# STUDIES ON THE GENE EXPRESSION, DEVELOPMENT AND PHYSIOLOGY OF HETEROCYSTS OF *ANABAENA VARIABILIS*

By

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# STUDIES ON THE GENE EXPRESSION, DEVELOPMENT AND PHYSIOLOGY OF HETEROCYSTS OF ANABAENA VARIABILIS

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# PREFACE

This study was intended as a varied approach to many aspects of the physiology, biochemistry and molecular biology of a developmental model system, the heterocyst of the filamentous cyanobacterium Anabaena variabilis. The regulation of heterocyst development and nitrogen fixation in response to various nitrogen sources has been investigated and the changes in gene expression that occur during the vegetative cell to heterocyst transition have been estimated. In addition, several parameters of the nondividing heterocyst following isolation from the filament, in particular RNA synthesis, have been studied. These varied approaches have provided a diverse expansion to existing knowledge of cyanobacterial nitrogen fixation and heterocyst development.

This dissertation is divided into three chapters which are separate and complete manuscripts prepared for submission to the Journal of Bacteriology. A review of the literature is provided by the extensive introductions which are part of each individual chapter.

I am indebted to the Samual Roberts Noble Foundation for the Predoctoral Fellowship awarded to me for the final year of my study. In addition to providing a stipend, the fellowship also provided invaluable supply money. A portion of this work was also supported by the National Science Foundation Grant

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My advisor, Dr. James D. Ownby, has worked closely with me for the entire project and I am indebted to his invaluable scientific discussions, criticisms and advice, as well as the moral encouragement that he continually provided. Dr. John A. Bantle has donated a generous portion of his time, energy and equipment to the completion of this project and deserves a special commendation. My committee members, Dr. David A. Francko, Department of Botany and Microbiology, Dr. Ulrich Melcher, Department of Biochemistry, and Dr. John A. Bantle, Department of Zoology, have all provided useful criticism and discussion when necessary. Dr. George V. Odell, Department of Biochemistry, generously provided the gas chromatograph used in a portion of the study. Dr. Margaret S. Ewing, Department of Zoology, and Dr. David Meinke, Department of Botany and Microbiology, have provided helpful discussion and comments throughout the study in addition to editorial help and friendship. Ms. Brenda L. Knox is acknowledged for help with graphics design and the long and arduous task of dissertation preparation.

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TABLE OF CONTENTS

# LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

# GROWTH, HETEROCYST PRODUCTION AND NITROGENASE ACTIVITY IN RESPONSE TO VARIOUS NITROGEN SOURCES IN *ANABAENA VARIABILIS*

by

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# ABSTRACT

Heterocyst differentiation and nitrogenase activity are induced in some filamentous cyanobacteria in response to media lacking combined nitrogen. In our studies with Anabaena variabilis, both nitrate and ammonia act to suppress heterocyst differentiation in nitrogen-fixing cultures; however, the effect of these two sources of combined nitrogen on the specific activity of nitrogenase is different. Ammonia rapidly inhibited activity (within 10 h in a culture whose doubling time is 25 h) while nitrogenase activity in the presence of exogenous nitrate is unaffected over the same time interval. The enhanced growth rate observed following addition of nitrate with respect to either exogenously applied ammonia or continued growth in N-free media indicates that this organism has the capacity to fix $N_2$ and utilize exogenous nitrate simultaneously.

# INTRODUCTION

The cyanobacterium Anabaena variabilis preferentially uses combined nitrogen during growth, but in nitrogen-free media both nitrogenase and specialized cells called heterocysts are induced which allow aerobic growth on elemental nitrogen. In spite of the initial controversy over heterocyst function (reviewed by 7,19,25), these cells are now known to provide an anaerobic chamber for nitrogenase and to transport reduced nitrogen, probably in the form of glutamine, to vegetative cells in exchange for carbon skeletons (7). The differentiation of a vegetative cell to a heterocyst involves a wide array of morphological and physiological changes (1).

The inverse relationship between nitrogen content of the growth media and heterocyst frequency has been well established (6), yet controversy still exists over whether heterocyst development and nitrogenase induction are coordinantly regulated. The regulatory mechanism has been especially difficult to determine since heterocyst frequency and nitrogenase activity vary depending upon species, physiological state of the cell, and even light and temperature (1). Mutagenesis studies of A. variabilis, using growth on reduced nitrogen as a selection factor, suggested that heterocysts were required for nitrogenase activity; all nif- mutants (incapable of reducing $N_2$) possessed

phenotypically altered heterocysts, and revertants to the nif+ phenotype invariably returned as well to the normal heterocyst phenotype (het+)(5). It has been postulated that transcription of the nif structural genes is one of the programmed steps in heterocyst differentiation (12) but the fact that the nif genes can be induced in an anaerobic environment without simultaneous heterocyst differentiation (20) makes this seem unlikely.

The effect of ammonia on cyanobacterial nitrogenase and heterocyst differentiation has been studied in detail. Koolasooriya and Fay (9) demonstrated that 1 mM $NH_4Cl$, added to A. cylindrica shortly after the appearance of mature heterocysts, did not change the heterocyst frequency; i.e. mature proheterocysts did not regress to vegetative cells at this point in development. The inability of mature proheterocysts to regress to vegetative cells has been demonstrated by other investigators (4,12) and loss of heterocyst frequency following addition of ammonia is generally thought to be due primarily to dilution during continued growth and suppression of subsequent differentiation. In contrast, ammonia has a marked inhibitory effect on nitrogenase activity, although the effect is not a direct inhibition of the enzyme (13,19). In one report, the $NH_4$+-induced loss of $C_2H_2$-reduction activity within 48 h in A. cylindrica was attributed to nitrogenase turnover (19), while in another report 50% of the nitrogenase activity was lost within 3 h in the same species (14). In the latter case, the authors suggested that $NH_4^+$ not only supressed nitrogenase

biosynthesis, but in addition, lowered the supply of energy and/or reductant available for the nitrogenase complex resulting in depressed levels of enzyme activity. More recently, activity-independent measurement of nitrogenase using an immunoelectrophoretic technique indicated that both protein biosynthesis and heterocyst frequency become insensitive to repression by $NH_4^+$ as heterocysts mature (13). Therefore, $NH_4^+$ or its metabolites must regulate nitrogen fixation by repressing heterocyst differentiation and inhibiting nitrogenase acivity through competition for reductant and/or ATP, but nitrogenase biosynthesis in heterocysts does not seem to be directly regulated by this compound. In marked contrast, in free-living bacteria such as Klebsiella, nitrogenase synthesis is regulated at the transcriptional level in the presence of ammonia via the adenylation and deadenylation of glutamine synthetase (21).

The effects of other sources of nitrogen, in particular nitrate, on nitrogenase activity and heterocyst development are variable. Stewart et al. (19) showed a decrease in $C_2H_2$-reduction activity in steady-state cultures of A. cylindrica of only 20% following addition of 150 mg/l nitrate. The decline occurred rapidly and the slightly depressed rate of $C_2H_2$-reduction remained constant for more than two days. Calothrix sp. when grown either on nitrate or ammonia displayed unusual morphological alterations (15). Growth on nitrate resulted in tapered filaments that lacked heterocysts, while $NH_4^+$-grown cells had uniformly shaped filaments. Many of the variable effects of $NO_3^-$ may be due to differing rates

of uptake by different species and under different growth conditions (11). Nitrate uptake by Nostoc muscorum has been shown to be ATP-dependent, enhanced by prior growth on elemental nitrogen, and inhibited by $NH_4^+$ (16); however, if ATP is added to the medium, nitrate and ammonia are taken up at the same rate.

The regulation of nitrogenase biosynthesis and activity is fairly well understood in the free-living bacteria (21) but in Anabaena, where the induction of this enzyme under aerobic conditions strictly requires concurrent heterocyst differentiation, the mechanism of regulation appears to be quite different. The expression of the nif and het genes must be coordinately regulated by some means, at least in certain cases, but the factors involved are poorly understood. The nif genes in Anabaena have been extensively studied (8) and their regulation is beginning to be understood (20); however, the much more complex, yet equally important, process of heterocyst differentiation, its regulation, and interaction with nitrogenase biosynthesis remains a mystery in many respects. We have used two sources of combined nitrogen (nitrate and ammonia) to show that heterocyst frequency and nitrogenase activity are affected in a different manner and to a different extent under various conditions.

# MATERIALS AND METHODS

Anabaena variabilis (ATCC 29413) was grown on Allen and Arnon's media (AA media) (2) supplemented with 0.5 mM $NaNO_3$/1.0 mM $NH_4Cl$ in 0.5 - 1.0 l flasks on a reciprocating shaker illuminated from above with cool white fluorescent tubes (80-100 uEinsteins/$m^2$/sec). When cultures in log phase growth reached a density of approx. 1 ug chl a/ml, the cells were collected in large plastic centrifuge bottles (2000g, 5 min) and transferred to AA media lacking a combined nitrogen source or, in one experiment, containing 1 mM $NaNO_3$. Heterocysts were allowed to develop for 2 days at which time either 0.5 mM $NaNO_3$ or 1.0 mM $NH_4Cl$ was added to the flasks. Growth was monitored by chlorophyll determinations according to Wetzel and Likens (22) and by following the absorbance at 650 nm. A total of 600-1000 cells were counted to determine heterocyst frequency. Proheterocysts were identified by their larger size, refractive envelopes, and the agranular cytoplasm. Heterocyst maturity was verified by the appearance of polar nodules.

Nitrogenase activity was measured by an acetylene reduction technique (24). Two-ml aliquots of culture were placed into 30-ml bottles filled with 23:23:4 argon:hydrogen:acetylene and serum stoppered. Individual samples were incubated for 1 and 2 h before termination by

injection of 0.4 ml of 30% trichloroacetic acid. Ethylene production was measured using a Perkin-Elmer 990 gas chromatograph equipped with a Porapak N column (Alltech) and a flame ionization detector.

# RESULTS

Combined nitrogen effects on log phase cultures. Undifferentiated cultures of A. variabilis ($A_{650}$=0.10 to 0.15) were transferred to nitrogen-free medium for 2 days. Heterocyst frequency and nitrogenase activity are maximal 36-48 h following transfer to inducing medium (unpublished observation). In addition, although completely synchronous populations of heterocysts cannot be achieved in this organism, the heterocyst:proheterocyst ratio is maximal during this period (Fig. 1b). Spiking these cultures with a source of combined nitrogen (both nitrate-nitrogen and ammonia-nitrogen) during this period resulted in a rapid loss of nitrogenase activity and proheterocyst frequency (within 10 h) and a decrease in heterocyst frequency to 25% of control within 48 h (Fig. 1, b and c). If it is assumed that heterocysts contain 1.6-times the chlorophyll a of vegetative cells (18), this apparent loss of heterocysts can be completely accounted for by dilution due to continued growth. Although log phase growth was maintained by both the $N_2$-grown and $NO_3^-$/$NH4^+$-grown cultures, the growth rate was slightly enhanced in the presence of combined nitrogen (Fig. 1a).

Effect of nitrate on undifferentiated cells. Cells were allowed to grow to 0.45 ug chl a/ml in AA medium+ 0.5 mM $NaNO_3$

and 1.0 mM $NH_4Cl$, at which time the cells were collected and transferred to media containing only nitrate (0.5 mM) as a source of nitrogen. Control cultures were simultaneously transferred to media lacking combined nitrogen. During the first 18 h in nitrogen-free media, growth virtually ceased, as has been reported previously (1,12), but cells resume log-phase growth concurrently with the induction of nitrogenase activity (Fig. 2, open symbols). Cells transferred to $NO_3^-$-containing media experienced no significant depression of growth, although nitrogenase activity was induced to a low level during the first 18 to 42 hours (17% and 19%, respectively, of N-free cultures)(Fig. 2,closed symbols). During the following 24 hours, nitrogenase activity increased 6-fold in the nitrate-grown cultures, probably due to depletion of nitrate. Growth rate measurements, based on total chlorophyll a content, indicate that the nitrogen-starved cultures have larger doubling times following maximum nitrogenase activity (from 18 to 70 h following removal to N-free media) than the $NO_3^-$-grown cells (32.7 vs. 26.3), which are apparently using both nitrate and elemental $N_2$.

Ability of $NO_3$- and $NH_4$+ to reverse nitrogen starvation effects after a 48 hour induction. In this set of experiments, log-phase undifferentiated cultures were transferred to nitrogen-free media for 48 hours. At this time, cells were collected by centrifugation and placed into either AA-N, AA + 0.5 mM $NaNO_3$, or AA + 1.0 mM $NH_4Cl$.

Nitrogenase acitvity, growth and heterocyst frequency were monitored for the following 24 h. As shown in Table 1, two separate experiments indicated that although both $NO_3^-$- and $NH_4^+$-treated cultures experienced a similar decline in heterocyst frequency (a decrease in heterocyst frequency to 4.4% $\pm$ 1.1 and 4.2% $\pm$ 0.5, respectively), the growth rate of $NO_3$-spiked cultures markedly exceeded that of the $NH_4^+$-spiked cultures in both experiments (20.3 h $\pm$ 4.4 and 26.2 h $\pm$ 0.1, respectively). The growth rate of cells supplied with exogenous ammonia slowed markedly 10 to 26 h following addition of ammonia. Although nitrogenase acitvity was rapidly inhibited during the first 2-5 hours following addition of ammonia, nitrate had virtually no effect on nitrogenase activity for up to 26 h following addition (Fig. 3). We suspect that the slowed growth rate observed in ammonia-treated cultures may have been primarily due to the presence of the non-dividing and now supposedly non-functional (due to inactivated nitrogenase) heterocysts which comprise 5% (expt. 1) and 4% (expt. 2) of the population. In contrast, the nitrate-treated heterocysts (5% of the population in both experiments) continue to function in supplying vegetative cells with at least a small fraction of their nitrogen demand; therefore, their presence would not be expected to affect the growth rate of the culture to the same extent.

# DISCUSSION

In nitrogen-starved cultures of Anabaena, nitrogenase is continually turned over and resynthesized (12,17), while fully mature heterocysts cannot morphologically revert to vegetative cells if another nitrogen source is supplied (4,9). In A. variabilis under the conditions of this study, a combination of ammonia-nitrogen and nitrate-nitrogen resulted in a response in which the loss of nitrogenase activity preceeded the disappearance of heterocysts, the frequency of which decreased in an inverse manner with continued growth. When differentiated cultures were treated with either nitrate-nitrogen or ammonia-nitrogen, we observed that nitrate did not have the immediate inhibitory effect on preformed nitrogenase that ammonia did (Fig. 3). In fact, growth in nitrate induced nitrogenase in undifferentiated filaments (Fig. 2), but to only a limited extent. We suspect that these cultures are using both nitrate-nitrogen and elemental nitrogen simultaneously since they seemed to attain higher rates of growth than cells that have been subjected to nitrogen-free media for a period of time sufficient to induce maximum nitrogenase activity. Although there is speculation that cyanobacterial species have highly varied ability to take up nitrate and ammonia (11), nitrate appears to be assimilated efficiently by this organism since enhanced growth rates are

achieved by: 1)growth on nitrate alone as compared to elemental nitrogen; 2) addition of nitrate to $N_2$-fixing cultures as compared to addition of ammonia. Ammonia has been proposed to regulate nitrogenase activity and biosynthesis indirectly through C:N ratio of the cells (3,10) or the pools of stored nitrogen (26); our results suggest that nitrate exerts its regulatory effects on nitrogenase differently from that of ammonia. Since levels of $NH_4^+$ in surface waters of both oligotrophic and eutrophic lakes are low (23), surface-living cyanobacterial species may be selected for simultaneous utilization of both organic nitrogen as $NO_3^-$ and elemental nitrogen.

The specific activity of nitrogenase (on the basis of ug heterocyst chl a) decreases drastically upon addition of $NH_4^+$, but not upon addition of nitrate (Fig. 3). Although it is well established that ammonia does not directly inhibit nitrogenase (13,26), the activity of this enzyme is rapidly curtailed in the presence of $NH_4^+$ which may be due to rapid inhibition of further synthesis coupled with preferential shunting of reductant and energy demand to ammonia assimilation rather than nitrogen-fixation processes, as has been speculated previously (13). If this is true, however, nitrate utilization would be expected to put similar demands on the organism and curtail nitrogenase activity, which is not the case. We suspect that the regulatory mechanism differs for nitrate and ammonia which results in the different morphological and biochemical responses seen in both cases.

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Table I. Effect of 0.5 mM nirate or 1.0 mM ammonia on heterocyst frequency and growth rate in differentiated cultures of A. variabilis added 48 h following heterocyst induction.

| | $+NO_3-$ | | | | $+NH_4+$ | | | |
|---|---|---|---|---|---|---|---|---|
| | Expt. 1 | | Expt. 2 | | Expt. 1 | | Expt. 2 | |
| h following addition of nitrogen source | $t_D$(h)[a] | Δ %het[b] | $t_D$(h)[a] | Δ %het[b] | $t_D$(h)[a] | Δ %het[b] | $t_D$(h)[a] | Δ %het[b] |
| early (+5 to +10 h) | 21.7 | -0.4 | 15.1 | -2.4 | 21.2 | -1.3 | 20.0 | -1.3 |
| late (+10 to +26 h) | 24.4 | -3.2 | 18.7 | -2.8 | 28.2 | -3.3 | 32.2 | -3.9 |
| overall (+5 to +26 h) | 23.4 | -3.6 | 17.2 | -5.2 | 26.2 | -4.6 | 26.3 | -3.9 |

[a]Generation times ($t_D$) are calculated on the basis of chlorophyll increase according to the equation $t_D=t_2-t_1/3.3 \log(c_2/c_1)$; c= ug chlorophyll/ml.
b Δ %het is the loss in heterocyst frequency over the time interval specified.

Fig. 1. Effect of addition of combined nitrogen on growth rate (a), heterocyst frequency (b), and nitrogenase activity (c) of nitrogen-fixing cultures of A. variabilis. Cultures were transferred to nitrogen-free media at -47 h. At t=0, control flasks (solid line) continued growth in -N media; sample flasks (dashed line) were given both $NaNO_3$ (0.5 mM) and $NH_4Cl$ (1.0 mM).

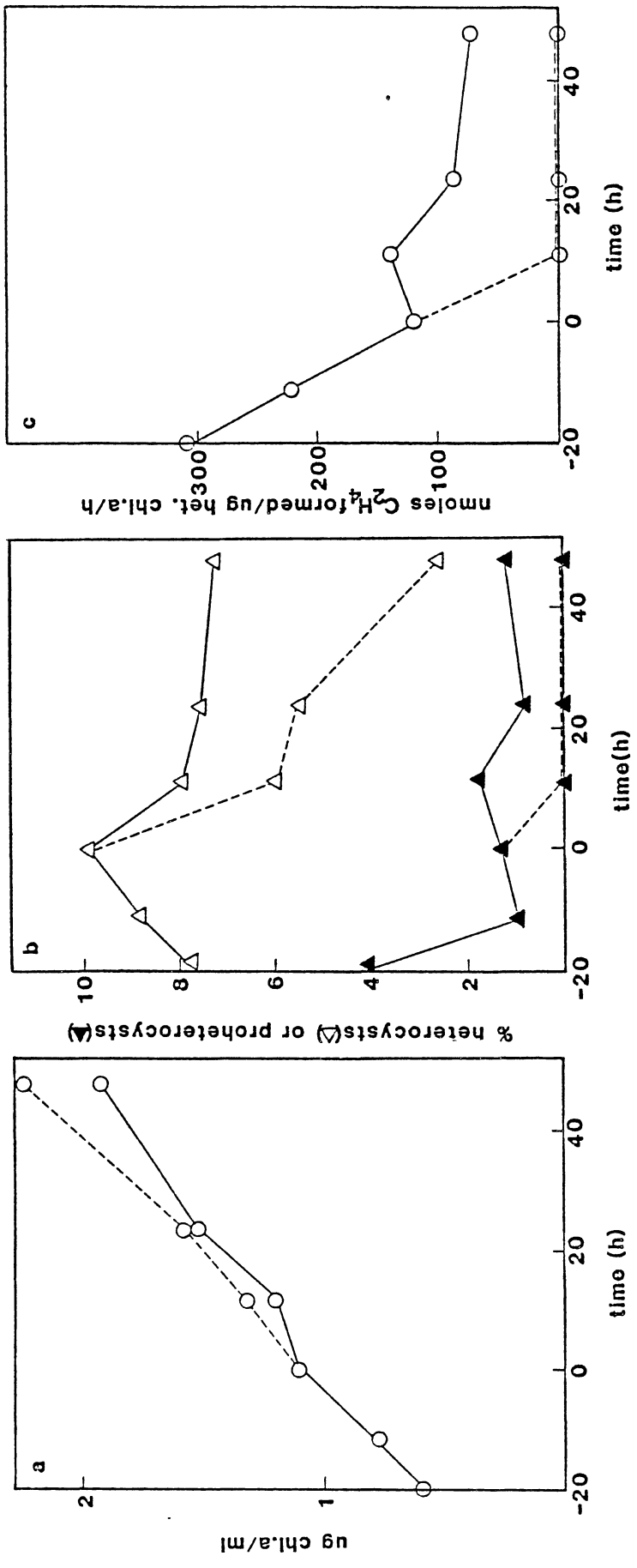

a
ug chl.a/ml
time (h)
b
% heterocysts(△) or proheterocysts(▲)
time(h)
c
nmoles C2H4formed/ug het. chl.a/h
time (h)

Fig. 2. Growth and nitrogenase activity of nitrogen-starved and nitrate-grown cultures of A. variabilis. At t=0, undifferentiated cultures were transferred to AA-N (open symbols) or AA + 0.5 mM $NaNO_3$ (closed symbols). The slope of the line representing growth in nitrate is 0.034 ($r^2$=0.979); the slope of the line representing growth in $N_2$ is 0.029 ($r^2$=0.990)(based on the 18-69 h interval only).

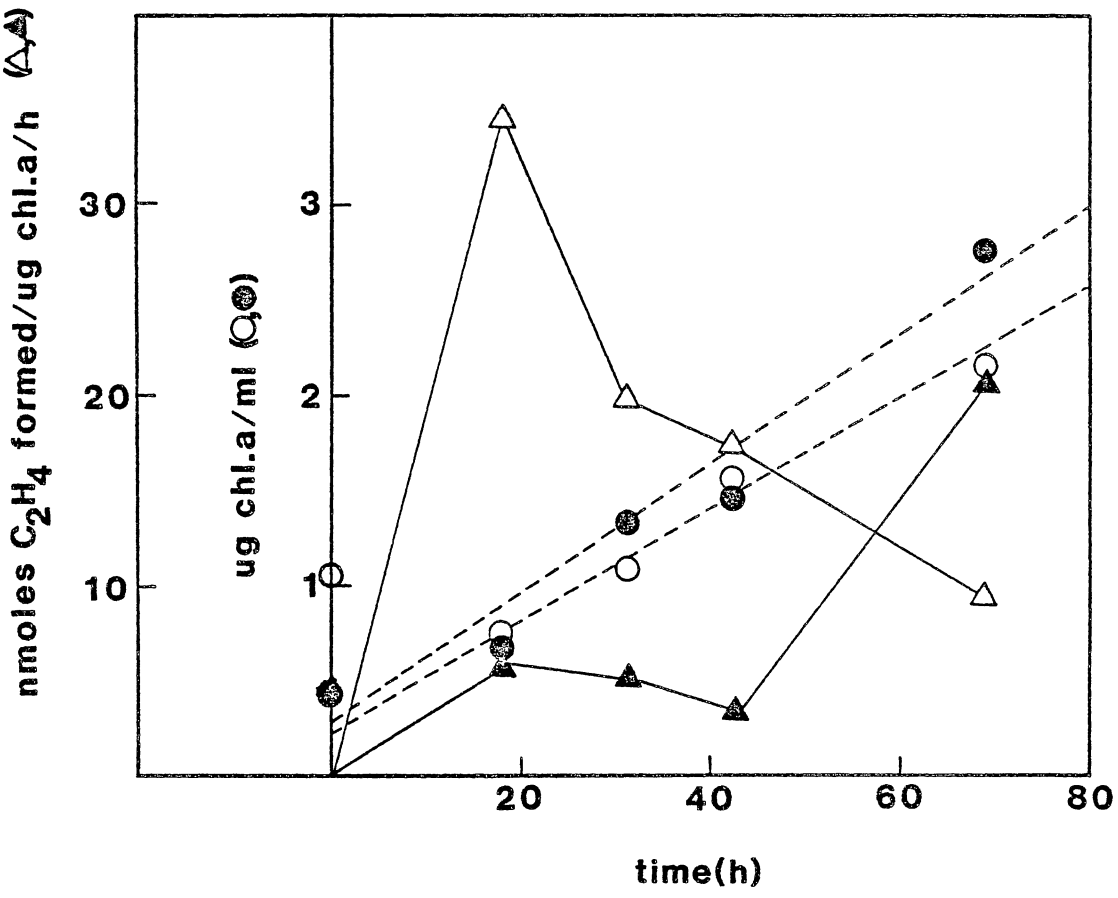
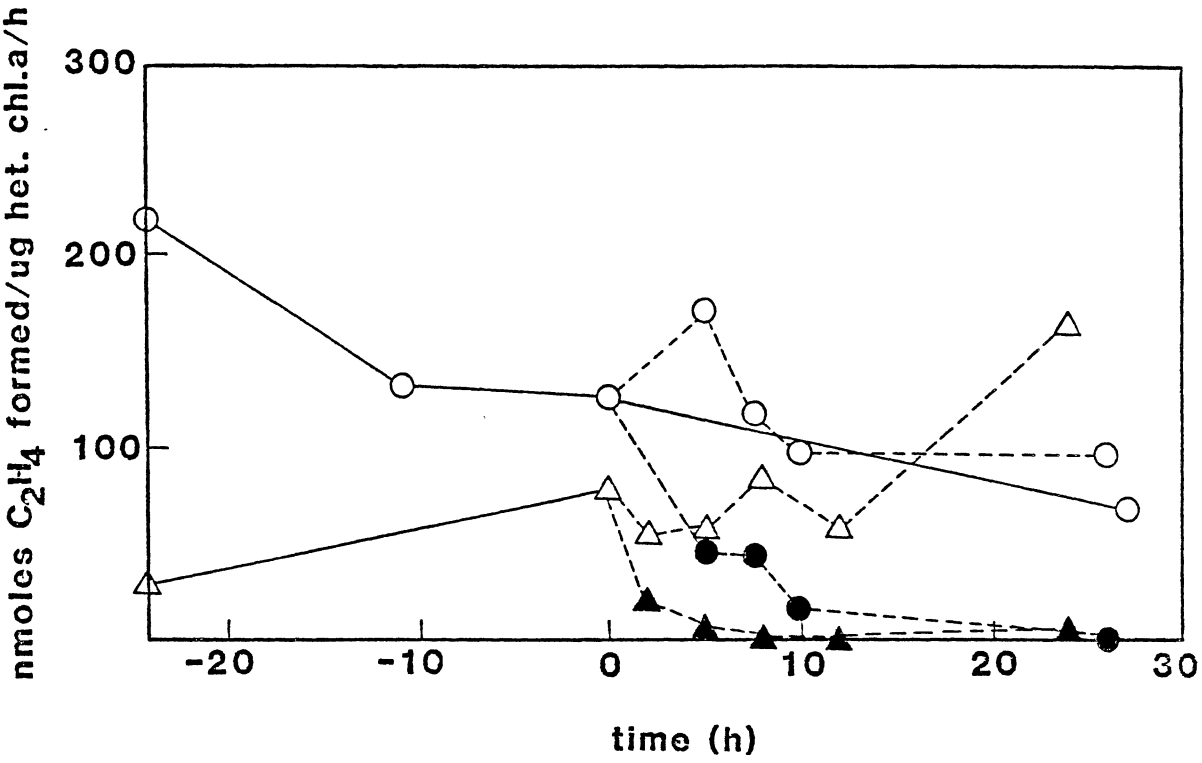

nmoles $C_2H_4$ formed/ug chl.a/h (△-▲)
ug chl.a/ml (○-●)
30
20
10
3
2
1
20
40
60
80
time(h)

Fig. 3. Effect of addition of 0.5 mM $NaNO_3$ or 1.0 mM $NH_4Cl$ on $C_2H_2$-reduction activity of A. variabilis. At t=-48, undifferentiated, log-phase cultures were transferred to N-free media; at t=0, 0.5 mM $NaNO_3$ (open symbols) or 1.0 mM $NH_4Cl$ (closed symbols) was added to flasks (dashed lines) or growth in N-free media was allowed to continue (solid line). Data presented are from two different experiments (expt.1, circles; expt.2, triangles).


nmoles $C_2H_4$ formed/ug het. chl.a/h
300
200
100
-20
-10
0
10
20
30
time (h)

# CHAPTER II

# ESTIMATION OF GENE EXPRESSION IN *ANABAENA VARIABILIS* USING DNA:RNA HYBRIDIZATION

by

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# ABSTRACT

The filamentous cyanobacterium Anabaena variabilis responds to nitrogen starvation by differentiating specialized cells called heterocysts that occur in a regular pattern along the filament and are the sites of nitrogen fixation. We have used two different types of DNA:excess RNA hybridization techniques to estimate the number of genes required for heterocyst differentiation. In the first, RNA and DNA were incubated in a phosphate buffer at 60°C and the hybrids were separated from the unhybridized material by hydroxylapatite chromatography; in the second, the nucleic acids were incubated at 50°C in a buffer containing 50% formamide and the fraction of DNA in duplexes was assayed by $S_1$ nuclease digestion. Both techniques revealed that approximately 65% of the A. var. genome was expressed in vegetative cells and 45% of the genome was expressed in heterocysts. A null DNA probe (DNA not transcribed in vegetative cells) was used to determine the fraction of the genome expressed only in heterocysts. Heterocyst-specific transcripts were encoded by 25% of the genomic coding capacity representing a possible 1500 genes (assuming an average mRNA length of 1500 nucleotides). The remaining ca. 1200 transcripts expressed by the heterocyst were constitutively produced in both vegetative cells and heterocysts. Furthermore, the heterocyst-specific

transcripts were present in abundant copies in the cell while the transcripts that are expressed by both cell types were present at much lower frequency.

# INTRODUCTION

The cyanobacterium Anabaena variabilis is a filamentous, autotrophic prokaryote capable of reducing atomspheric nitrogen wnen grown in the absence of combined nitrogen ($NO_3$-,$NH_4$+). The onset of nitrogen fixing capacity is accompanied by the differentiation of specialized cells called heterocysts which fix atmospheric $N_2$ and transport it to neighboring vegetative cells in exchange for carbohydrates (1,4,19,38 for reviews). Heterocysts are structurally and physiologically modified from their parent vegetative cells, are formed in a regular pattern along the filament, and export amino acids to vegetative cells in exchange for carbon skeletons, all characteristics which allow us to consider this a truly multicellular organism unique to the prokaryotic kingdom. In addition, several features of this organism make it particularly amenable to laboratory studies: 1) the genome is small and lacks repetitive DNA; 2) morphological development of the heterocyst can be monitored microscopically; 3) the mature heterocyst develops only 48 hours following nitrogen starvation; 4) heterocyst $N_2$-fixation can be assayed by an $C_2H_2$-reduction technique; 5) the fully differentiated state consists of only two cell types which can be readily separated.

Heterocyst physiology and morphology has been

extensively studied in the last decade due in part to a gentle method for the isolation of these cells (26) that allowed the recovery of physiologically active heterocysts. Electrophoresis of proteins extracted from developing and mature heterocysts and vegetative cells indicate that the protein systhesis of the two cell types is qualitatively different and that this difference is established very early after heterocyst induction, implicating transcriptional and/or translational regulation of heterocyst differentiation (9,10). In addition, certain vegetative cell proteins are rapidly degraded in those cells destined to become heterocysts.

Although mutants of several Anabaena strains have been isolated (7,36) no means of classic prokaryotic genetic analysis exists for this organism. As an alternative to this approach, Haselkorn and coworkers have used Klebsiella pneumoniae nif genes as heterologous probes to isolate DNA restriction fragments containing nitrogen fixation (nif) genes from Anabaena 7120 (24,28). Their most recent work indicates that extensive genetic rearrangement of the nif genes occurs in the heterocysts during differentiation (14). As a complement to studies that concentrate on single genes or groups of genes, our approach to the molecular biology of heterocyst differentiation has emphasized shifts in global gene expression during development.

In bacteria such as Klebsiella, the number of genes required for nitrogen fixation is relatively small (20). In Anabaena, however, aerobic nitrogen fixation requires concommitant heterocyst differentiation and, presumably,

induction of many more genes. In the work described here, we have attempted to measure mRNA complexity of vegetative cells and heterocysts in A. variabilis using DNA:excess RNA hybridization techniques. This approach extends protein synthesis studies (9,10) which only detect the expression of high copy number sequences and has allowed us to estimate the shift in total gene expression that occurs during the transition from vegetative cell to heterocyst. Our findings indicate that genes involved in formation and maintenance of heterocysts represent a significant portion of the total Anabaena genome.

## Materials and Methods

Culture conditions. A. variabilis (ATCC 29413) was grown aerobically in a New Brunswick MF-114 fermentor in 6 liters of Allen and Arnon's media (2) supplemented with 3 mM $NH_4Cl$ and 1mM $NaNO_3$ to an $A_{650}$ of 0.35-0.45. To induce heterocyst formation, the filaments were allowed to settle for several hours, the medium was drained off, and the cells were diluted to an $A_{650}$ of 0.15-0.20 with Allen and Arnon's medium lacking nitrogen . Forty-eight hours following dilution, cells were collected by a Sorvall KSB-R continuous flow apparatus.

Isolation of Heterocysts. The heterocyst isolation procedure of Peterson and Wolk (26) was followed with modifications. The fermentor containing 10 liters of culture was purged with argon for 30 minutes prior to harvest; all subsequent buffers were deoxygenated by thorough bubbling with either argon or hydrogen. The vegetative cell walls were selectively ruptured by a 45-minute lysozyme treatment (50 mM Tris, pH 8.1, 1 mM EDTA, 7% sucrose, 2 mg/ml lysozyme) on an illuminated reciprocating shaker under argon. Cells were collected by centrifugation (2000g, 5 min) and washed once in 0.01 M Hepes, 0.01 M Pipes, 5 mM $MgCl_2$, 0.18 M mannitol buffer, pH 7.2 (HP/M + mannitol) and finally suspended in 30 mls of the above

buffer. This mixture was probe sonicated using a Branson cell disruptor #185 (5-10 10-second bursts at 40% duty cycle) in an anaerobic, chilled chamber. Disruption of vegetative cells was determined microscopically. Heterocysts were separated from cell debris by 3 successive centrifugations in a Sorvall HB-4 rotor (350g, 150g, 50g). Less than 4% contamination by vegetative cells was considered acceptable. Heterocysts isolated in this manner routinely retained 40-70% of the nitrogenase activity of the intact filaments as determined by measuring acetylene reduction with a Perkin-Elmer gas chromatograph fitted with an Alltech Associates Poropak N column (37).

DNA and RNA Isolation. RNA from vegetative cells was isolated by gently disrupting the vegetative cells with lysozyme and detergents according to the method of Cattolico (6). Isolated heterocysts were disrupted by vortexing a dense slurry of the cells in HP/M + mannitol buffer and glass beads for 3-5 minutes. Nucleic acids were extracted with phenol:chloroform (27). For DNA purification, the nucleic acids were treated with RNase (15 ug/ml RNase A and 20 units/ml RNase T1, both pretreated by boiling to remove contaminating DNase activity), self-digested proteinase K (1 mg/ml) and subjected to another phenol:chloroform extraction. DNA was spooled from the aqueous phase and precipitated in 2 volumes of 100% ethanol. DNA was radiolabelled using a Bethesda Research Lab nick translation kit and [$^3$H]-dCTP from New England Nuclear to a specific activity of $10^6$-$10^7$ cpm/ug. The nick-translated

probe was sized on alkaline agarose gels prior to use in hybridization experiments (21).

RNA was purified from DNA by a Na-acetate precipitation (27), which selectively precipitates ribosomal and messenger RNA sequences. The resulting pellet was subjected to a DNase and protease treatment in the presence of placental ribonuclease inhibitor (21), and finally phenol:chloroform extracted again. The isolated nucleic acids were routinely electrophoresed on a 2.4% polyacrylamide/0.5% agarose gel to confirm that the material was undegraded and pure (21).

Hybridization Reactions. The hybridization reactions were performed using two slightly different methods. In the first (subsequently referred to as the HAP method), cold RNA was mixed in 10-30,000 fold-mass excess to nick-translated DNA in a phosphate buffer (0.12-0.42 M, pH 6.8, 0.05% SDS, 1 mM EDTA) and aliquots (5-10 ul) of the mixture were placed in silanized glass capillary tubes, the ends of which were flame sealed. All samples were incubated at 60°C to various $C_o t$ values ($C_o t$= moles per liter unlabeled nucleotides times seconds of incubation) after which time tubes were removed to -20°C ethanol until processed. Hybrid formation was assayed by hydroxylapatite chromatography according to Galau et al. (13). $C_o t$ values were converted to $eC_o t$ values by correcting for $Na^+$ concentration using the data of Britten et al. (3).

In the second method (the $S_1$ method), the excess cold RNA and [$^3$H]-DNA were mixed in a formamide buffer (50% formamide, 0.6 M NaCl, 0.01 M Pipes, pH 7.2, 1 mM EDTA, 0.05% SDS) and

5-10 ul aliquots were prepared and incubated as described for the HAP method. The samples were incubated at 50°C which is the optimum temperature for DNA:RNA heteroduplex formation but does not allow DNA:DNA reassociation (5). Hybrid formation was assayed by digesting the unhybridized material with $S_1$ nuclease (10 units/ul)(23) and precipitating the undigestied material with 4% trichloroacetic acid in the presense of 6 ug/ml salmon sperm DNA. The precipitate was collected by filtration on Whatman GF/C filters which were washed with 10-15 ml ice-cold 0.5% TCA, dried and the radioactivity was determined by liquid scintillation spectrometry (29). Since the rate of hybrid formation is slowed by the viscosity of formamide, there has been no attempt to make kinetic comparisons between the two methods or to previously reported experiments for other organisms.

The amount of [$^3$H]-DNA reassociation that occurred in the DNA:RNA hybridization reactions was routinely assayed by either digesting the hybrids with RNase A(50 ug/ml) under low salt conditions (0.05 M) and assaying by HAP (12,13) or by incubating the probe with a comparable mass excess of E. coli tRNA and digesting the mixture with $S_1$ nuclease (35). The double-stranded nicking activity of the $S_1$ nuclease was determined by digesting relaxed, supercoiled, and single stranded pBR322 and electrophoresing the products on a 1% agarose gel.

The hybrids that formed under the incubation conditions were routinely checked for mismatching by determining the $T_m$ on HAP and comparing the $T_m$ to the value determined for DNA

duplexes according to Martinson (22). Mismatching of the hybrids never exceeded 4-5% (decrease in $T_m$=5°C).

Preparation of null DNA. Null DNA was prepared by hybridizing the DNA probe to RNA isolated from vegetative cells (12,000-fold mass excess) in a 50% formamide buffer to a $C_o t$ value of 50,000. The mixture was diluted to 1.0 ml of 0.1 M phosphate buffer, pH 6.8, and passed over an hydroxylapatite column at 60°C. The material that eluted in the single-strand, low-salt washes was treated with alkali overnight to degrade the RNA, desalted by passage over G-50 Sephadex, and stored at 4°C over chloroform. Probe degradation was routinely monitored by electrophoresis on a 2% alkaline agarose gel (21) with BRL ΦX174 RF DNA-Hae III fragments as markers.

Data analyses. All data points were analyzed using one of two non-linear least-squares computer fitting programs configured to analyze DNA:RNA hybridization reactions. One program was written for a microcomputer (15), the other for a PDP-11 timesharing computer (25); both programs gave virtually identical results for the same set of data.

# RESULTS

DNA Reassociation. In order to assess the mRNA complexity of Anabaena variabilis, it was necessay to first determine the genomic complexity. Unlabeled DNA from vegetative cells was sheared by probe sonication to a mass average single-strand size of $1.85 \times 10^5$ daltons and reassociated in a 12-15,000-fold mass excess to labeled probe in 0.14M phosphate buffer containing 1% SDS. The sequences renatured as a single theoretical component with a rate constant of 0.091 L $mol^{-1} sec^{-1}$ (data not shown). The genome was calculated to be $9.07 \times 10^6$ nucleotide pairs, or about twice the size of the E. coli genome calculated using similar techniques (16). The genome size we determined for A. variabilis was slightly larger (approx. 30%) than those determined for other species of Anabaena by Herdman et al. (18). This discrepancy, however, may be due to the different methodology, differences in fragment sizes, or slight differences in the E. coli standards used in each case.

mRNA complexity classes of vegetative cells and heterocysts. When vegetative RNA was hybridized to DNA and the hybrids fractionated on HAP, a total of 32% of the input probe was found as RNA:DNA heteroduplexes at saturation (Fig. 1).

Assuming assymmetric transcription, this indicated that 64% of the sense strand was transcribed by A. variabilis during vegetative growth. Based on the kinetics of the hybridization reaction, the best fit of the data showed there were two relative abundance classes of transcripts in the vegetative cells: a high abundance class that forms hybrids at low $C_0t$ values and is transcribed from 8.1% of the sense strand, and a low abundance class that hybridized at higher $C_0t$ values but is transcribed from 56% of the sense strand. Using the procedure of Hahn et al. (16), the highly abundant class was calculated to comprise ∿95% of the mRNA mass. With a genome size of $9.07 \times 10^6$ base pairs, this class could contain 500 different messages 1500 nucleotides in mean length. To calculate the predicted copy number per cell of each mRNA species, we used values of $66.7 \times 10^{-14}$ g RNA/vegetative cell and $263 \times 10^{-14}$ g RNA/heterocyst (30,31). Using these estimates, the abundant class messages in vegetative cell RNA populations were present on average 40 copies per cell, while the rare class was found in 4 copies per 10 cells. These numbers are much higher than those reported for E. coli (16) and this may be due largely to the extreme dissimilarities in reported values for RNA content of the two bacteria ($2.41 \times 10^{-14}$ g total RNA/cell in E. coli, 16). We cannot account for this unexpected discrepancy; however, the reports for Anabaena RNA content are highly variable and growth conditions are known to make rapid changes in RNA content of logarithmically growing cyanobacterial cells (17).

Similar experiments with RNA isolated from heterocysts showed that the mRNA from the differentiated cell hybridized to DNA as a single kinetic component of an abundancy intermediate to the 2 vegetative cell abundancy classes (Fig. 2). In addition, the heterocyst contains fewer messages, since only 45.5% of the sense strand was transcribed as compared to 64% in the vegetative cell. This is sufficient genomic information to code for 2700 different mRNA species 1500 nucleotides in mean length. These 2700 putative mRNA species must include products of genes induced specifically during heterocyst development while the remainder would be mRNA constititutively produced in both heterocysts and vegetative cells. Although the 4% vegetative cell contamination of our heterocyst preparations may have resulted in overestimation for heterocyst mRNA complexity, we suspect that this problem is negligible due to the fact that heterocysts contain four-times the RNA of vegetative cells (30,31) and the bulk of the mRNA is transcribed from only a small fraction of the genome (8.1%).

mRNA complexity in vegetative cells and heterocysts as assayed by $S_1$ nuclease. The saturation hybridization value is of prime importance in determining total gene expression in each cell type. In order to verify the values obtained from the HAP columns, we have also carried out RNA excess hybridization experiments under different conditions. The same hybridization plateau should result although the kinetics of

the reactions may differ. In this approach, the RNA/ssDNA mixtures were incubated in a 50% formamide buffer and samples were analyzed using $S_1$ nuclease. The viscosity of formamide slows the nucleation events (5) and for this reason, $C_o t_{1/2}$ values cannot be compared to values obtained incubation in phosphate buffer. However, abundance classes are qualitatively evident and, assuming high enough $eC_o t$'s can be achieved, the saturation plateaus should be unchanged by this method (5).

As seen in Fig. 3, the reaction proceeded as a two component reaction, again indicating two mRNA abundance classes as in Fig. 1. At $C_o t$ values greater than 20,000, the hybrids began to dissociate which we attribute to probe breakdown after extended incubations. These high $C_o t$ values represent the hybridization of the extremely rare sequences that may only represent "leaky" genes and are unlikely to be of biological significance (16). The computer-predicted plateau for the data indicated that 66.5% of the sense strand was transcribed, which is close to the value obtained by the HAP method (64.1%). Unlike the HAP method, the two complexity classes determined by this method appeared to contain approximately equal numbers of transcripts (1900 for the abundant, 2100 for the rare class).

Hybridization experiments using RNA from heterocysts ($S_1$ method) were analyzed as both a single component reaction and a two component reaction. Analysis of these data as a 2 component reaction resulted in the most statistically accurate fit (RMS error=$4.2 \times 10^{-4} \pm 0.01$ for the 2 component fit; RMS

error=$4.2 \times 10^{-3} \pm 0.03$ for the 1 component fit). Although this is not consistent with the single complexity calculated from the HAP method (see Fig. 2 and Table 1), we have chosen to present two complexity classes here (shown in Fig. 4) for ease in comparison to the data presented in the following section concerning heterocyst-specific gene expression. The saturation value from Fig. 4 indicated that the heterocyst transcribes ca. 2600 possible mRNA species from 44.2% of the sense strand.

Heterocyst-specific gene expression. To determine the fraction of heterocyst genomic coding capacity expressed only in heterocysts, we prepared a null DNA probe as described. This probe, representing sequences not expressed during growth in combined nitrogen, was hybridized to total cell RNA from heterocysts (Fig. 5). The hybridization plateau (7.2% of input probe formed hybrids), when corrected for the presumed asymmetric transcription and enrichment for the nonsense strand that occurs in the fractionation procedure, indicated that 24.5% of the sense strand codes for a possible 1500 genes of 1500 nucleotides in mean length that are heterocyst-specific transcripts. The terminally differentiated heterocyst, then, expresses 1500 genes that are presumably derepressed during heterocyst development and 1200 genes that are expressed by both cell types.

The $C_o t_{1/2}$ of the reaction ($C_o t_{1/2}$=200; Fig. 5 and Table 2), corresponded to the $C_o t_{1/2}$ of the more abundant class of transcripts in heterocysts as assayed by the $S_1$ nuclease

method (see Table 2). This indicated that the heterocyst-specific sequences are present in high copy number, while the so-called "house-keeping" sequences are in lower copy number in the cell.

In order the substantiate the data defining heterocyst-specific gene expression, we hybridized a 1:1 mixture of total cell RNA from vegetative cells and heterocysts to the total genomic [$^3$H]-DNA probe (Fig. 6), anticipating that the saturation value would be the sum of the vegetative RNA saturation value (64%) and the heterocyst-specific RNA saturation value (24.5%). The actual saturation (81.2%)(Table 2) was slightly less than expected (91%) but this may be because the rare sequences were diluted to the point that the highest $C_o t$ values achieved were not sufficient to drive their hybridization. The expected saturation plateau may also be slightly overestimated due to vegetative cell contamination of heterocyst preparations as previously discussed.

# DISCUSSION

There is a correlation between genomic size and organizational and/or developmental complexity for all organisms for which this information is known (11). A detailed study of 128 strains of cyanobacteria carried out by Herdman and colleagues (18) suggested that even within this restricted group of photosynthetic prokaryotes, there was a good correlation between genome sizes and morphological complexity. Those strains capable of heterocyst and akinete formation have chromosomes generally larger than those of unicellular strains. The supposition was made that the more complex morphological forms arose by genomic duplication of a simpler ancestral genome, whose redundant genomic material could then produce mew genetic information through mutation. This theory is supported by our data which indicate that a considerable portion of the genome is devoted to the development and maintenance of the heterocyst. While E. coli has been shown to transcribe virtually 100% of the coding strand (16) and certain mammalian cells contain less than 1% transcriptionally active DNA (11), our results for Anabaena variabilis indicate that 85% of the sense strand is transcribed by the vegetative cells and heterocysts combined. The remainder of the DNA whose transcription cannot be detected under the conditions of these experiments may

represent sequences which our assays would not detect, for example, vegetative cell-specific sequences expressed under nitrogen limiting conditions or genomic information for the formation of the spore-like akinetes which would be expected to require novel gene expression (1). In addition, the computer-predicted saturation plateaus may be an underestimate since the probe inevitably tends to degrade after the long incubations required to achieve the highest $C_0t$ values. However, the sequences detected at the very high $C_0t$ values may represent only extremely rare sequences that are of little biological significance (16). We conclude that Anabaena, like E. coli, probably contains little DNA that is not transcriptionally active in some phase of its life cycle.

Although only 17 genes are required for nitrogen fixation in the facultative anaerobic bacterium Klebsiella pneumonia (20), the genes required to form the heterocyst, basically an anaerobic chamber that houses the nif gene products, are far more numerous; our data indicate that nearly 45% of the Anabaena genome (2600-2700 different mRNA species) is transcribed by the terminally differentiated heterocyst and more than one-half of the transcribed species are heterocyst-specific. That the copy frequency of the heterocyst-specific transcripts was high is not surprising, considering that many inducible systems show high rates of transcription under the inducing conditions. For example, there may be less that 1 copy per 100 cells of the lac operon under fully repressed conditions as compared to ~10 lac mRNA's per cell under fully induced conditions (34). The apparent

high rate of heterocyst-specific transcription may prove to be especially interesting in light of the recent evidence concerning "nif-promoters" for genes in the nif complex of Anabaena that differ both from the conventional E. coli-like promoter and the Klebsiella nif promoters (32). It would be interesting to know whether the heterocyst-specific genes are all transcribed from similar promoters, which may explain the coordinate and high level of expression of these genes. Alternatively, there is some evidence that heterocyst gene transcription may be in part regualted by the σ factor of Anabaena RNA polymerase (33).

Heterocyst differentiation is somewhat analogous to sporulation induction in Bacillus subtilis, a system which has been extensively studied both by hybridization techniques and mutagenesis mapping. Over 50 sporulation loci have been mapped on the Bacillus chromosome and competition hybridization studies indicate that 40% of the hybridizable mRNA from developing spores is qualitatively of the sporulation-specific type (8). In spite of the analogies between sporulation and heterocyst formation, the heterocyst is quite unique in its interdepentancy on neighboring vegetative cells and its active physiological functions. The induction of heterocyst formation thus represents one of the few well-studied developmental systems for which we have the potential of understanding the regulation of a wide array of genes that contribute in concert to a morphologically and physiologically differentiated cell.

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Table 1. Complexity of mRNA transcripts from vegetative cells and heterocysts as assayed by hybridization in phosphate buffer and fractionated on HAP columns.

| cell type | %genomic[a] complexity | eCot½[b] | $K_{obs}$[c] | $K_{exp}$[d] | frac. of[e] mRNA mass | est. #[f] species | ave. #[g] copies/cell |
|---|---|---|---|---|---|---|---|
| vegetative | | | | | | | |
| abundant | 8.1 | 45.6 | 0.61 | 0.65 | 93-95% | 500 | 40 |
| rare | 56.0 | 4328.1 | 0.0064 | 0.0935 | 6-7% | 3400 | 0.4 |
| heterocyst | 45.5 | 2640.0 | 0.0105 | - | - | 2700 | 32 |

[a] Calculated assuming asymmetric transcription and corrected for probe reactibility.

[b] The value at which ½ of the given complexity class forms heteroduplex.

[c] $K_{obs} = \frac{\ln 2}{\text{mRNA eCot}}$ (mRNA eCot=eCot x 0.025; assuming 2.5% of the RNA is mRNA and responsible for driving the reaction)

[d] $K_{exp} = \frac{2 \times C_e \times 0.091}{C_a} \times \frac{450}{1000}$ ; $C_e$=complexity of transcriptionally active genome
$C_a$=complexity of class of mRNA
0.091=rate constant (K) for A. var. DNA renaturation
2=correction for RNA driven reaction
450/1000=correction factor for length of reannealing molecules

[e] $K_{obs}/K_{exp} \times 100$

[f] average mRNA length of 1500 nucleotides assumed

[g] total RNA content of cell=66.7 x $10^{-14}$ g/veg. cell; 263 x $10^{-14}$ g/heterocyst (30,31)

Table 2. Complexity of mRNA transcripts from vegetative cells and heterocysts as assayed by hybridization in 50% formamide buffer and S1 nuclease digestion.

| source of RNA | mRNA complexity class | % genome complexity | $Cot_{\frac{1}{2}}$ | est. # of species |
|---|---|---|---|---|
| vegetative cells | abundant | 31.7 | 168.6 | 1900 |
| | rare | 34.8 | 3120.5 | 2100 |
| | total | 66.5 | | 4000 |
| heterocysts | abundant | 19.5 | 216.1 | 1200 |
| | rare | 24.7 | 3516.5 | 1500 |
| | total | 44.2 | | 2600 |
| heterocyst-specific RNA | total | 24.5 | 199.5 | 1500 |
| vegetative + heterocyst cells | abundant | 35.7 | 115.5 | 2200 |
| | intermediate | 20.7 | 1397.1 | 1300 |
| | rare | 24.8 | 3484.8 | 1500 |
| | total | 81.2 | | 5000 |

Fig. 1. Hybridization of trace amounts of [$^{3}$H]-ssDNA probe with total cell RNA isolated from vegetative cells. Each sample was incubated in a phosphate buffer for varying amounts of time and the hybridized material was fractionated by hydroxylapatite chromatography at 60°C. The curve represents the best least square fit to the data assuming two theoretical complexity classes of mRNA. In all calculations it was assumed that only one strand equivalent of the Anabaena genome was transcribed and therefore, hybridization to 4% of the input probe in the abundant class represented transcription from 8% of the sense strand (see Table 1). The root-mean-square (RMS) error for the curve was 0.00223.

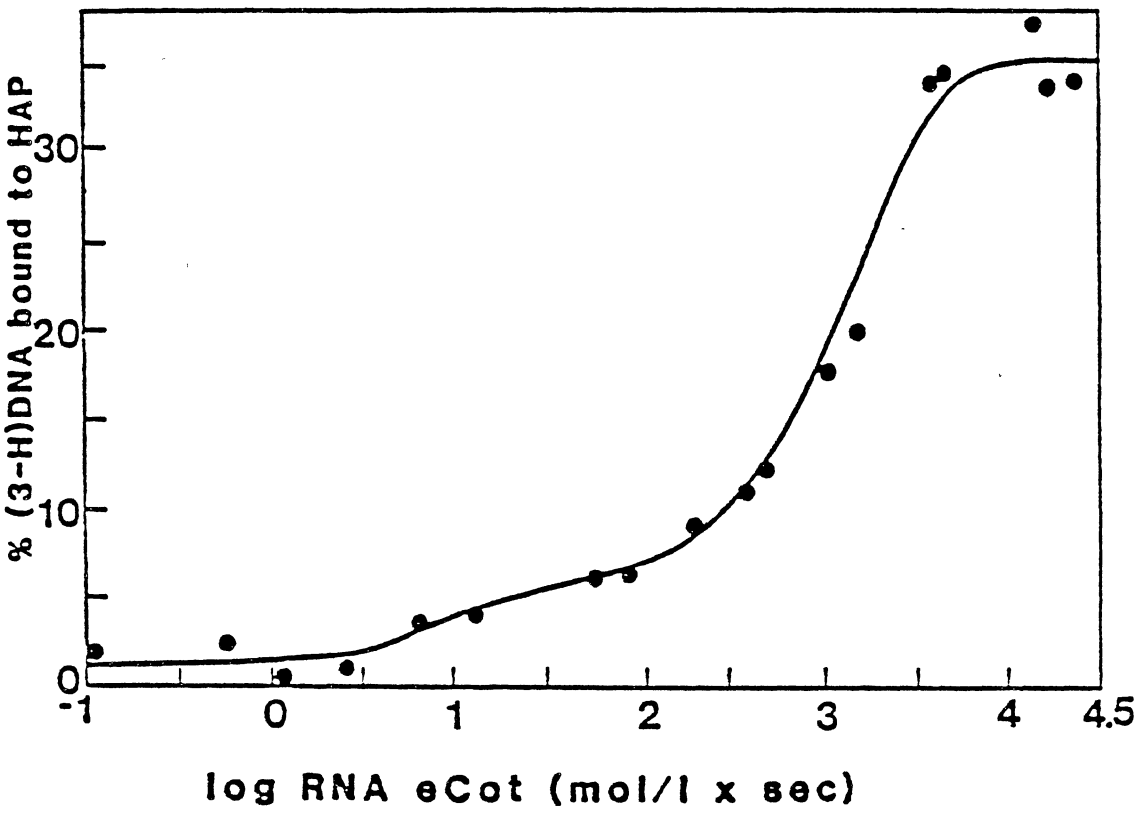

% (3-H)DNA bound to HAP
0
10
20
30
-1
0
1
2
3
4
4.5
log RNA eCot (mol/l x sec)

Fig. 2. Hybridization of trace amounts of [$^{3}$H]-ssDNA probe with total cell RNA isolated from heterocysts. All samples were incubated and assayed as in Fig. 1. The best least squares computer fit of these data (RMS error = 0.00115) indicated a single complexity class of mRNA transcribed from 45.5% of the sense strand.

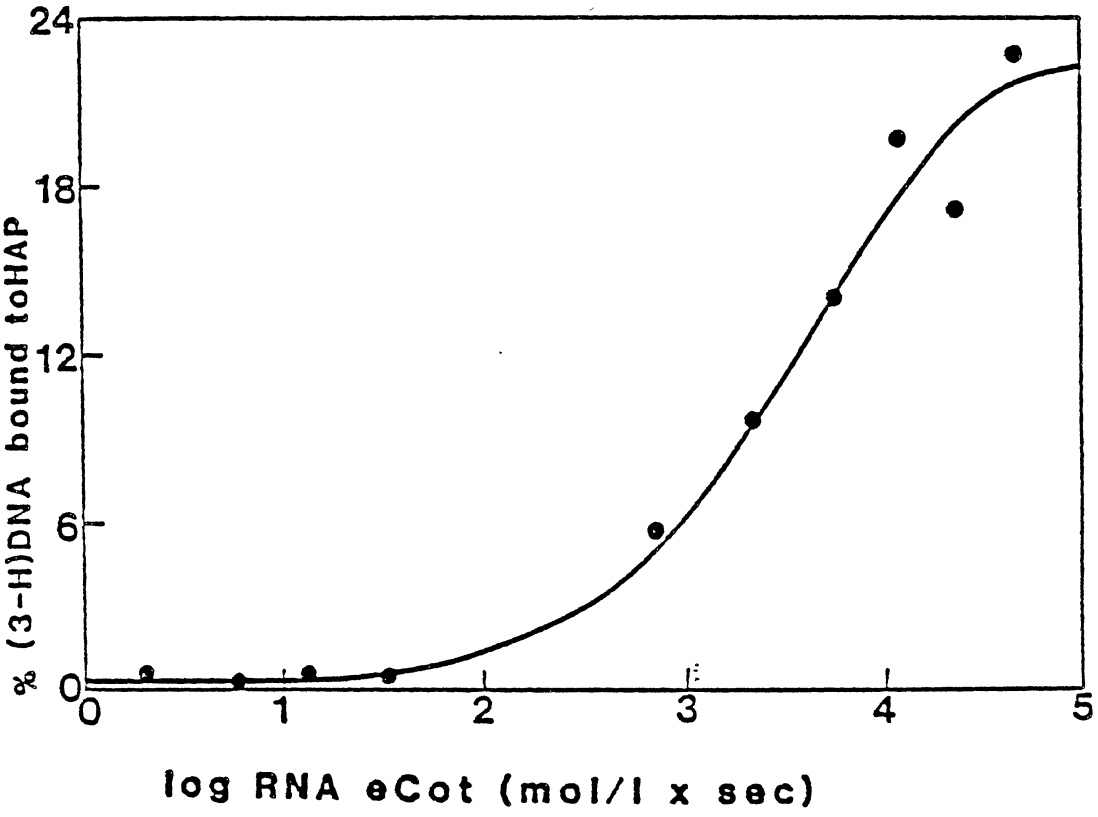

24
18
12
6
0
% (3-H)DNA bound toHAP
0
1
2
3
4
5
log RNA eCot (mol/l x sec)

Fig. 3. Hybridization of trace amounts of [$^3$H]-ssDNA probe with total cell RNA isolated from vegetative cells. Each sample was incubated in 50% formamide buffer and duplex material was assayed using S1 nuclease digestion. The best least squares fit of the data (RMS error = 0.0029) indicated two theoretical complexity classes. In calculating the $C_o t$ value for these reactions, the monovalent cation concentration ($eC_o t$) was not considered.

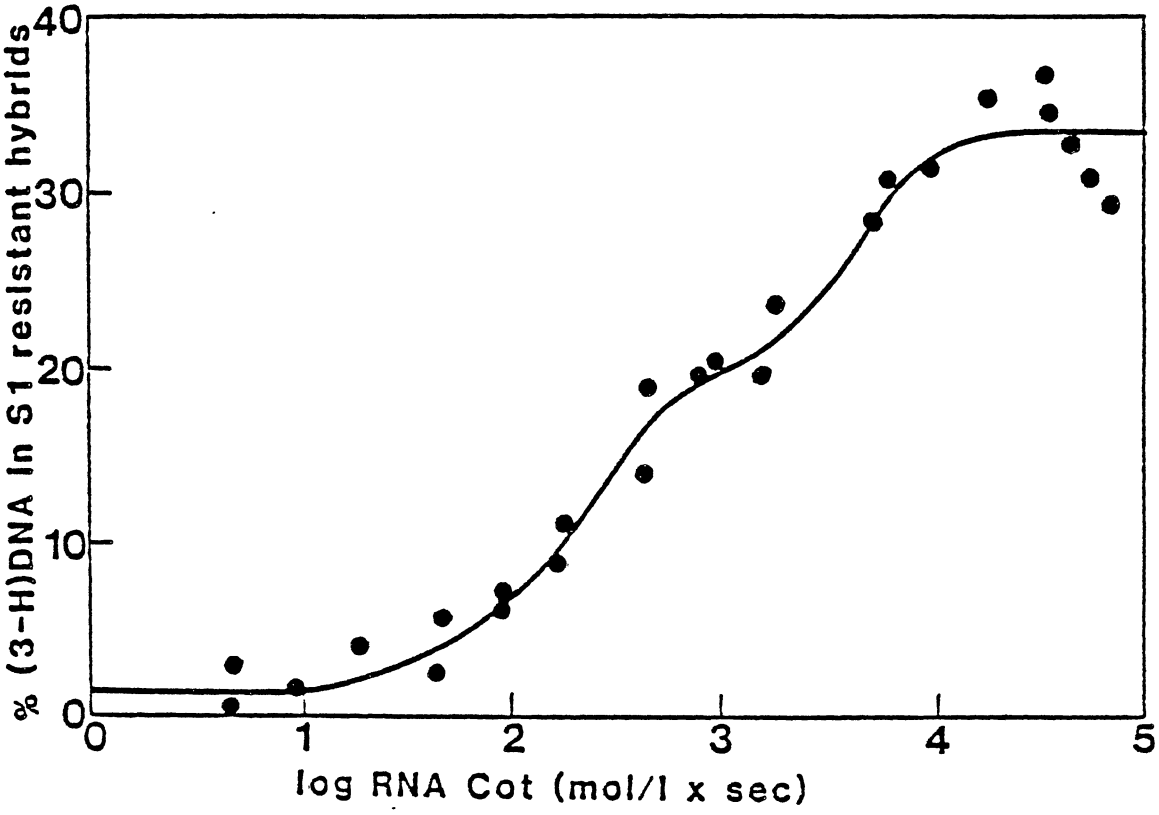

% (3-H)DNA In S1 resistant hybrids
0
10
20
30
40
0
1
2
3
4
5
log RNA Cot (mol/l x sec)

Fig. 4. Hybridization of trace amounts of [$^{3}$H]-ssDNA probe with total cell RNA isolated from heterocysts. Samples were incubated and assayed as described for Fig. 3. The computer fit of the data (RMS error = 0.0042) indicated two complexity classes of mRNA transcribed from 44% of the sense strand.

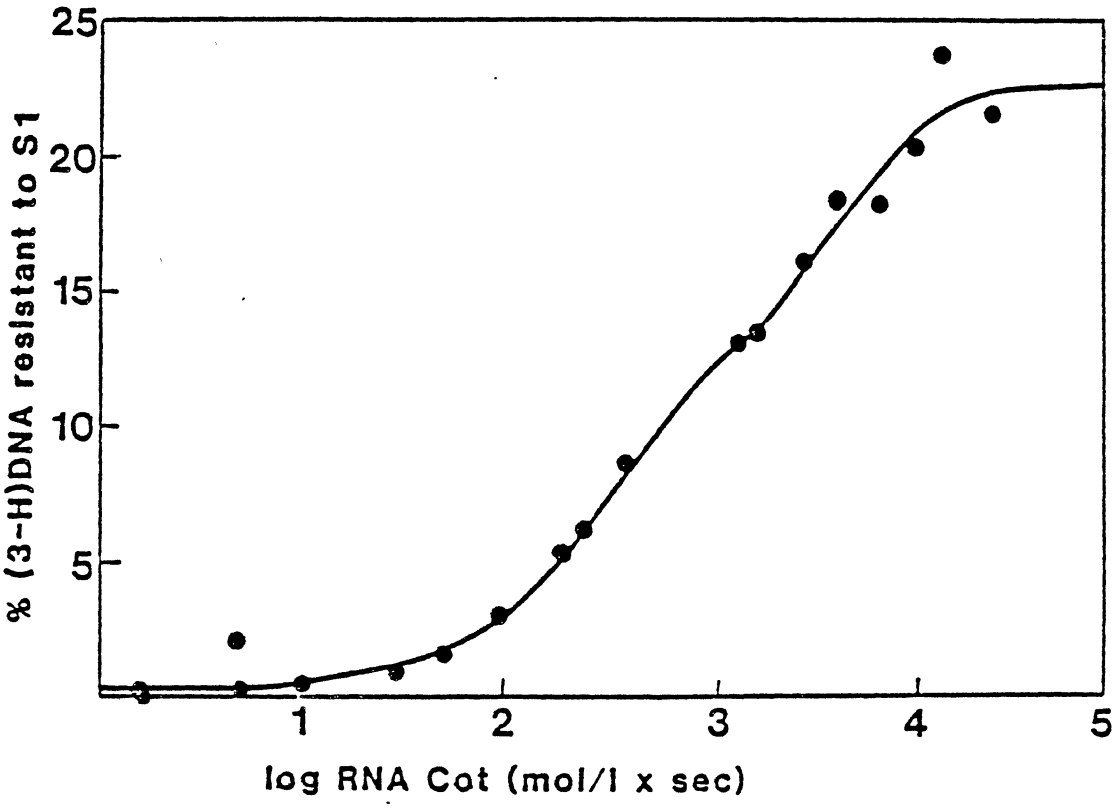

25
20
15
10
5
% (3-H)DNA resistant to S1
1
2
3
4
5
log RNA Cot (mol/l x sec)

Fig. 5. Hybridization of trace amounts of null ssDNA probe with total cell RNA isolated from heterocysts. The null DNA probe was prepared as described in Materials and Methods. Samples were incubated in 50% formamide and assayed using S1 nuclease digestion. The best fit for the data yielded an RMS error of 0.00024.

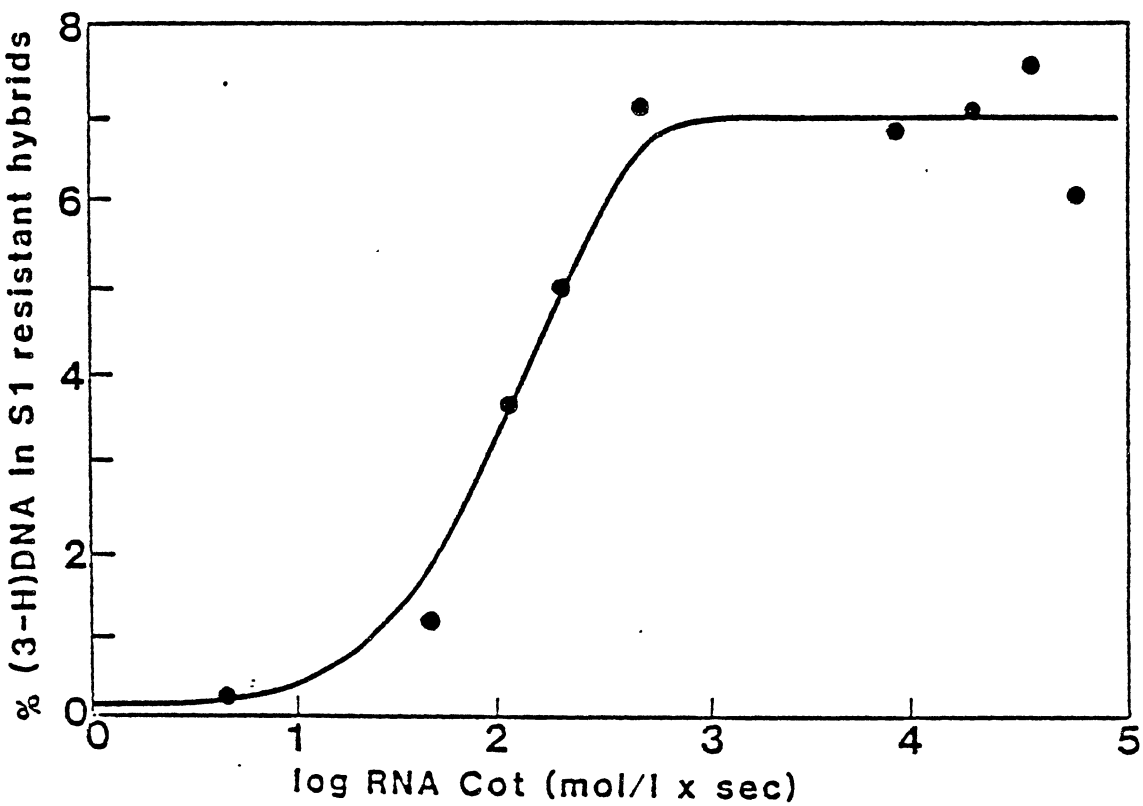
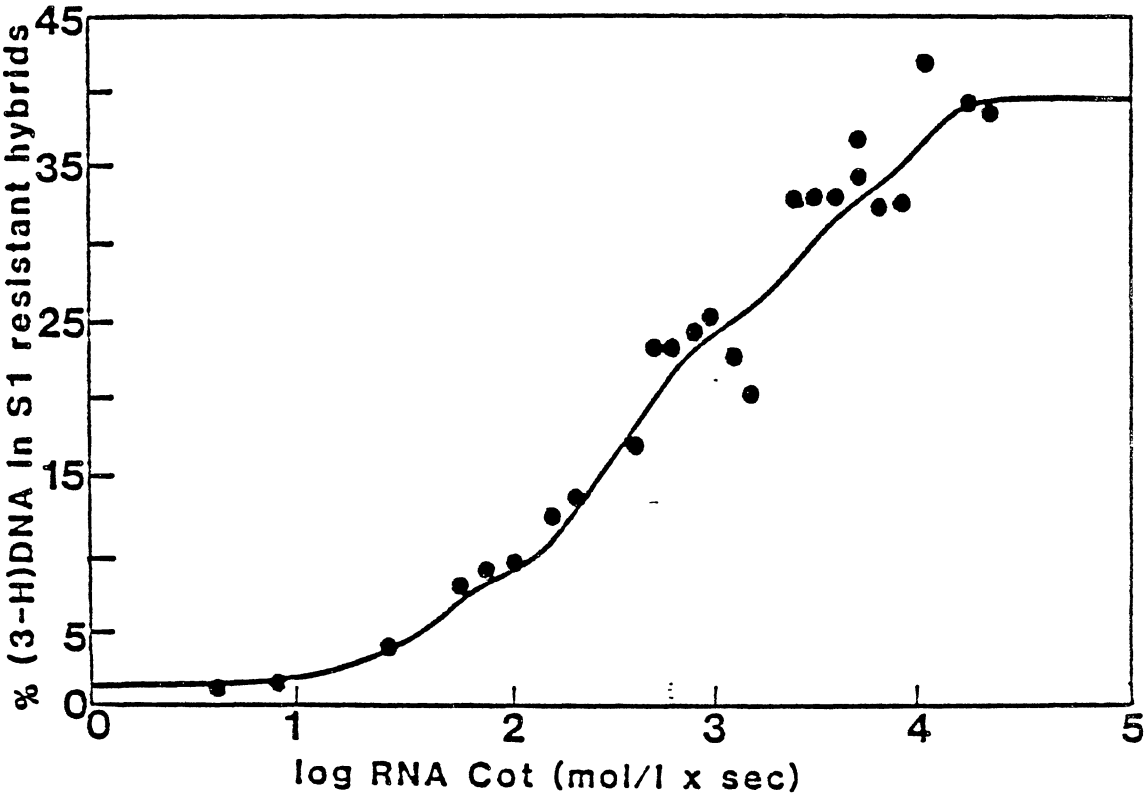

% (3-H)DNA In S1 resistant hybrids
0
2
4
6
8
0
1
2
3
4
5
log RNA Cot (mol/l x sec)

Fig. 6. Hybridization of trace amounts of [$^{3}$H]-ssDNA probe with total RNA from both heterocysts and vegetative cells mixed in a 1:1 ratio. Samples were incubated in a 50% formamide buffer and assayed using S1 nuclease digestion. The least squares analysis of these data (RMS error = 0.00536) indicate that the combined transcripts segregate into three theoretical components and are transcribed from 81.2% of the sense strand.


% (3-H)DNA In S1 resistant hybrids
45
35
25
15
5
0
0
1
2
3
4
5
log RNA Cot (mol/l x sec)

CHAPTER III

# TRANSCRIPTIONAL ACTIVITY OF HETEROCYSTS ISOLATED FROM *ANABAENA VARIABILIS*

by

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# ABSTRACT

The nondividing,terminally-differentiated heterocysts of Anabaena variabilis are specialized for aerobic nitrogen-fixation. They reduce acetylene to ethylene, synthesize nitrogenase, and carry out a host of other functions following isolation from the filament. Many of these functions cannot be maintained for extended periods of time and require light, anaerobic conditions, and a reducing environment. We found that nitrogenase activity of isolated heterocysts was inhibited by incubation in the dark and by RNA or protein synthesis inhibitors. Although protein synthesis is maintained in heterocysts for up to 4 h following isolation, RNA synthesis occurs at only about 10% the rate of vegetative cells and ceases approx. 80 min after isolation. Pulse-chase experiments indicate that the high molecular weight RNAs synthesized during a 30 min incubation were slowly turned over during a 2 h chase. We speculate that heterocysts may require some precursor for RNA synthesis that intact heterocysts import from vegetative cells. The fact that isolated heterocysts can maintain continuous protein synthesis in the absence of detectable RNA synthesis suggests that heterocysts contain either long-lived messages or low levels of nucleases with respect to the parent vegetative cell. It appears that diminished heterocyst transcriptional activity

following isolation may be one factor that contributes to the eventual loss of nitrogenase activity in isolated heterocysts.

# INTRODUCTION

Anabaena variabilis is one of several species of cyanobacteria that produces heterocysts, cells specialized for aerobic $N_2$ fixation induced in response to a medium that lacks a combined nitrogen source (1,25,27,32). Nitrogenase, the enzyme complex that reduces $N_2$ to $NH_3$, can reduce $C_2H_2$ to $C_2H_4$ as well and this is the standard assay for the detection of this enzyme (31). Although as early as 1949 heterocysts were hypothesized to be the sole site of nitrogen fixation (9), for many years the low yield of acetylene reduction by heterocysts isolated from filaments of Anabaena prevented definitive localization of nitrogenase (24). In 1974, studies using protein extracts from heterocysts and the undifferentiated vegetative cells clearly showed that heterocysts housed the vast majority of nitrogenase and any evidence of nitrogenase activity in vegetative cell extracts was probably due to leakage from heterocysts (7,8). Eventually, Peterson and Wolk (19) were able to recover 91% of the nitrogenase and 69% of the nitrogenase reductase in $^{55}Fe$-labeled protein extracts from heterocysts. Their isolation procedure, which involved gentle disruption of vegetative cells and maintenance of the heterocysts in a strict anaerobic and reducing environment, allowed recovery of 60-70% of the $C_2H_2$-reduction activity of the intact filaments.

The DNA content of heterocysts is virtually identical to that of vegetative cells (23) and DNA synthesis is apparently not required for heterocyst development (4,27). Transcription, however, is vital to at least certain stages of development (28). Messenger RNA complexity of heterocysts from A. variabilis is 71% that of vegetative cells (see Chapter 2), and both types of cells incorporate [$^3$H]-uracil as detected by autogradiophy at the same rate in Cylindrospermum (28). Bradley and Carr (4) demonstrated that RNA synthesis inhibitors (rifampicin and proflavin) could block heterocyst development in A. cylindrica following transfer to nitrogen-free media but the time of addition of inhibitor was critical. Inhibitor added 1-2 hours following induction failed to prevent proheterocyst development, whereas mature heterocysts were not committed to their developmental fate until 8-9 hours following induction, at which time their development becomes refractory to RNA synthesis inhibitors.

The importance of protein synthesis to heterocyst development has been studied by numerous investigators. Protein patterns on one dimensional gels differ for vegetative cells, heterocysts, and even immature heterocysts (proheterocysts)(8), indicating that translation is important for both early and late stages of differentiation. In addition, the protein synthesis inhibitor chloramphenicol has been shown to block virtually any stage of heterocyst development (1,14).

The mutual dependance of heterocysts and vegetative cells is well established (18,30) and heterocysts are not long-lived

following isolation from the filament; i.e., $C_2H_2$ reduction rates can only be maintained for 3 to 6 hours (10). In addition to the high rates of $C_2H_2$-reduction by isolated heterocysts (10,19), these cells can also synthesize nitrogenase and other proteins (13), synthesize heterocyst-specific envelope polysaccharides (5) and carry out many processes such as photophosphorylation and respiratory $O_2$ consumption (3,26). In spite of the interest given to the aerobic, nitrogen-fixing cyanobacteria in recent years and the large number of studies which have involved isolated heterocysts, we are still far from a full understanding of factors from vegetative cells that contribute to the maintenance of heterocyst activity and the importance of physiological processes within the heterocyst such as transcription and translation to the maintenance of nitrogenase activity (33). We have attempted to address some of these questions by studying the transcriptional activity of isolated heterocysts and by using various specific inhibitors to identify functions of heterocysts necessary to maintenance of biological activity; i.e., nitrogenase activity in particular.

# MATERIALS AND METHODS

Heterocyst Induction and Isolation. A. variabilis (ATCC 29413) was grown aerobically to an $A_{650}$ of 0.35-0.45 in a New Brunswick MF-114 fermentor in 6 liters of Allen and Arnon's media (2) supplemented with 2mM $NH_4Cl$ and 1 mM $NaNO_3$. To induce heterocyst formation, the filaments were allowed to settle for several hours, the medium was drained off and the cells were diluted to an $A_{650}$ of 0.15-0.20 with Allen and Arnon's medium lacking combined nitrogen.

Heterocyst induction and development occurred over the next 48 h following which time the cells were collected by a Sorvall KSB-R continuous flow apparatus. Heterocysts were isolated essentially according to the method of Peterson and Wolk (19) with some modifications. The fermentor, which normally contained 10 liters of culture, was purged with argon for 30 min prior to harvest and all subsequent buffers used in the isolation procedure were deoxygenated by thorough bubbling with either argon or hydrogen. Vegetative cell walls were selectively ruptured with a 45-min incubation in 2 mg/ml lysozyme at room temperature and the vegetative cells were disrupted by probe sonication (5-10 10-second bursts at 40% duty cycle with a Branson cell disruptor #185). Heterocysts were separated from vegetative cell debris by three successive centrifugations in a Sorvall HB-4 rotor (350g, 150g, 50g)

and finally resuspended in a HP/M buffer (0.03 M Hepes, 0.03 M Pipes, pH 7.2, 0.001 M $MgCl_2$)(19) + 0.18 M mannitol. In one series of experiments, heterocysts were isolated by rupturing lysozyme-treated vegetative cells with 0.4% sodium dodecyl sulfate, followed by a 15-fold dilution and three 15-min centrifugation spins (2 x 1200g, 2000g). Vegetative cell contamination, as determined microscopically, was always less than 4%.

Chlorophyll a determinations were routinely obtained for both intact filaments and isolated heterocysts according to Wetzel and Likens (29). In addition, cell counts for the isolated heterocysts were carried out using a Levy corpuscle counting chamber.

$C_2H_2$-Reduction Assays. For the nitrogenase assay, isolated heterocysts were diluted to 0.5-1.5 ug chl a/ml with 7/8 HP/M + mannitol buffer and 1/8 AA-N media; intact filaments were incubated in AA-N media. Two-ml volumes were placed in 30-ml incubation bottles in an atmosphere of 46% argon/46% hydrogen/8% $C_2H_2$ and incubated on a reciprocating shaker illuminated with cool-white fluorescent tubes at 80-100 $uE/m^2/s^1$.

The reaction was stopped by adding 0.4-mls 30% trichloroacetic acid to each bottle. Ethylene production was measured by flame ionization detection with a Perkin Elmer 990 gas chromatograph fitted with a Porapak N column (31)and calibrated with ethylene standards from Alltech Associates.

Radiolabeling of Isolated Heterocysts. Isolated heterocysts were labeled with 1-10 uCi/ml [5,6-$^{3}$H]-uracil (40 Ci/mmol), 5 uCi/ml [2-$^{3}$H]-glycine (17 Ci/mmol), or 10 uCi/ml [$^{32}$P] as orthophosphate. In a separate experiment, differentiated filaments were labeled with [2-$^{14}$C]-uracil (0.125 uCi/ml) for 4 h prior to heterocyst isolation. Although cultures from which heterocyts were isolated were axenic, 10 ug/ml ampicillin was added to isolated heterocyst buffers to prevent radioisotope uptake by low-level bacterial contamination which might have been introduced during heterocyst isolation. A control experiment indicated that ampicillin did not alter the $C_2H_2$-reduction activity of the isolated heterocysts for up to 11 hours. All radiochemicals were purchased from ICN Biomedicals, Inc. with the exception of [5,6-$^{3}$H]-uracil (New England Nuclear).

Radiolabel uptake by isolated heterocysts was determined by filtration of the cells over Millipore 0.45 um membrane filters and extensive washing with 1mM cold glycine, uracil, or phosphate. Incorporation of radiolabels by isolated heterocysts was determined by placing an aliquot of the cells into 4% cold TCA, and after 10-20 minutes on ice filtering over Whatman GF/C filters and washing with 15-20 mls of cold 0.5% TCA + 1 mM glycine, uracil, or phosphate (17).

Nucleic Acid Isolation. Nucleic acids from vegetative cells were isolated by gently disrupting the vegetative cells with lysozyme and detergents according to the method of Cattolico

(6). Isolated heterocysts were disrupted by vortexing a dense slurry of the cells in HP/M + mannitol buffer and glass beads for 3-5 minutes. Nucleic acids were purified from cell debris by extraction with phenol:chloroform (20).

The isolated nucleic acids were electrophoresed on 2.4% polyacrylamide/0.5% agarose cylindrical gels (6) and scanned with a ISCO UV monitor model UA-5 and gel scanner model 1310. To measure radioactivity in each fraction, the gels were sliced into 2 mm sections with a Bio-Rad gel slicer model 190, and the sections were solublized in 200 ul 30% $H_2O_2$ (12 hours, 60°C) and counted in 4.0 mls BioCount.

# RESULTS

Characterization of heterocyst formation. Maximum heterocyst frequency and nitrogenase activity were attained in cultures 30-48 hours following transfer to nitrogen-free media (Fig. 1). The chlorophyll content of these cultures ranged from 1.0-1.5 ug chl a/ml culture and had an $A_{650}$ that ranged from 0.2-0.35.

$C_2H_2$-Reduction of isolated heterocysts. Heterocysts isolated by the techniques described above typically retained 30-50% of the $C_2H_2$-reduction activity of the intact filaments on a per ug heterocyst chlorophyll a basis, assuming that each heterocyst has ca. 1.6 times the chlorophyll a of a vegetative cell (22). Heterocysts incubated in HP/M buffer containing 0.18 M mannitol as an osmoticum retained $C_2H_2$-reduction rates 2.5-3 times that of heterocysts incubated in HP/M buffer alone after 3 hours (data not shown, see also 10); for this reason all assays were performed in the presence of mannitol.

Incubation in the dark resulted in a 75% and 74% depression in the $C_2H_2$-reduction activity of isolated heterocysts and intact filamants, respectively, after one hour (Table 1). Aerobic conditions eliminated the activity of isolated heterocysts and had only a slight effect on the activity of the intact filaments after a one-hour incubation

period. $C_2H_2$-reduction rates of isolated heterocysts were depressed in the presence of the RNA synthesis inhibitor rifampicin (100 ug/ml) and the protein synthesis inhibitor chloramphenicol (10 ug/ml), although the inhibition was most marked after extended incubation periods. It appears that synthesis of new RNA templates and continued protein synthesis may be required to maintain activity in heterocysts following isolation. The early inhibition on $C_2H_2$-reduction activity (Table 1) in the presence of rifampicin may be due to a previously reported direct interference by rifampicin on the nitrogenase complex itself (28).

Translational activity of isolated heterocysts. To further investigate the inhibitory effect of chloramphenicol on $C_2H_2$-reduction, heterocysts isolated as described were incubated anaerobically with 0.8 uCi [$^3$H]-glycine/ug heterocyst cholorphll a under various conditions (Fig. 2). After a four-hour incubation period, heterocysts maintained anaerobically in the light incorporated 84% of the [$^3$H]glycine taken up into TCA-insoluble molecules as measured by filtration of cells over membrane filters. The heterocysts did become leaky to unincorporated label in all treatments during long incubation times (>2 h), which probably limited incorporation. Incubation in the dark resulted in a 32% inhibition of incorporation, while the RNA polymerase inhibitor proflavin slowed glycine incorporation by 76% that of the control after four hours. Rifampicin had little effect on protein synthesis for the initial 75 minutes; however, for

longer periods of time, the rifampicin-treated cells virtually ceased protein synthesis while the control continued to assimilate the exogenous glycine. The differential effect of these two inhibitors of RNA synthesis may be due to their different mechanisms of action. In addition, proflavin was approximately 10-fold more concentrated than rifampicin. The protein synthesis inhibitor chloramphenicol markedly inhibited [$^{3}$H]-glycine incorporation by isolated heterocysts from the outset, as has been described previously (13).

Transcriptional activity of isolated heterocysts. The inhibition of both nitrogenase activity and amino acid incorporation of isolated heterocysts by the RNA polymerase inhibitors proflavin and rifampicin led us to hypothesize that transcription may be necessary for heterocyst activity in vivo. A 4 h in situ [$^{14}$C]uracil labeling of filaments followed by immediate chilling, heterocyst isolation, and nucleic acid extraction resulted in RNA species from heterocysts and vegetative cells with similar specific activities (5280 and 4340 cpm/ug, respectively). As shown in Fig. 3, the stable species of RNA isolated from heterocysts contained the majority of the label. Spectrophotometric scans and count profiles of total RNA from vegetative cells were essentially identical.

Isolated heterocysts maintained in an anaerobic environment were able to take up and incorporate [$^{3}$H]uracil into a TCA-insoluble fraction to a limited extent. During a 4 h incubation in 1.2-10 uCi [$^{3}$H]uracil/ml, 30% of the label was

taken up by the heterocysts and approximately 5% of this was TCA-insoluble, indicating that RNA systhesis is not limited by uptake. Both incorporation and assimilation virtually ceased after 90 minutes of incubation (data not shown), quite different from results for [$^3$H]glycine incorporation by isolated heterocysts which continued for up to 240 minutes (Fig. 2).

RNA from isolated heterocysts labeled with [$^3$H]uracil for 30 minutes had a specific activity of 44 cpm/ug, which declined to 22 cpm/ug after a 2-h chase with 1mM cold uracil. By comparison, vegetative cells labeled for 30 min with approximately the same concentration of [$^3$H]uracil typically achieve a specific activity of 400-700 cpm/ug (unpublished observation). Although the incorporated radioactivity was low, the labeled material appeared to migrate as high molecular weight RNA ($3 \times 10^4$ - $10^6$ daltons) and UV scans indicate that RNA from both pulsed and chased samples was undegraded (Fig. 4). There is no relative increase in specific activity of the stable species during the chase, which suggests that stable and unstable RNA are being both degraded and resynthesized at extremely slow rates in isolated heterocysts.

The heterocysts routinely isolated in our lab are subjected to a brief probe sonication treatment to rupture the lysozyme-treated vegetative cells. This has proved an effective method for obtaining large quantities of highly purified heterocysts. To determine the possible inhibitory effects of the sonication treatment on the transcriptional

apparatus of heterocysts, we also isolated heterocysts using a detergent treatment followed by extensive dilutions and centrifugations (12). Heterocysts isolated by this more gentle method showed similar low rates of [$^3$H]uracil uptake and incorporation. PEI-cellulose chromatography of acid-soluble extracts from heterocysts after a 2 h incubation period showed that all detectable counts remained in the uracil fraction. Heterocysts may lack the uracil scavenging pathway. Efforts to circumvent this by labeling heterocyst RNA with radiolabeled phosphate resulted in incorporation of the label into an ethanol-insoluble, TCA-soluble fraction and count profiles on polyacrylamide gels virtually identical to those obtained by electrophoresis of RNA labeled with [$^3$H]uracil (data not shown).

# Discussion

Heterocysts represent a situation unique among prokaryotes in that they have lost certain features characteristic of parent vegetative cells and gained other features unique to the differentiated state while maintaining physiological interdependency with vegetative cells. Furthermore, the fact that these cells never divide again, yet retain DNA content similar to that of vegetative cells (28) raises the question of whether or not their physiological functions depend on continued transcriptional and translational activity.

We have shown that both RNA and protein synthesis inhibitors curtail nitrogenase activity in isolated heterocysts, indicating that both synthesis and turnover of macromolecules are required to maintain biological activity *in situ*. Although the inhibitors may be exerting a direct effect on nitrogenase as Simon (28) suggests for nitrogenase inhibition in intact filaments of *C. licheniforme*, this seems unlikely in our studies. The effect of the inhibitors on nitrogenase activity was greatest after prolonged incubation periods (2-3 h) which is consistent with the idea that heterocyst RNA and proteins are turned over (21). In addition, it seems unlikely that three chemically different inhibitors (rifampicin, proflavin, chloramphenicol) with

totally different modes of action, would each specifically affect the nitrogenase enzyme complex.

The translational activity of isolated heterocysts was studied by their ability to incorporate radiolabeled glycine. Continued glycine incorporation by illuminated heterocysts maintained in an anaerobic, reducing environment as shown by other investigators (13) was verified by this study (Fig. 3). Addition of the RNA synthesis inhibitors rifampicin and proflavin resulted in cessation of protein synthesis in isolated heterocysts 75 and 30 min, respectively, following addition of the inhibitor. The DNA intercalating agent proflavin would be expected to immediately interrupt RNA synthesis while rifampicin only inhibits initiation of new RNA chains. Thus, heterocysts seem to turnover messenger RNA and require continued synthesis of these molecules to maintain translational activity.

Data from in situ labeling studies indicate that heterocysts and vegetative cells incorporate uracil at approximately the same rate, which confirms an earlier report for Cylindrospermum (28). Since heterocysts do not divide, it seems puzzling that they would continue to accumulate rRNA and tRNA. The accumulation of label in stable RNA species could be attributed to turnover of unstable RNA during isolation but the fact that the these cells incorporate glycine in a chloramphenicol sensitive manner indicates that RNA templates are available within the heterocyst. The phenomenon of stable RNA accumulation has been recognized for the differentiating cells of Dictyostelium discoideum which continue to synthesize

rRNA at 10-15% the rate of undifferentiated cells (16). Heterocysts of this strain of Anabaena are approx. twice the size of vegetative cells and are reported to contain 4 times the RNA (22), thus extensive RNA synthesis may continue to occur in the recently matured heterocysts used in this study.

Following isolation from filaments, heterocysts were shown to assimilate uracil into RNA species which migrated to high molecular weight regions of gels, but at a much reduced rate as compared to heterocysts in situ or vegetative cells. We have shown that neither DNA damage by sonication during isolation nor the apparent inability to scavenge uracil by isolated heterocysts can account for the low levels of RNA biosynthesis. The fact that glycine incorporation seems to proceed relatively unperturbed in isolated heterocysts may indicate that heterocysts contain RNA molecules that are stable with respect to the messages of vegetative cells ($t_{1/2}$= 10-12 min, 15) or that ribonucleases which normally contribute to the short half-life of bacterial messages are limited in heterocysts. The inhibition of [$^3$H]glycine incorporation by RNA synthesis inhibitors makes this less likely. It seems possible that the special proteases formed during the initial stages of nitrogen starvation (34,35) may limit some of the degradative enzymes in heterocysts since these terminally differentiated, non-dividing cells would not be expected to undergo rapid metabolic changes in response to environmental conditions. However, this is incongruent with the high rates of synthesis by intact heterocysts. The idea that heterocysts must import essential precursors for RNA biosynthesis from

vegetative cells cannot be dismissed. Giddings et al. (11) showed that differentiated heterocysts lose the ability to reduce $SO_4$ following isolation and apparently must import $S^{2-}$ from vegetative cells but these authors suggest that heterocyst reducing potential may be monopolized by nitrogenase. When isolated heterocysts were labeled with $^{35}S^{2-}$, cysteine, or methionine, a portion of the $^{35}S$ was found in protein; however, quantitative determinations could not be made by these investigators. Janaki and Wolk (13) isolated heterocysts of A. variabilis that had been labeled in situ with [$^{35}$S]methionine. Both quantity and quality of translation products on polyacrylamide gels were much altered by the isolation procedure, leading the authors to conclude that the program of protein synthesis in heterocysts depends upon interaction with vegetative cells. In our studies, RNA biosynthesis by A. variabilis seemed similarly affected by isolation from vegetative cells. Previous work by this lab (J. Ownby, unpublished observation) indicates that stable species of RNA in vegetative cells of A. variabilis are virtually not turned over; yet, these studies show that in isolated heterocysts all pulse-labeled RNA molecules are degraded at approx. the same rate (Fig. 4). The vegetative cells may be providing ribonucleotides for continued synthesis by the non-growing heterocysts, as was hypothesized for protein turnover (13). With the increased interest in the molecular biology of the cyanobacteria it is hoped that heterocyst-specific probes can be used to study the regulation, synthesis, and in vivo stability of specific RNA

molecules involved in heterocyst function.

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Table 1. Effect of various treatments on $C_2H_2$-reduction by intact filaments and isolated heterocysts of A. variabilis.

| treatment | Intact Filaments | | Isolated Heterocysts | | | |
|---|---|---|---|---|---|---|
| | | | $C_2H_4$ formed (nmol·ug chl. $a^{-1}$·$h^{-1}$) | | | |
| | $C_2H_2$ formed (nmol·ug het chl $a^{-1}$·$h^{-1}$) | % of control | 1-2 h* | % of control | 2-3 h* | % of control |
| anaerobic | 192.4 | 100 | 35.8 | 100 | 73.0 | 100 |
| dark | 49.2 | 26 | 8.8 | 25 | N.D. | |
| aerobic | 173.7 | 90 | 0.0 | 0 | 0.0 | 0 |
| 100 ug/ml rifampicin | N.D. | | 22.2 | 62 | 19.1 | 26 |
| 10 ug/ml chloramphenicol | N.D. | | 37.1 | 104 | 15.1 | 21 |

*For isolated heterocysts, separate rates are given for 1-2 and 2-3 h after incubation in $Ar/H_2$ (1:1) containing 8% $C_2H_2$. N.D.: not determined.

Fig. 1. Heterocyst frequency and nitrogenase activity of A. variabilis following transfer to nitrogen-free medium. Data are averages of 4 experiments.

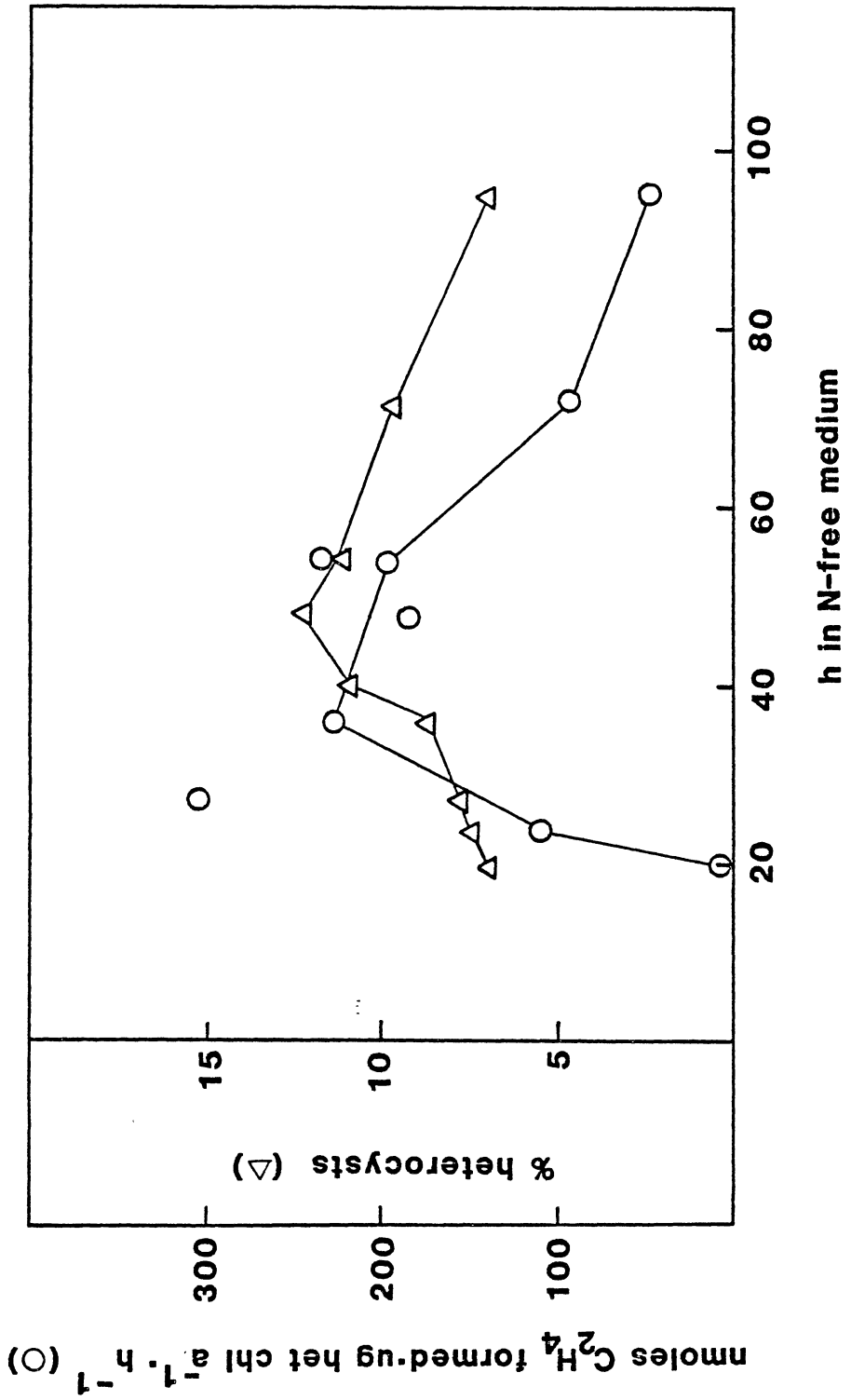

nmoles $C_2H_4$ formed.ug het chl $a^{-1}$. $h^{-1}$ (○)
300
200
100
% heterocysts (△)
15
10
5
20
40
60
80
100
h in N-free medium

Fig. 2. Incorporation of [$^3$H]glycine (5 uCi/ml) into trichloroacetic acid-precipitable material by isolated heterocysts. The time course of incorporation was measured in a 1:1 Ar:$H_2$ atmosphere in the light (○), dark (●), or in the light with 5 ug rifampicin/ml (▲), 50 ug proflavin/ml (□), 50 ug chloramphenicol/ml (△) added at t=0.

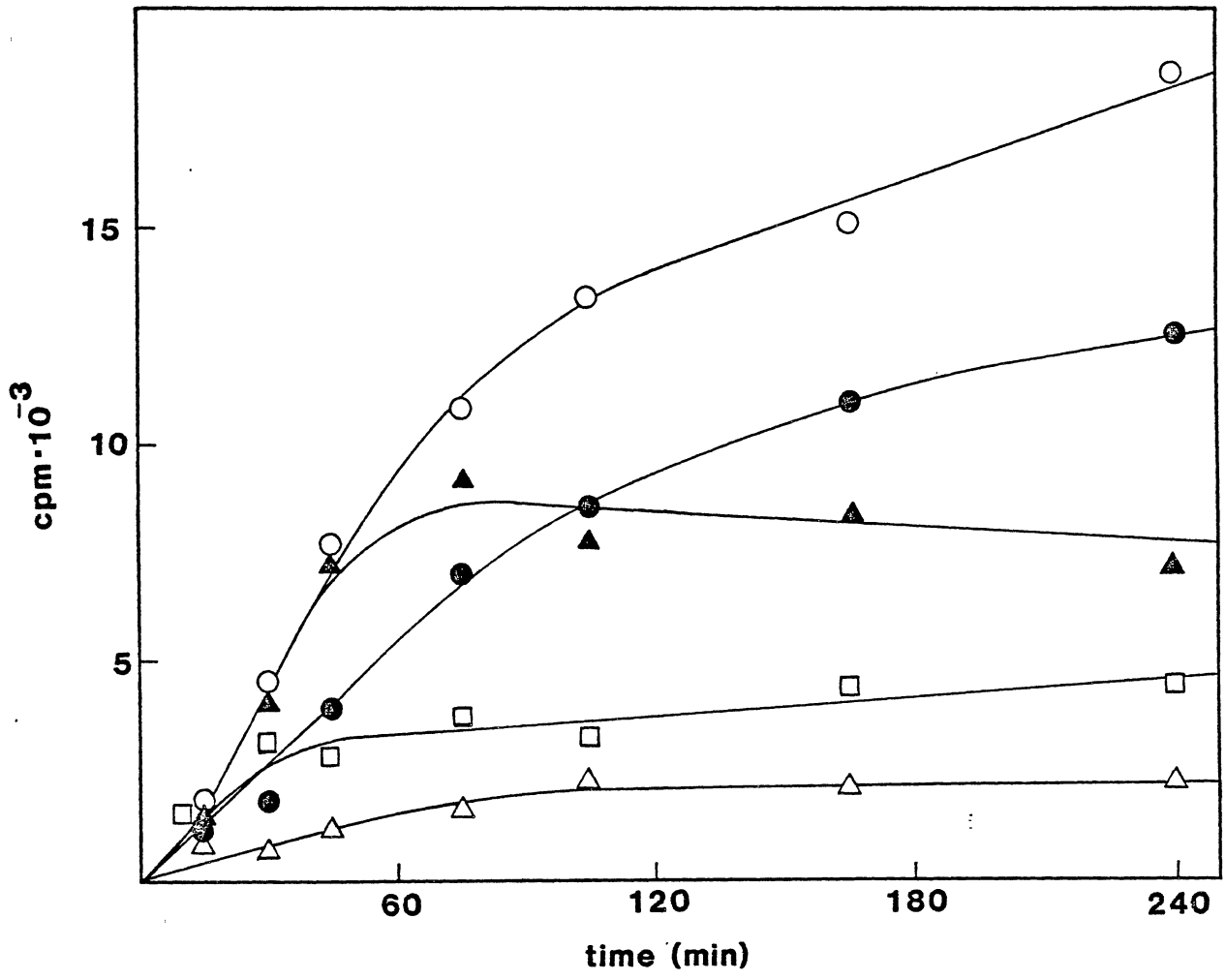

15
10
5
cpm·10⁻³
60
120
180
240
time (min)

Fig. 3. Spectrophotometric scan (solid line) and count profile (dashed line) of total nucleic acids (13 ug) from intact heterocysts labeled for 4 h with 0.125 uCi [$^{3}$H]uracil/ml and isolated as described.

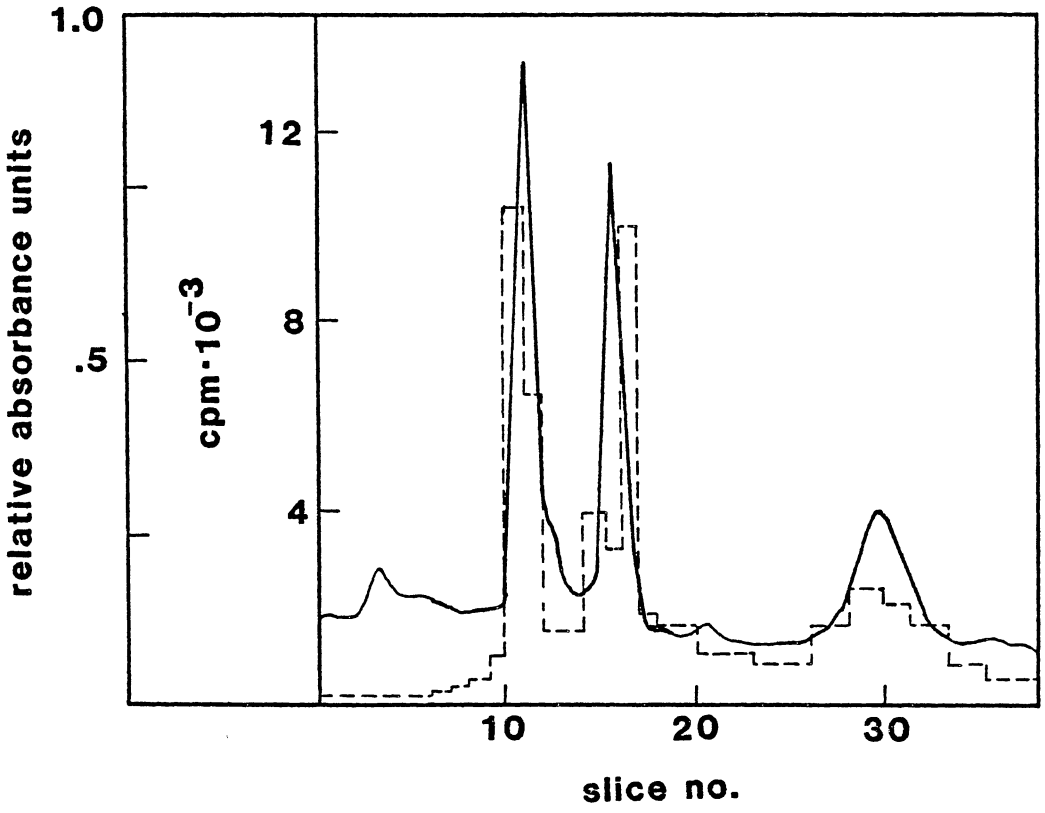
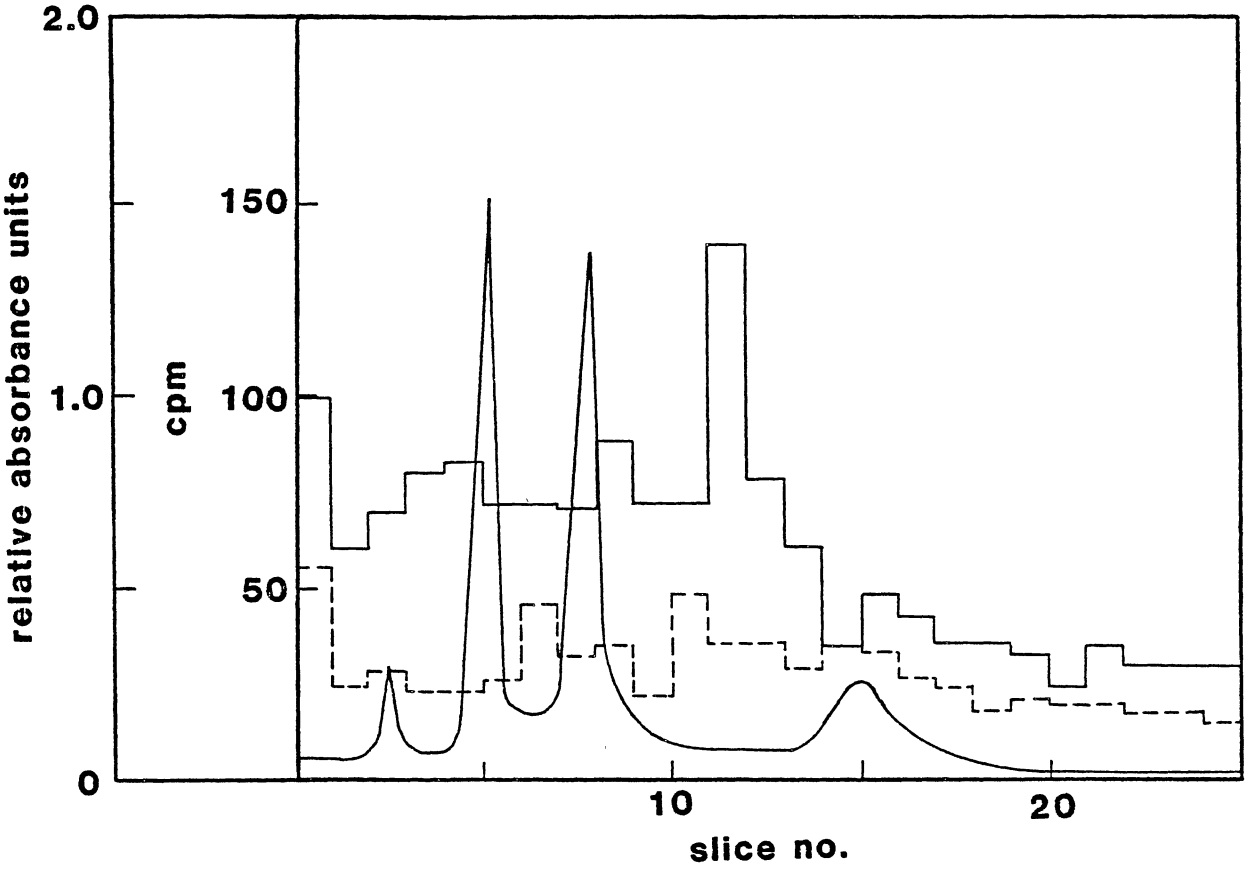

1.0
.5
relative absorbance units
12
8
4
cpm·10⁻³
10
20
30
slice no.

Fig. 4. Spectrophotometric scan and count profile of total nucleic acids (34 ug) from isolated heterocysts labeled for 30 min with 3.75 uCi [$^{3}$H]uracil/ml (solid bar graph) and chased for 2 h with 1 mM cold uracil (dashed bar graph). Spectrophotometric scans were essentially identical for both cases; only pulse-labeled scan is presented.


2.0
1.0
0
relative absorbance units
150
100
50
cpm
10
20
slice no.

# VITA

Melanie Elizabeth Lynn

Candidate for the Degree of

Doctor of Philosophy

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