-lysine by cells growing in MS lysine was virtually unchanged throughout growth (Fig. 3B).

Effects of vitamins on growth in mineral media. When A. atrocyaneus was grown in 0.5% w/v MS succinate medium only biotin stimulated growth (Fig. 4). When the same vitamins were tested for their effect on the growth of the organism in 0.5% w/v MS lysine medium, several stimulated growth but biotin had a significantly greater effect (Fig. 5).

The effect of biotin addition on the growth of the organism in minimal medium differed with the carbon source. For most carbon sources

the addition of biotin increased growth. For some amino acids the cell density after 48 hrs incubation was maximum without the addition of biotin. Biotin had its greatest stimulatory effect when added to a medium containing glycerol, fructose or lactate as the sole carbon source. Biotin as a carbon source did not support growth or induce rod formation. The addition of biotin inhibited reversion of A. atrocyaneus grown in MS alanine, MS glycine, MS histidine and MS serine (Table 3).

When biotin was added to a minimal medium containing glucose or pyruvate (Fig. 6) growth ensued with induction.

Avidin did not affect growth of A. atrocyaneus in MS succinate or MS lysine but did inhibit growth in MS lactate, MS serine and MS gluconate.

Tricarboxylic acid cycle intermediates. Five intermediates of the TCA cycle supported growth with induction. One supported a complete morphogenic cycle. Two intermediates, citrate and isocitrate, did not support growth (Table 4).

NADP specific isocitrate dehydrogenase activity was present during coccoidal growth of A. atrocyaneus in MS lysine and throughout the morphogenic cycle in the diauxic medium. NADP specific isocitrate dehydrogenase activity was greater in cocci than rods. The greatest specific activity was present in TGY grown stationary cocci. Changes in specific activity correlated with growth rather than the morphogenic cycle (Fig. 7).

Glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase specific activity was greater in cocci than in rods.

Specific activity for A. atrocyaneus grown in diauxic growth medium decreased during induction, then rose gradually through the remainder of growth (Fig. 8). During coccoidal growth in MS lysine, the specific activity remained low for the exponential phase of growth then rose steadily during the stationary phase (Fig. 10).

Malate enzyme. During growth of A. atrocyaneus in diauxic growth medium the malate enzyme specific activity dropped sharply during induction then rose gradually through the remainder of growth (Fig. 8). During coccoidal growth in MS lysine medium, the specific activity was low. No changes in specific activity were noted during the exponential or stationary phases of coccoidal growth (Fig. 9).

DISCUSSION

Succinate and other inducing carbon sources not only support growth and induction of A. atrocyaneus (CBRI 21001) but, when present in excess, inhibit complete fragmentation and reversion of rods to cocci. The non-inducing carbon source L-lysine supported the fragmentation of rods and their subsequent reversion to cocci. These data indicate that succinate and other carbon sources not only induce the coccus to rod morphological change but also inhibit its reversal. Reversion of rods to cocci in A. atrocyaneus (CBRI 21001) is therefore not a constitutive endproduct of induction but a controlled morphogenic step.

A chemically defined medium which supported good growth and a complete morphogenic cycle resulted in a diauxic growth curve. Succinate was preferentially used and inhibited the transport of L-lysine. The inhibition of the transport of a second carbon source by a TCA cycle intermediate is an often reported occurrence in Arthrobacter (11, 20, 21, 22). The ability of all TCA cycle intermediates except citrate or isocitrate to support growth of A. atrocyaneus (CBRI 21001) and the activity of NADP specific isocitrate dehydrogenase during growth indicate that the entire TCA cycle is functional in this bacterium. The inability of Arthrobacter species to grow in MS citrate is the rule rather than the exception (14).

Many strains of Arthrobacter exhibit a biotin requirement (10). For A. atrocyaneus (CBRI 21001) growing in MS medium with a sole carbon source, the addition of biotin usually stimulated growth. Added biotin enabled A. atrocyaneus (CBRI 21001) to utilize some carbon sources it

could not utilize without added biotin. Conversely, the addition of avidin totally inhibited growth of the organism in some carbon sources which previously had supported growth. Pyruvate and catabolic precursors of pyruvate; glucose, gluconate, fructose and lactate could not support growth without added biotin. Of the amino acids tested as nutrients Alanine, serine and glycine supported only a low level of growth without added biotin, but high levels with added biotin. Alanine, serine and glycine all enter the TCA cycle via pyruvate.

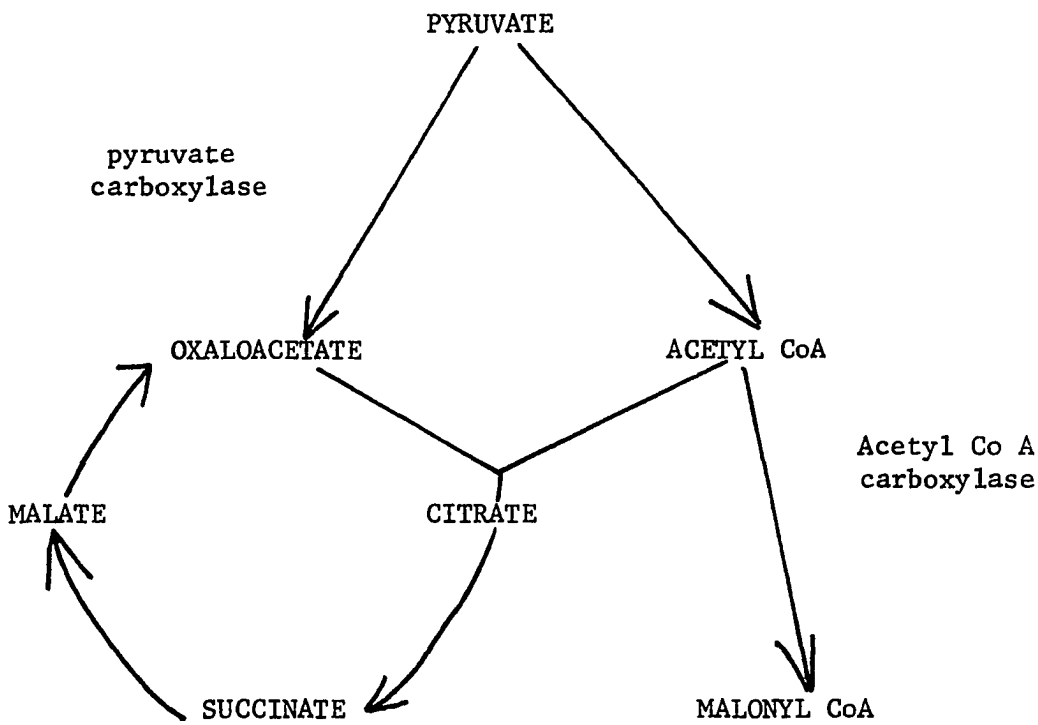
Pyruvate carboxylase and acetyl Co A carboxylase are biotin coupled enzymes. Pyruvate carboxylase is the major allosteric enzyme for the amphibolic pathway synthsizing TCA cycle intermediates. Likewise, acetyl Co A carboxylase is the major allosteric enzyme in fatty acid synthesis (13). There is a high potential for allosteric control interaction between the two carboxylases (13). How many of these allosteric controls are active in A. atrocyaneus (CBRI 21001) is unknown, but acetyl Co A is known to be a critical positive effector of pyruvate carboxylase in A. globiformis (2).

Since some carbon sources support growth of A. atrocyaneus (CBRI 21001) without added biotin, the organism must have a biotin synthetic capability. The following model could explain the biotin effect in A. atrocyaneus (CBRI 21001).

Pyruvate carboxylase (3) and acetyl Co A carboxylase (23) have been shown to exist as inactive apoenzymes in organisms lacking biotin. With the addition of biotin the apoenzymes were converted to functional holoenzymes through the action of a biotin-activating enzyme (4, 5).

1) ATP + biotin $\xrightleftharpoons{Mg^{++}}$ biotinyl-AMP + PPi

2) Biotinyl-AMP + apocarboxylase $\xrightleftharpoons[\text{synthetase}]{\text{holocarboxylase}}$ holocarboxylase + AMP



If in A. atrocyaneus (CBRI 21001) the acetyl Co A carboxylase enzyme system has a higher affinity for biotin than the pyruvate carboxylase system, the pyruvate carboxylase would exist as an inactive apoenzyme and acetyl Co A carboxylase as an active holoenzyme under conditions of biotin limitation.

If the biotin synthetic capabilities of the organism are limited, the acetyl Co A carboxylase apoenzyme would be activated in preference to the pyruvate carboxylase. Therefore, A. atrocyaneus (CBRI 21001)

would grow without added biotin if the nutrient allowed synthesis of TCA cycle intermediates by a pathway which did not require pyruvate carboxylase. Conversely, the organism could not utilize nutrients which required pyruvate carboxylase activity unless biotin was added.

In this model the allosteric controls of pyruvate carboxylase would only function after the intracellular biotin level reached a critical level. The inhibition of growth of A. atrocyaneus (CBRI 21001) on carbon sources (pyruvate and precursors) which would drain biotin away from the critical synthesis of fatty acids would have survival value for an organism with an impaired biotin synthetic capability.

Some reports in the literature which attribute crypticity of A. pyridinolis towards glucose to a transport deficiency (21, 22) could also be explained by this model.

The specific activities for NADP specific isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and malic enzyme, during morphogenic growth of A. atrocyaneus (CBRI 21001), did not correlate with the data obtained in our laboratory for A. crystallopoietes (8) or A. globiformis (26). In all three organisms the patterns of specific activities differed. This indicated that the specific activities of these enzymes during morphogenic growth correlated with the utilization of different carbon sources rather than cellular morphological changes.

A correlation with earlier reports was noted for the specific activity of the malic enzyme during coccoidal growth. Ferdinandus (8) found that A. crystallopoietes growing coccoidally in MS glucose medium possessed no malic enzyme activity. The same was true for A. atrocyaneus growing without induction in MS lysine medium. The lack of

malic enzyme activity during coccoidal growth of a second strain of Arthrobacter utilizing a different carbon source may be a coincidence, but at present it does lend support to the consideration of the malic enzyme as an important enzyme of Arthrobacter morphogenesis (9).

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TABLE 1

CARBON SOURCES FOR ARTHROBACTER ATROCYANEUS

GROWTH WITHOUT INDUCTION

L-Lysine

GROWTH WITH INDUCTION BUT WITHOUT REVERSION

FRUCTOSE	MANNOSE
GALACTOSE	SUCCINATE
GLUCONATE	SUCCROSE
GLYCEROL	L-ALANINE
LACTATE	L-ARGININE
LACTOSE	L-ASPARAGINE
MALATE	L-GLUTAMINE
	GLYCINE

GROWTH WITH INDUCTION AND REVERSION

L-ASPARTATE	L-HISTIDINE
L-GLUTAMATE	L-PHENYLALANINE
	L-SERINE

TABLE 2

Reversion of MS succinate grown[1] A. atrocyaneus rods.

Treatment	% Rods 1.5μ[2]	% Cocci[3]	Viability[4]
No treatment[5]	100	4	less than 1%
MS Succinate[6]	92	10	less than 1%
MS[7]	90	6	100%
MS Lysine[8]	42	62	100%

1. A. atrocyaneus grown for 4 days @ 30C and 200 rev min^{-1} in MS medium with 0.5% succinate and 2 μg/liter biotin. The culture then consisted solely of rods 94% of which 1.5 μ in length.

2. 4 days post-treatment incubation.

3. 11 days post-treatment incubation.

4. 11 days post-treatment incubation. Viability determined using TGY streak plates. MS plate used as 100 % viability standard.

5. Continued incubation of original sample.

6. Cells collected, washed and resuspended to same cell density in new MS succinate medium with 2 μg/liter biotin.

7. Cells collected, washed and resuspended to same cell density in MS base with 2 μg/liter biotin.

8. Cells collected, washed and resuspended to same cell density in MS lysine medium with 2 μg/liter biotin.

TABLE 3

CARBON SOURCES	MAX. O.D. W/O BIOTIN	REVERSION	MAX. O.D. WITH BIOTIN[1]	REVERSION
NON-NITROGEN .5%				
GALACTOSE	.28	no	1.6	no
GLUCONATE	.33	yes	1.6	yes
GLYCEROL	.09	no*[2]	1.6	no*
FRUCTOSE	.08	no	1.6	no*
LACTATE	.08	no*	1.5	no
LACTOSE	.30	no	1.6	no*
MALATE	.54	no	1.3	no*
MANNOSE	.15	no*	1.6	no*
SUCCINATE	.70	no	1.4	no*
SUCROSE	.20	no	1.4	no*
AMINO ACIDS .5%				
ALANINE	.10	yes	1.2	no*
ARGININE	1.4	no	1.3	no

TABLE 3 Continued

CARBON SOURCES	MAX. O.D. W/O BIOTIN	REVERSION	MAX. O.D. WITH BIOTIN[1]	REVERSION
ASPARAGINE	.48	no	1.3	no*
ASPARTATE	.54	no	1.2	no
GLUTAMATE	.65	yes	1.7	yes
GLUTAMINE	.32	no	1.4	no*
GLYCINE	.16	yes	1.0	no
HISTIDINE	1.4	yes	1.4	no*
PHENYLALANINE	1.7	no	1.7	no
PROLINE	1.7	no	1.7	no*
SERINE	.30	yes	1.3	no*

BIOTIN mg/ml	INITIAL O.D.	MAX. O.D.	INDUCTION
$4X10^{-6}$	.025	.035	no
$2X10^{-4}$	.03	.04	
$2X10^{-2}$	.03	.055	no

1. 4 μg/l biotin

2. Gram positive coccoidal inclusions in Gram negative rods.

TABLE 4

TCA Intermediates

Carbon Source	Growth	Induction	Reversion
Oxaloacetate	+	+	-
Citrate	-	-	-
Isocitrate	-	-	-
Alpha Ketogluterate	+	+	+
Succinate	+	+	-
Fumerate	+	+	-
Malate	+	+	-

Fig. 1. Diauxic growth curve of A. atrocyaneus in MS 0.4% w/v lysine, 0.006% w/v succinate and 0.1 μg/l biotin.

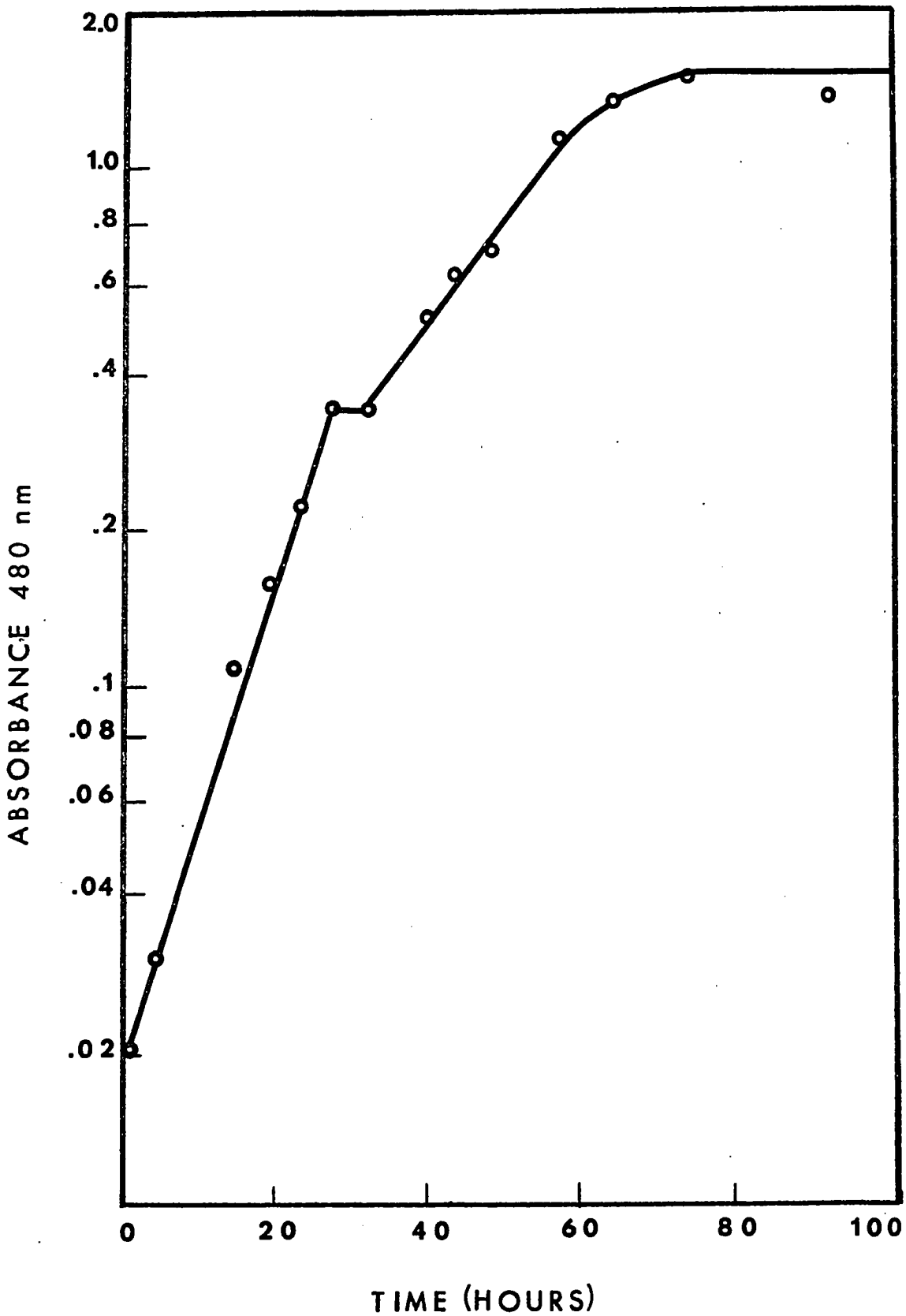

2.0
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
ABSORBANCE 480 nm
0
20
40
60
80
100
TIME (HOURS)

Fig. 2a. Diauxic growth curve of A. atrocyaneus in MS 0.5% w/v lysine, 0.005% w/v succinate, 0.1 μg/l biotin medium.

Fig. 2b. ^{14}C L-lysine uptake by A. atrocyaneus during diauxic growth.

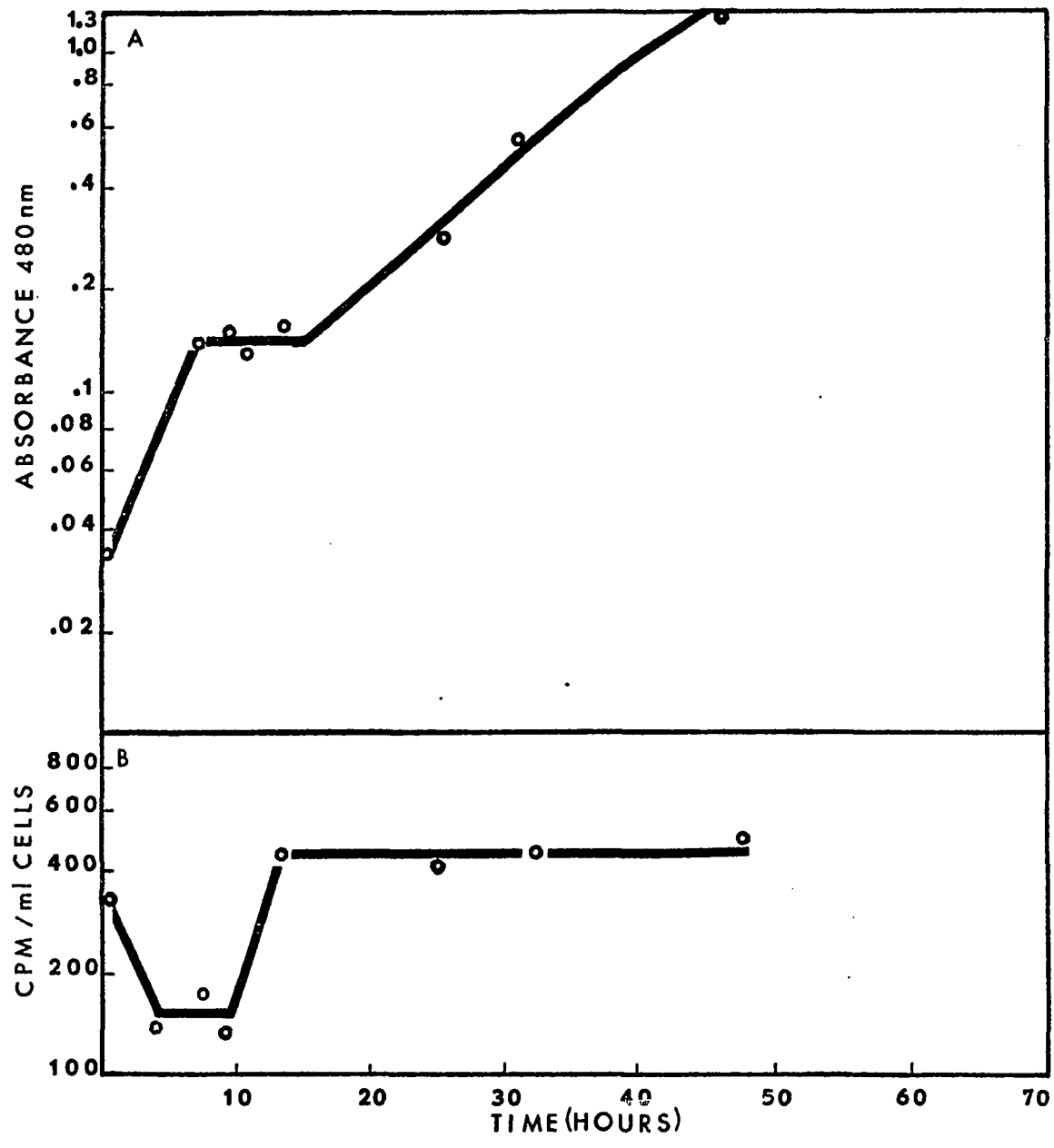

A
B
ABSORBANCE 480nm
1.3
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
CPM / ml CELLS
800
600
400
200
100
10
20
30
40
50
60
70
TIME (HOURS)

Fig. 3a. Growth of A. atrocyaneus in MS lysine medium.

Fig. 3b. ^{14}C L-lysine uptake by A. atrocyaneus during coccoidal growth in MS lysine.

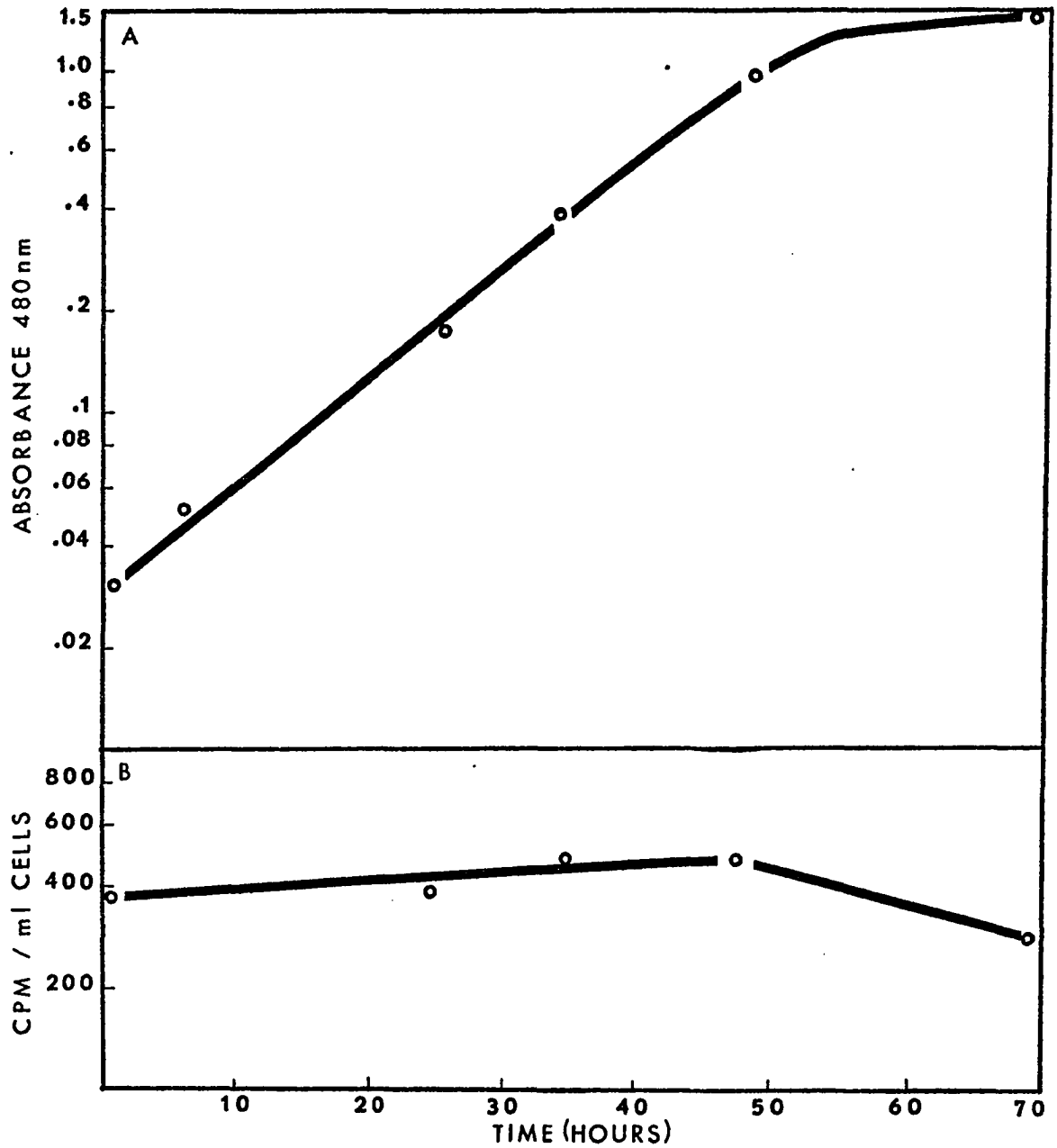

A
B
ABSORBANCE 480nm
CPM / ml CELLS
TIME (HOURS)
1.5
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
800
600
400
200
10
20
30
40
50
60
70

Fig. 4. Growth of A. atrocyaneus in MS succinate medium. (◘) mean value for growth of A. atrocyaneus for all vitamins except biotin, (0) growth with the addition of 0.04 μg/l biotin. All (0) reading from the 38th h on are 2 standard deviations from the mean.

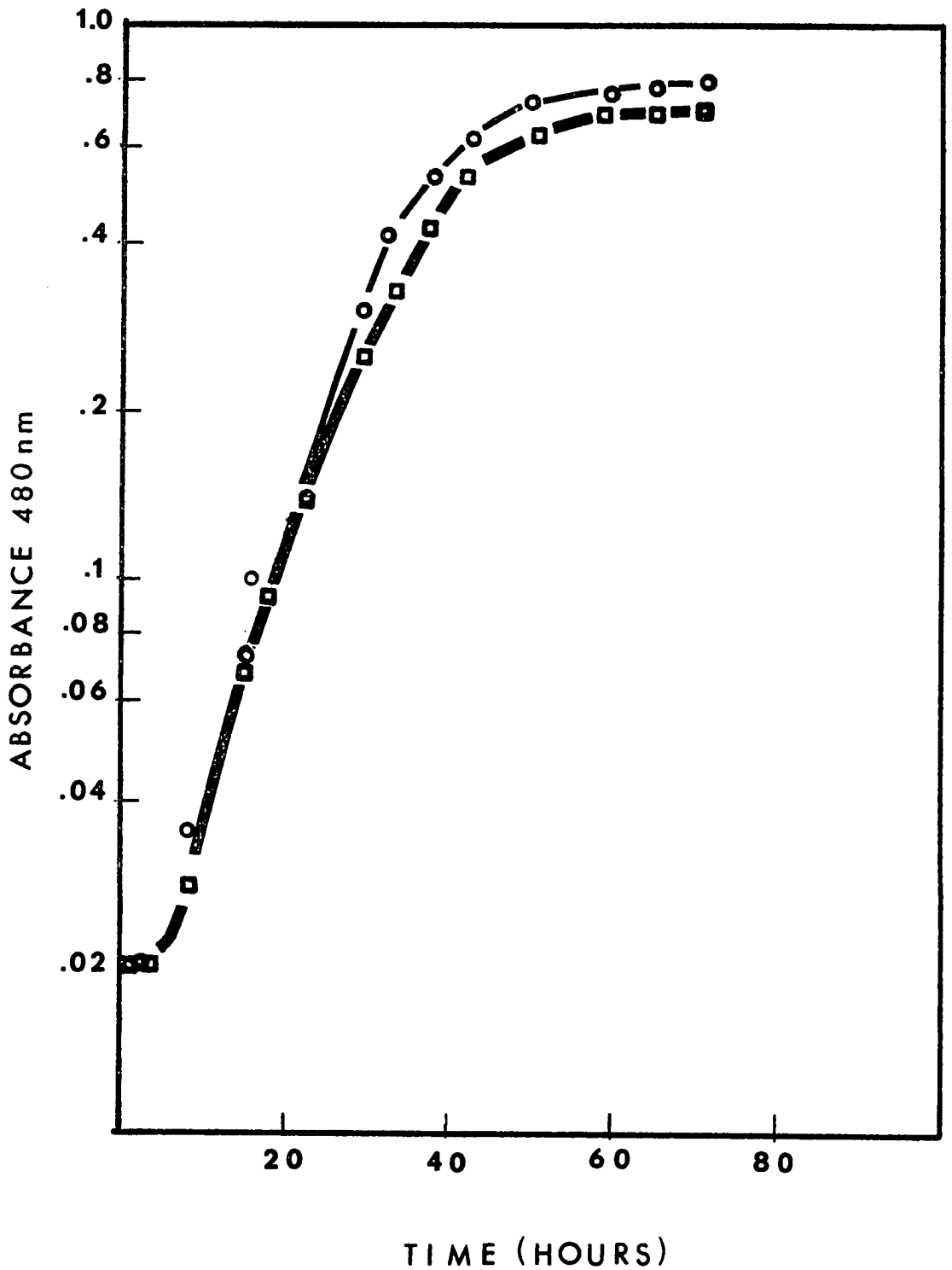

1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
ABSORBANCE 480 nm
20
40
60
80
TIME (HOURS)

Fig. 5. Growth of A. atrocyaneus in MS lysine medium. (X) growth without added vitamins, (□) mean and 1 standard deviation of the mean for all samples with and without added vitamins, (0) growth with 0.04 μg/l added biotin.

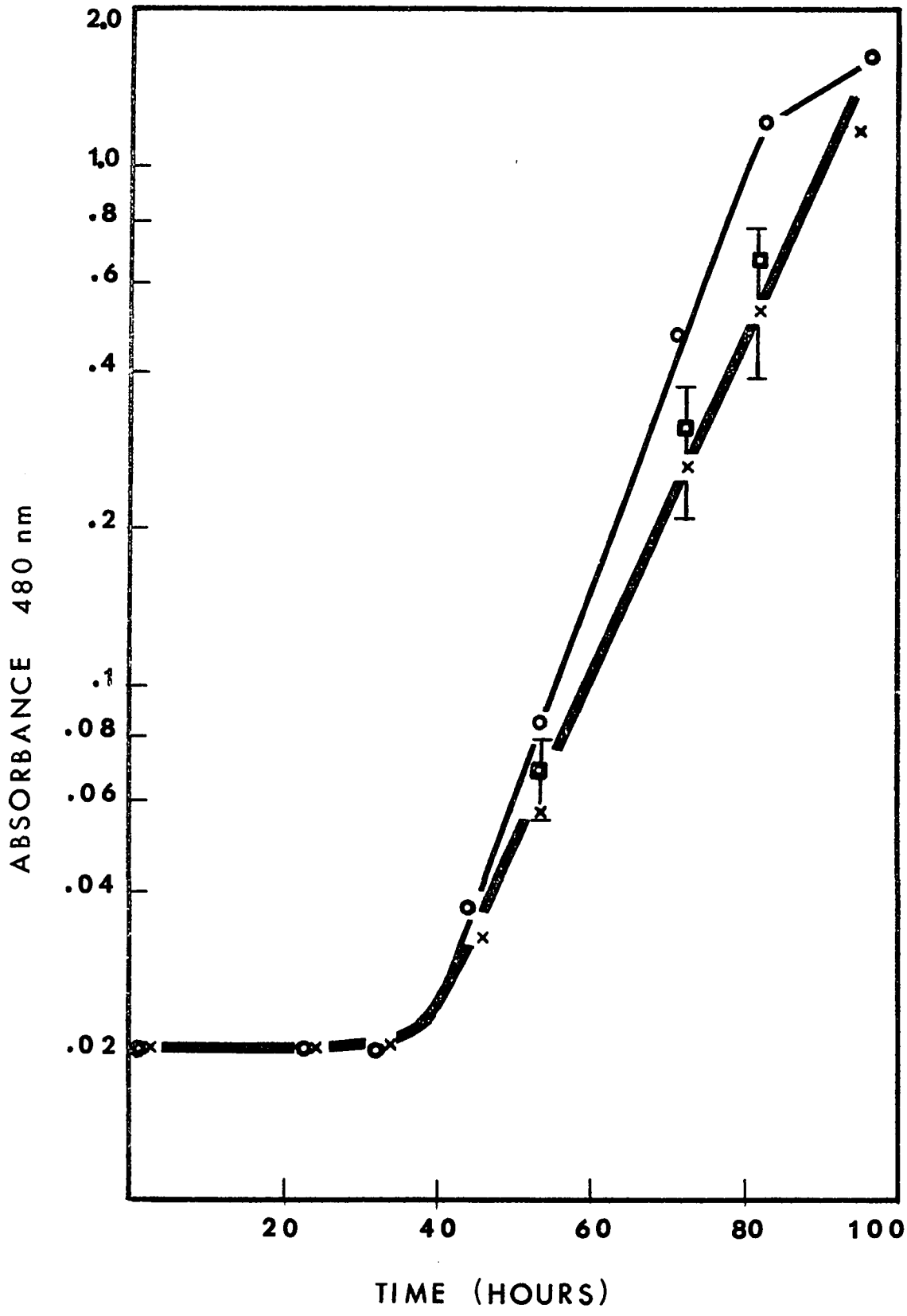

2.0
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
ABSORBANCE 480 nm
20
40
60
80
100
TIME (HOURS)

Fig. 6. Comparison of growth of A. atrocyaneus in MS pyruvate medium without (□) and with (0) 0.1 μg/l added biotin.

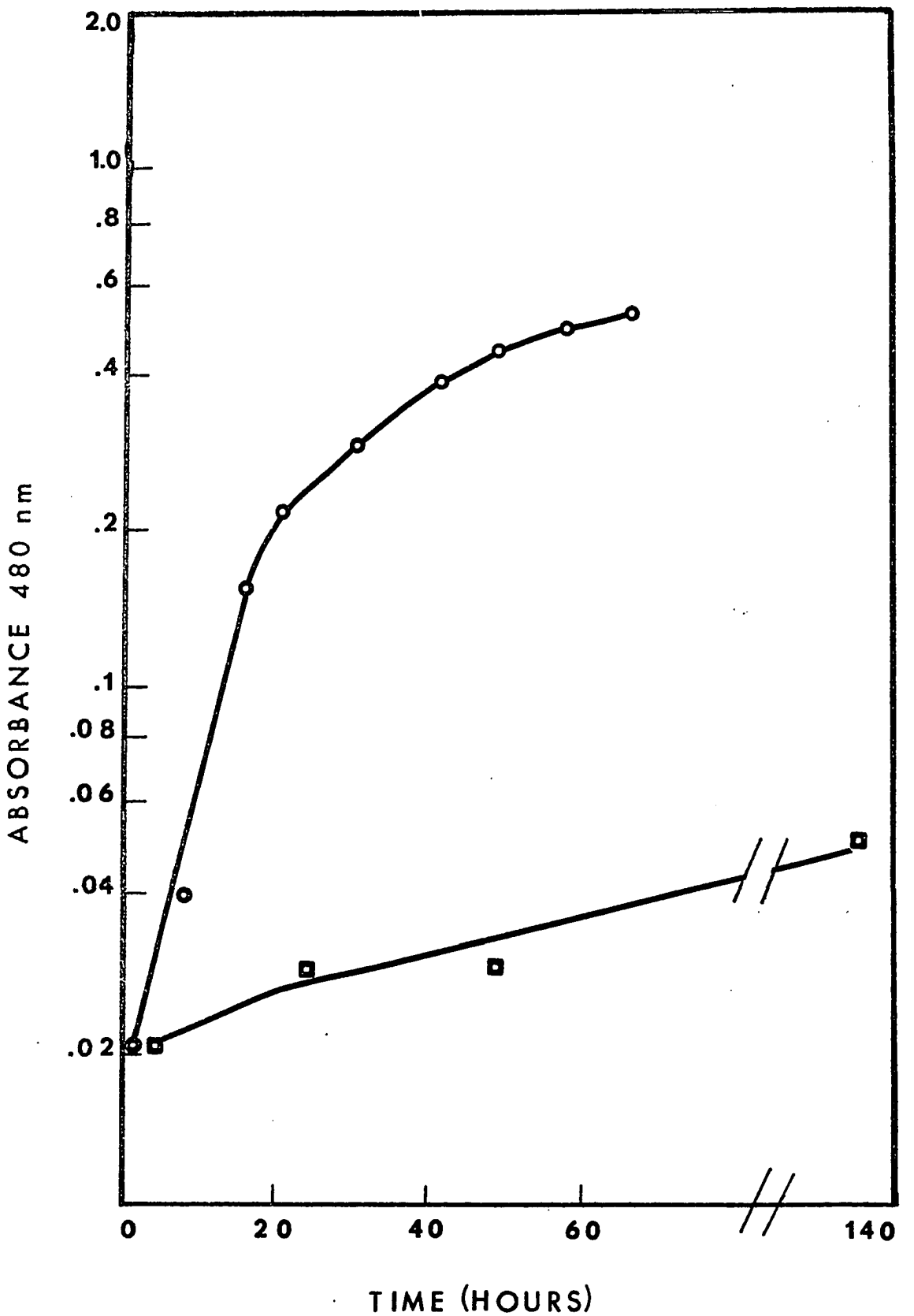

2.0
1.0
.8
.6
.4
.2
.1
.08
.06
.04
.02
ABSORBANCE 480 nm
0
20
40
60
140
TIME (HOURS)

Fig. 7. Specific activities of NADP specific isocitrate dehydrogenase during coccoidal (□) and morphogenic (0) growth. Morphological stages of A. atrocyaneus grown in diauxic growth medium are depicted at the bottom of the graph. Sampling times for coccoidal growth were, TGY cocci, log phase in MS lysine and stationary phase in MS lysine.

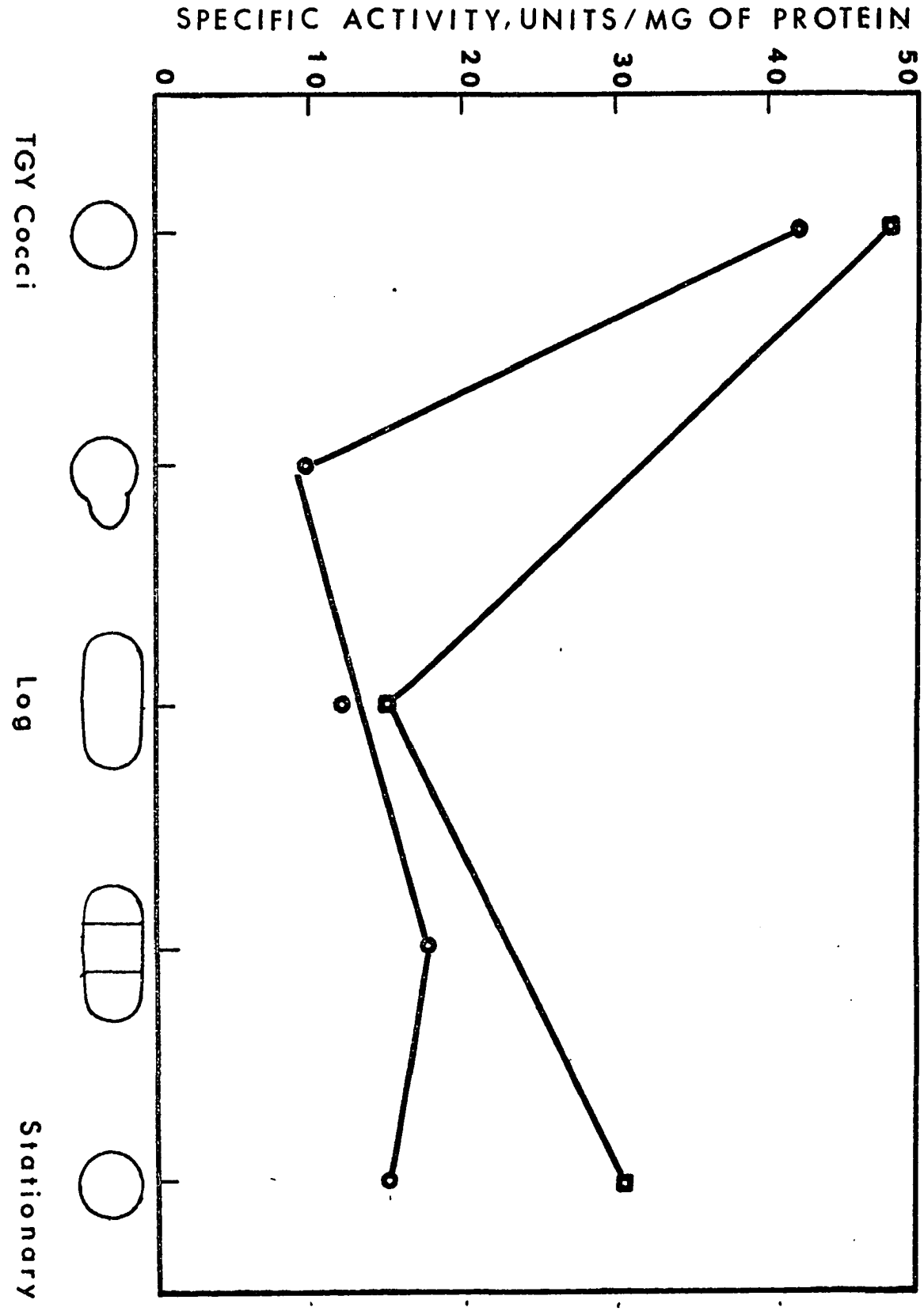

SPECIFIC ACTIVITY, UNITS / MG OF PROTEIN
0
10
20
30
40
50
TGY Cocci
Log
Stationary

Fig. 8. Specific activities of glucose-6-phosphate dehydrogenase (0) and malic enzyme (□) during growth of A. atrocyaneus in diauxic growth medium. 2 h induction, 3 h long rods, 6 h fragmentation, 14 h reversion.

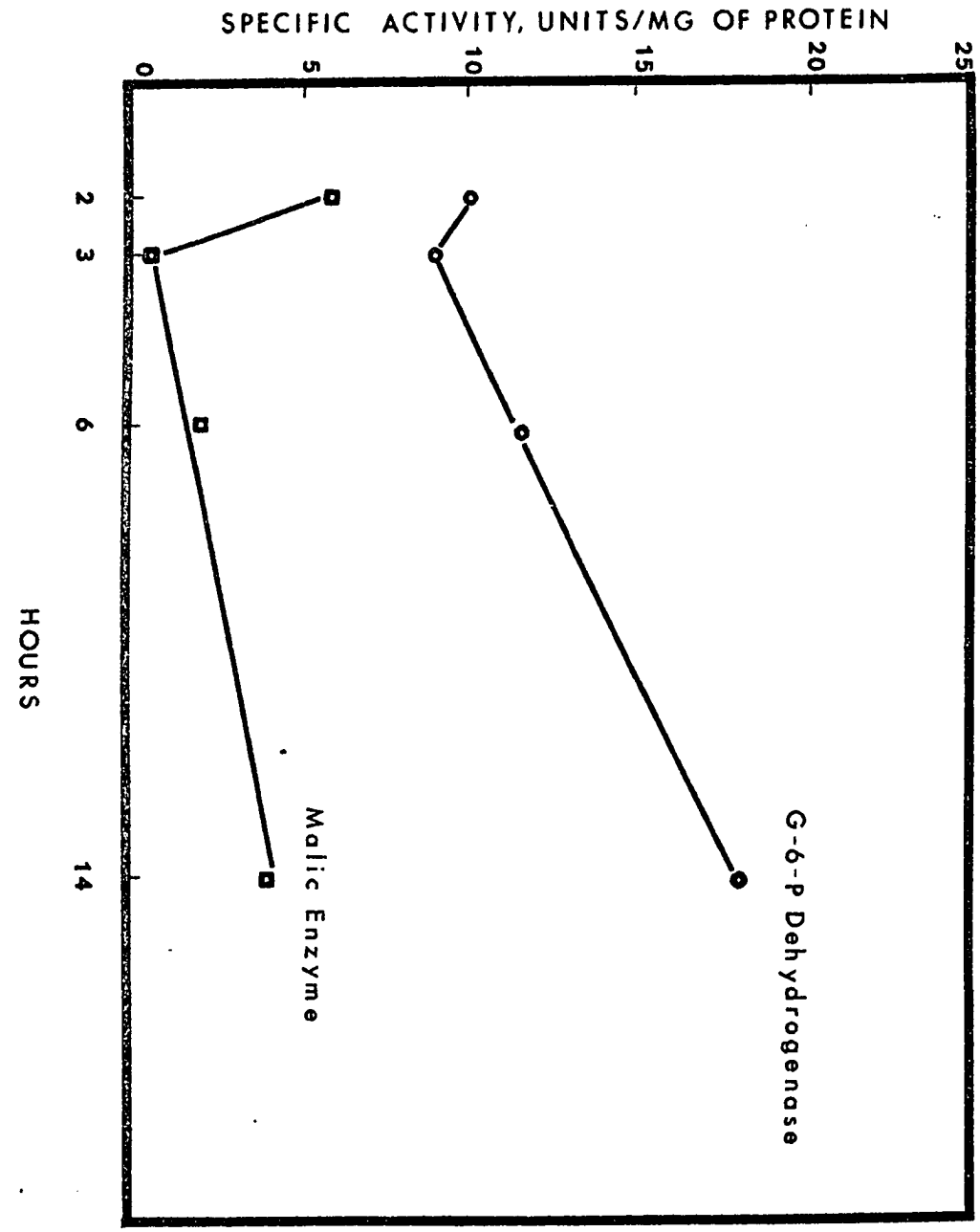
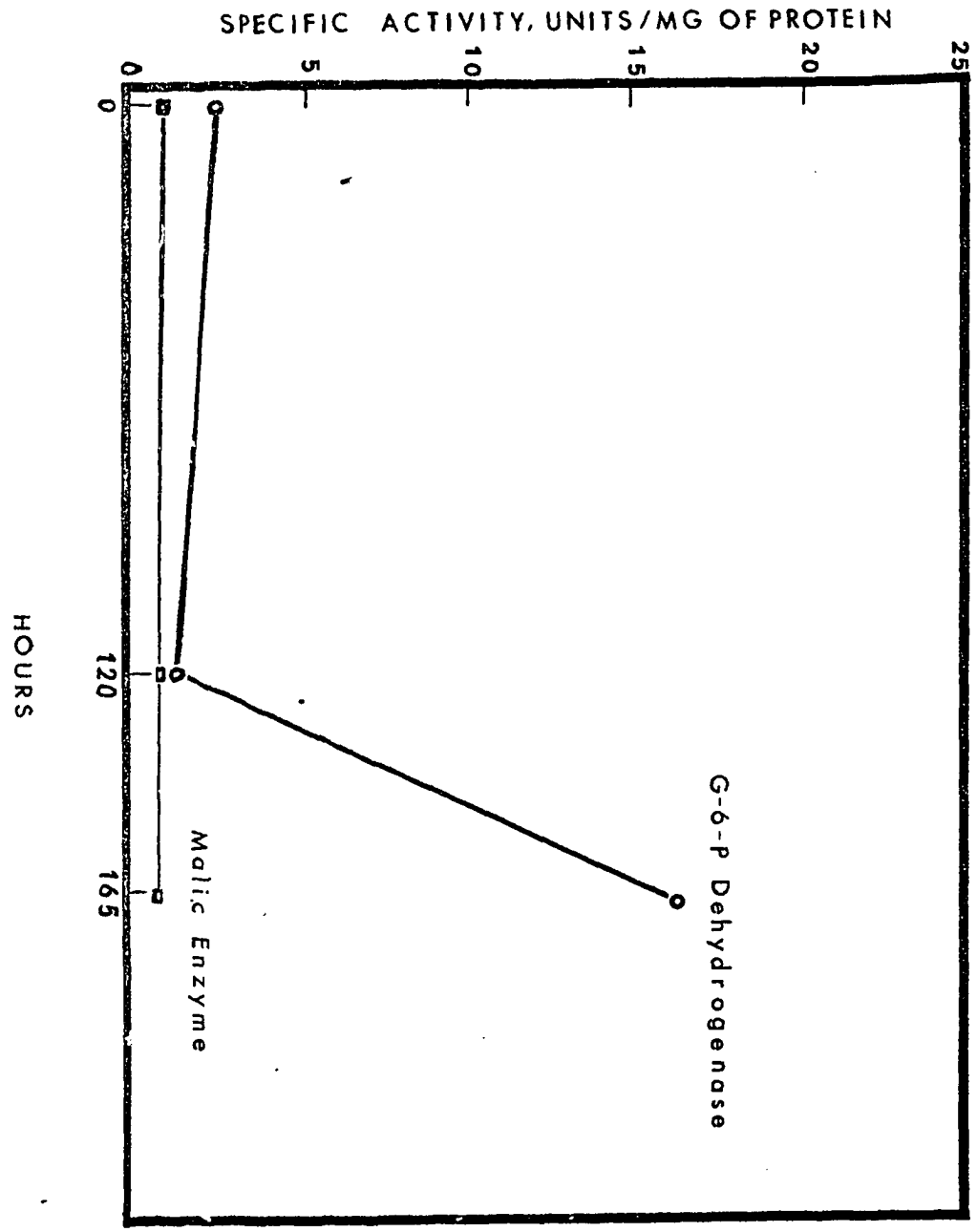

SPECIFIC ACTIVITY, UNITS/MG OF PROTEIN
0
5
10
15
20
25
HOURS
2
3
6
14
Malic Enzyme
G-6-P Dehydrogenase

Fig. 9. Specific activities of glucose-6-phosphate dehydrogenase (0) and malic enzyme (□) during coccoidal growth of A. atrocyaneus in MS lysine medium. 0 h TGY cocci, 120 h exponential growth phase, 165 h stationary growth phase.


SPECIFIC ACTIVITY, UNITS/MG OF PROTEIN
0
5
10
15
20
25
HOURS
0
120
165
Malic Enzyme
G-6-P Dehydrogenase