CHARACTERIZATION OF FACTORS CONTROLLING THE FORMATION OF AKINETES IN THE CYANOBACTERIUM, *CYLINDROSPERMUM LICHENIFORME* KÜTZ.

By

Takayasu Hirosawa

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1978

ABSTRACT

CHARACTERIZATION OF FACTORS CONTROLLING THE FORMATION OF AKINETES IN THE CYANOBACTERIUM, *CYLINDROSPERMUM LICHENIFORME* KÜTZ.

By

Takayasu Hirosawa

Substances which stimulate the formation of akinetes (spores) in *Cylindrospermum licheniforme* Kütz. are secreted into a phosphate-free sporulation medium by filaments of that cyanobacterium. One such substance, which is able to initiate sporulation in a phosphate-containing culture medium, was purified from the centrifugal supernatant fluid of sporulating cultures. High resolution mass spectrometry showed that the chemical formula of the substance is C_7H_5OSN. Other peaks of high intensity found at m/e = 123 and m/e = 96 in the mass spectrum were produced by loss of CO from the molecular ion, and by additional loss of HCN. Proton nuclear magnetic resonance spectroscopy of the substance showed a complex of peaks in the region of δ = 7.19 to δ = 7.29 ppm. Peaks in the infrared absorption spectrum were attributable to methylene C-H bonds and

to C=S and cyclic —C(=O)-N(H)— groups. The most probable structure consistent with the above findings embodies two fused, five-membered rings, one of which is a lactam and the other of which has a thioketone group.

The addition of 12.5% H_2 stimulated sporulation in air or under CO_2:O_2:Ar (0.1:19.9:80, v/v) by up to 2.5-fold. The same concentration of hydrogen did not significantly affect the reduction of acetylene by intact filaments. These data indicate that the stimulation of sporulation by hydrogen was not mediated by an effect upon the fixation of nitrogen. Hydrogen uptake in a cell-free suspension derived from whole filaments was detected manometrically with phenazine methosulfate as electron acceptor, at a rate of 5.8 μmoles H_2 (mg chlorophyll a)$^{-1}h^{-1}$. The uptake hydrogenase activity derived from isolated heterocysts accounted for 84 ± 2% of the uptake hydrogenase activity from whole filaments, on a per heterocyst basis, whereas no activity was detected in a fraction derived from the vegetative cells.

The formation of the pattern consisting of spores contiguous with heterocysts may be controlled by either, or a combination of, (i) a sporulation-stimulatory substance, if that substance is synthesized solely in heterocysts, or (ii) some substance which is reduced by the uptake hydrogenase in heterocysts.

ACKNOWLEDGMENTS

The author wishes to express his sincere gratitude to his major professor, Dr. C. Peter Wolk, for his patience and guidance throughout these investigations. The author also wishes to extend his gratitude to Dr. Philip Filner, Dr. Michael Jost, Dr. Kenneth Poff, and Dr. Harold L. Sadoff for their participation in guidance committee.

The assistance of Mr. Bernd Soltmann with the mass spectrometer and of Dr. Frank J. Bennis with the NMR spectrometer is warmly acknowledged.

The author wishes to thank all of the members of the PRL community, whom I cannot name individually, for friendship and assistance; Dr. Thomas Currier for his correction of the English of this thesis; and Dr. Richard B. Peterson for his assistance during assays of hydrogenase.

The continuous encouragement by Dr. Masayuki Takeuchi and Dr. Hisashi Matsushima is gratefully appreciated.

Finally the author wishes to thank his wife Reiko for her continuous moral support throughout these years.

This work was supported by the U.S. Department of Energy under Contract EY-76-C-02-1338.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

AA/8	Medium of Allen and Arnon (1955), diluted eight-fold
c-AMP	Adenosine 3':5'-cyclic monophosphoric acid
Chl	Chlorophyll *a*
DCPIP	2,6-Dichlorophenolindophenol
Dibut-c-AMP	$N^6,O^{2'}$-Dibutyryl adenosine 3':5'-cyclic monophosphoric acid
Dibut-c-GMP	$N^2,O^{2'}$-Dibutyryl guanosine 3':5'-cyclic monophosphoric acid
Na_2EDTA	(Ethylenedinitrilo) tetraacetic acid, disodium salt
HEPES	N-2-Hydroxyethylpiperazine-N'—2-ethane-sulfonic acid
m/e	Mass to charge ratio
NBT	Nitro blue tetrazolium chloride, or 2,2'-diphospho-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride
PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PMS	Phenazine methosulfate
SSM	Standard sporulation medium
TES	N-Tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid
δ	Chemical shift relative to tetramethylsilane

INTRODUCTION

In many multicellular organisms, patterns are formed by temporally and spatially controlled differentiation of certain cells. Intercellular communication can play an important role in the control of differentiation. In most of these organisms, there is a large and heterogeneous population of cells, interactions among which are organized in three dimensions.

In certain filamentous cyanobacteria (blue-green algae) there are three distinct types of cells: the vegetative cell, the heterocyst, and the akinete (spore). The latter two types of cells arise by differentiation of vegetative cells. The three types of cells are arrayed in one-dimensional patterns along the filaments: the heterocysts are spaced apart, whereas the akinetes form either next to the heterocysts, as in *Cylindrospermum licheniforme* Kütz., or remote from the heterocysts.

Structure and Chemical Composition of Akinetes

Mature akinetes are spherical or cylindrical cells surrounded by a thick envelope. In *C. licheniforme*, they average 20 μm in diameter and 40 μm in length, i.e., their diameter is twice, and their length is three to four times, as great as the corresponding dimensions of vegetative cells.

Akinetes retain many of the cytological and chemical features of vegetative cells. Like vegetative cells, the akinetes may contain photosynthetic thylakoids. Fay (1969b) reported that there is lower content of photosynthetic pigments, per dry weight, in akinetes than in either heterocysts or vegetative cells, in Fogg's strain of *Anabaena cylindrica* Lemm. He also reported that chlorophyll is largely replaced by pheophytin and that the carotenoid composition is different from that of vegetative cells. However, because the akinete has a thick and dense envelope, it is unclear whether the content of photosynthetic pigments differs from the corresponding values for the other two types of cells per dry weight of protoplasm. Also, no controls were presented to test whether the degradation of chlorophyll to pheophytin and the difference in carotenoids were artifacts of the extraction procedure. Wolk and Simon (1969), working with a different strain of *A. cylindrica*,

found that the *in vivo* absorption spectra of isolated akinetes and of vegetative cells were virtually superimposable. On the other hand, a complete breakdown of photosynthetic pigments is a common feature of the differentiation of akinetes in other cyanobacteria (Geitler, 1932). Whether, in the latter cyanobacteria, there is also a disappearance of the thylakoids is unknown. It has also been reported that the akinetes of Fogg's strain of *A. cylindrica* have a lower content of lipids and fatty acids than have vegetative cells, but that their lipids are more saturated than those of vegetative cells (Yamamoto, 1972). These lipids are presumably present principally in the thylakoids, although lipid granules have been reported to be present in the cytoplasm of the akinetes of other species (Miller and Lang, 1968; Clark and Jensen, 1969).

The only known qualitative difference between the structures of akinetes and of vegetative cells is the occurrence of a thick envelope surrounding the cell wall in the akinete and, seemingly of lesser significance, the apparent absence of deposits of polyphosphate in the akinete (Talpasayi, 1963; Wildon and Mercer, 1963; Leak and Wilson, 1965; Miller and Lang, 1968; Jensen and Clark, 1969). The chemical composition of a wall-plus-envelope fraction from akinetes of *A. cylindrica* was

shown to be 41% carbohydrate, 24% amino compounds, 11% lipid, 2% ash and the balance unaccounted for. The sugar composition of the carbohydrate moiety is 70% glucose, 17% mannose, 4% xylose and 3% galactose (Dunn and Wolk, 1970). Cardemil and Wolk (1976) reported that the backbone polysaccharides from both akinetes and heterocysts consist of repetitions of the same structural unit, β(1→3)-linked glucosyl-glucosyl-glucosyl-mannose. The xylose and galactose, as well as part of the mannose and part of the glucose, are present in side branches.

Cyanophycin granules, consisting of copolymers of aspartic acid and arginine, are present in akinetes, as they are in vegetative cells, but are larger and more numerous in the akinetes. Unique to the cyanobacteria, these granules probably function as a nitrogenous reserve (Simon, 1971). The relative amount of DNA per cell in akinetes, compared with the vegetative cells, is either variable or controversial. On the basis of staining with the fluorescent dye coriphosphin, Ueda (1971) and Ueda and Sawada (1971, 1972) estimated that akinetes of *Cylindrospermum* sp. contain 30-fold more DNA than do vegetative cells. Simon (1977a), however, found no great difference between direct measurements of the amount of DNA per cell in isolated akinetes and in

intact filaments (consisting mostly of vegetative cells) of *Anabaena cylindrica*. Akinetes, like vegetative cells, appear to contain glycogen (Zastrow, 1953).

Metabolic Activities of Akinetes

Akinetes are considered to be resting cells. They have been shown to be capable of germination even after five years of storage in the dark in a desiccated condition (Yamamoto, 1975). Fay (1969a) reported that isolated akinetes from *A. cylindrica* fix carbon dioxide in the light at a lower rate and evolve carbon dioxide in the dark at a higher rate than do intact filaments, per mg dry weight. Nitrogenase activity was not detected in the isolated akinetes. Whether the measured activities had been affected by the isolation procedures employed — i.e., whether these might have been the activities of germinating or immature akinetes — was not determined.

Factors Controlling the Formation of Akinetes in Cyanobacteria

Nutritional factors

Although the effects of various environmental conditions on the formation of akinetes differ from species to species, one of the most important factors appears to be the concentration of phosphate in the

medium. Generally, the formation of akinetes is promoted by the absence of phosphate (Glade, 1914; Wolk, 1964, 1965; Gentile and Maloney, 1969). Glade (1914) reported that a medium consisting of 0.05-0.1% $Ca(NO_3)_2$, 0.02% $MgSO_4$, 0.02% K_2HPO_4, and trace of iron was best for formation of akinetes in *Cylindrospermum*, after growth. He suggested that the formation of akinetes was initiated by the depletion of the components of the medium. Sucrose and calcium nitrate inhibited the formation of akinetes in *Nostoc punctiforme* (Harder, 1917). Supraoptimal concentrations of nitrate and sulfate promoted the formation of akinetes in several species of *Anabaena* (Canabaeus, 1929). The formation of akinetes by *A. cylindrica* was optimal under the following conditions: concentration of phosphate less than 50 μM, presence of a buffer (ca. 5.7 mM DL-alanyl-DL-alanine or DL-alanyl-glycine), a high concentration of acetate (25 mM), a high concentration of calcium ions (2.5 mM), and a large amount of inoculum; light intensity of 80 fc; and a temperature of 25-30 C (Wolk, 1965).

In *A. doliolum*, however, the supply of nitrogen may be more important, because nitrate, nitrite and ammonium nitrogen inhibit the formation of akinetes (Singh and Srivastava, 1968; Tyagi, 1974). Glucose (25 mM) promotes the formation of akinetes in *A. doliolum* (Tyagi, 1974).

Genetic factors

Singh and Sinha (1965), working with *Cylindrospermum*, have reported that genetic recombination between a streptomycin-resistant mutant unable to form akinetes and a penicillin-resistant mutant was able to form akinetes resulted in a strain resistant to both antibiotics that was, in addition, able to form akinetes at low frequency. Singh (1967) reported that two distinct mutants of *A. doliolum* which were incapable of forming akinetes could recombine to form a strain which could form akinetes capable of germination. The author concluded that the formation of akinetes in that organism is under the control of more than one genetic determinant.

Extracellular products

Cyanobacteria secrete biologically active substances into culture media. Harder (1917) first described auto-inhibition of growth in an old culture of *Nostoc punctiforme*. Because addition of new salts and sugar did not reverse the inhibition, he suggested that the inhibition is due to a growth-inhibitory metabolite accumulated in the medium. Certain of the secreted substances inhibit the growth of other organisms (Prescott, 1960; Hartman, 1960). Jakob (1961) reported that it is a dihydroxyanthroquinone secreted by *Nostoc muscorum* which is responsible for inhibition of the

growth of *Cosmarium*, *Phormidium* and *Euglena*. Toxins from *Aphanizomenon flos-aquae* show chemical characteristics similar to those of the toxin from the marine dinoflagellate *Gonyaulax catenella* (Jackim and Gentile, 1968).

On the other hand, some of the secreted materials exert growth-stimulatory effects. Polypeptides secreted by *A. cylindrica* complex with metal ions (Fogg, 1952; Walsby, 1974a), reducing the toxicity of — in particular — copper. A substance with a high affinity for iron has been isolated from a culture filtrate of *Anabaena* sp. grown in iron-containing medium (Simpson and Neilands, 1976). In addition, the non-dialyzable extracellular products of *A. cylindrica* decrease the toxic effect of polymixin B against *A. cylindrica* and *Anacystis nidulans*. The same material showed no effect upon the formation of akinetes by the *Anabaena* or upon the uptake of phosphate by either of the organisms (Whitton, 1965, 1967).

Fisher and Wolk (1976) reported that the culture filtrate from an akinete-forming culture of *Cylindrospermum licheniforme* in phosphate-free standard sporulation medium (SSM) stimulates the formation of akinetes in a fresh inoculum of the cynobacterium, without affecting the growth of the organism. Supplementation of the culture filtrate with all of the

constituents of SSM did not affect the stimulation by the filtrate. They therefore concluded that the stimulation of sporulation by the culture filtrate was due not to depletion of the medium, but to a substance or substances released into the medium.

Heterocysts

The participation of heterocysts in the formation of akinetes has long been suggested for those instances in which there is a close spatial relationship between the two types of cells. Carter (1856), having recognized the relationship, first suggested that heterocysts were supplying substances to neighboring, developing akinetes. Other researchers have subsequently made similar suggestions (Fritsch, 1904, 1951; Bharadwaja, 1933; Wolk, 1965). Wolk (1966) provided the first experimental evidence in support of the idea that heterocysts play a role in the sporulation of contiguous vegetative cells. Having separated heterocysts from neighboring vegetative cells by gentle agitation, he observed that the vegetative cells detached from heterocysts failed to differentiate into akinetes. Although the detached vegetative cells appeared undamaged, it could not be excluded that the detachment procedure had — in fact — damaged the vegetative cells, and had thereby prevented sporulation.

Knowledge of the metabolic activities of heterocysts has led to elucidation of certain interactions between heterocysts and vegetative cells. Fay *et al.* (1968) proposed that heterocysts are sites of nitrogen fixation. Use of ^{15}N had provided no evidence in favor of nitrogen fixation by isolated heterocysts (Fay and Walsby, 1966), but subsequent work (Fay *et al.*, 1968) showed that nitrogenase activity had been lost completely during the initial stages of the isolation procedure employed. Using the more sensitive acetylene reduction technique, Stewart *et al.* (1969) demonstrated, nitrogenase activity in heterocysts in the presence of ATP and sodium dithionite. Wolk and Wojciuch (1971) interpreted certain kinetic experiments as implying the occurrence of light-dependent nitrogenase activity in heterocysts. In recent reports, 10 to 40% of the nitrogenase activity of intact filaments of *Anabaena* 7120 and *A. cylindrica*, and 60% of the nitrogenase activity of intact *Anabaena variabilis*, could be recovered in isolated heterocysts (Peterson and Burris, 1976; Thomas *et al.*, 1977; Peterson and Wolk, 1978b). Moreover, an average of 91% of the MoFe-protein and 70% of the more oxygen-labile Fe-protein of nitrogenase of the intact filaments could be recovered in the heterocysts isolated from *A. variabilis* (Peterson and Wolk, 1978b). Thus,

although it appears clear that nitrogenase can be present in vegetative cells under anaerobic or microaerobic conditions (Stewart and Lex, 1970; Rippka *et al.*, 1971; Rippka and Stanier, 1978), heterocysts are the major, and very possibly the sole, sites of nitrogen fixation by heterocyst-forming species under aerobic conditions. The initial pathways of assimilation of fixed nitrogen by nitrogen-fixing cyanobacteria have recently been elucidated (Wolk *et al.*, 1976; Meeks *et al.*, 1977, 1978). Fixed nitrogen is assimilated by glutamine synthetase into the amide group of glutamine; the amide group is then transferred to α-ketoglutarate by glutamate synthase, to form the α-amino group of glutamate. Glutamine synthetase is present in both heterocysts and vegetative cells, although at slightly higher specific activity in heterocysts (Dharmawardene *et al.*, 1973; Thomas *et al.*, 1977). However, the kinetics of solubilization of glutamate synthase indicate that little, if any, of that enzyme is located in heterocysts (Thomas *et al.*, 1977). These results suggest that nitrogen fixed in heterocysts leaves the heterocysts (Wolk *et al.*, 1974) as glutamine, and that some of the glutamate formed by glutamate synthase is transported back to the heterocysts.

Heterocysts lack two major photosynthetic functions. Fay (1969b) and Wolk and Simon (1969) have shown that heterocysts of *A. cylindrica* contain very little or no phycocyanin, a pigment characteristic of photosystem II. Microspectrophotometric analysis of individual vegetative cells and heterocysts confirmed this result for heterocysts *in situ* in *Anabaena* sp. (Thomas, 1970). A relatively high ratio of P700 to chlorophyll, low fluorescence by chlorophyll *a*, a low intensity of emission of delayed light, and lack of a Hill reaction (Donze *et al.*, 1972), further suggested that heterocysts lack photosystem II. The absence of photosystem II from heterocysts was further confirmed by the lack of O_2 evolution by heterocysts isolated from *A. cylindrica* (Bradley and Carr, 1971; Tel-Or and Stewart, 1977). The suggestion by Fay and Walsby (1966) that there is a deficiency of CO_2-fixing activity in heterocysts was confirmed by autoradiography (Wolk, 1968) and by the demonstration (Winkenbach and Wolk, 1973; Stewart and Codd, 1975) that ribulose-1,5-bisphosphate carboxylase is absent from heterocysts. Because of these deficiencies, heterocysts have to depend on vegetative cells for the electron donors and carbon skeletons necessary for nitrogen fixation. In fact, Wolk (1968) showed by autoradiography that part of the carbon fixed by photosynthesis in vegetative cells moves into heterocysts.

Jüttner and Carr (1976) concluded from pulse-labeling experiments with $H^{14}CO_3^-$ that carbon enters the heterocysts of *A. cylindrica* principally as maltose, whereas work of this laboratory (unpublished observations of Schilling and Wolk cited by Wolk, 1979) has been interpreted as suggesting that sucrose and glutamate are the major carbon compounds entering heterocysts of *A. variabilis*.

High "reducing activity" is also characteristic of heterocysts. Cytochemical studies showed that heterocysts reduce 2,3,5-triphenyl tetrazolium chloride more rapidly than do vegetative cells (Drews, 1955; Drawert and Tischer, 1956; Talpasayi and Bahal, 1967; Stewart *et al.*, 1969; Fay and Kulasooriya, 1972). Reductant can be generated in heterocysts by dehydrogenases: heterocysts exhibit at least seven-fold higher specific activity of glucose-6-phosphate dehydrogenase and of 6-phosphogluconate dehydrogenase, and two-fold higher specific activity of hexokinase, than do vegetative cells. Little or no activity of enzymes of the glycolytic pathway was detected (Winkenbach and Wolk, 1973; Lex and Carr, 1974). Thus, reducing power necessary for nitrogen fixation in heterocysts can be produced by oxidative metabolism of carbon compounds synthesized in vegetative cells (Bothe, 1970; Smith, Noy and Evans, 1971; Apte *et al.*, 1978; Lockau *et al.*, 1978).

Peterson and Wolk (1978a) showed that uptake hydrogenase activity is localized solely in heterocysts in aerobically grown *Anabaena* 7120. By reassimilating hydrogen released during nitrogen fixation, heterocysts may conserve reducing equivalents.

MATERIALS AND METHODS

Culture Conditions

Stock cultures of *Cylindrospermum licheniforme* Kütz. (ATCC 29412) were grown axenically in 50 ml of an eight-fold dilution (AA/8) of Allen and Arnon's medium (Allen and Arnon, 1955) in 125-ml Erlenmeyer flasks. The cultures were grown on a reciprocating shaker (107 RPM) in continuous light (2.0×10^4 erg $cm^{-2}s^{-1}$) from cool white fluorescent lamps (Sylvania, Winchester, KY) at 26 $\pm$ 1 C, and were subcultured 1% (v/v) weekly in order to prevent the formation of akinetes. Phosphate-free standard sporulation medium (SSM) was prepared as described previously (Wolk, 1965) except that N-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid (TES) was used as buffer in place of DL-alanyl-glycine, and the pH was adjusted to 7.5. For isolation of substances for chemical analysis, stock cultures were inoculated into five-gallon glass carboys containing 16 liters of SSM, and were aerated with compressed air and exposed to continuous light (8.8×10^4 erg $cm^{-2}s^{-1}$) from cool white fluorescent lamps

at 25 ± 2 C. For studies of hydrogenase, the carboys contained AA/8.

Bioassay of Substances Inducing the Formation of Akinetes

Bioassays were performed under the same conditions of light, temperature and agitation as described for the growth of stock cultures. An assay mixture consisted of 4 ml of the material being tested, dissolved in SSM, plus 1 ml of a suspension of filaments of *C. licheniforme* in SSM, in a 50-ml Erlenmeyer flask. The pH of the test solution was adjusted to 7.5, and the solution was sterilized by filtration through a 0.22-μm pore-size membrane filter (Millipore Corp., Bedford, MA). The cells to be resuspended in SSM for bioassay were collected from one week old stock cultures by centrifugation. The initial concentration of chlorophyll *a* (Chl) in an assay mixture containing cyanobacteria was 0.11 μg Chl/ml. After three and one half to four days, the numbers of akinetes attached to a sample of 200 heterocysts were counted. A cell was considered to be a heterocyst if it was present at a terminal position on a filament, had a thick envelope, and contained a polar granule (Clark and Jensen, 1969). Cells were considered to be akinetes only if they were at least twice as long as a normal vegetative cell and were wider than

heterocysts (Simon, 1977b).

When bioassays were conducted under a variety of gas phases, 20 ml of cell suspension in SSM (0.11 µg Chl/ml) were placed in 250-ml filter flasks (effective volume, 280 ml) modified as follows. The side arm of a flask was sealed with a rubber serum stopper. The mouth of the flask was fitted with a rubber stopper penetrated by glass tubing (1.5 mm i.d.) which reached the bottom of the flask. The glass tubing was connected via silicon rubber tubing to a 0.22-µm pore-size Millipore filter. The flask was then flushed with one gas or a mixture of gases via the Millipore filter and tubing, with gas efflux through a hypodermic needle inserted into the serum stopper in the side arm, needle then removed and the tubing clamped shut. Measured portions of other gases, sterilized with a Millipore filter, were then injected through the side arm with a gas-tight syringe. Excess pressure was released with the injecting syringe.

Chemical Fractionation

a) Gel filtration. Gel filtration was performed on a 2.5 cm i.d. x 40 cm long column of Sephadex G-25, fine (Pharmacia Fine Chemicals, Piscataway, NJ). The column was eluted with water at a flow rate of 0.5 ml/min. The void volume was 87 ml. Twenty-four 10-ml fractions were collected.

b) Charcoal-celite column fractionation. Activated charcoal (Darco G-60) was cleaned as follows. It was boiled in 20% acetic acid for ten minutes, and then washed extensively with (i) hot (95 C) double distilled water, (ii) 50% glass-distilled ethanol containing 2% NH_4OH, and (iii) distilled water. The charcoal was then dried at 120 C, and kept at 120 C until used. Celite 545 was cleaned in the same way except that it was boiled with 6 N HCl rather than with 20% acetic acid.

Charcoal-celite column fractionation was performed on a 0.9-cm long glass column packed with a 1:2 (w/w) mixture of charcoal and celite. The charcoal and celite were mixed in an Erlenmeyer flask by vigorous shaking. Samples were passed through the column at a flow rate of 100 ml/hr. The adsorbed materials were eluted first with 50% glass-distilled ethanol and then with 50% ethanol containing 2% NH_4OH.

c) Paper chromatography. Descending paper chromatography was performed on Whatman No. 1 filter paper (20 cm x 20 cm) in a solvent system of isopropanol: 58% NH_4OH:water (9:1:1, v/v).

d) Silica gel thin layer chromatography. Silica gel thin layer chromatography was performed on plates (E. Merck, Darmstadt, W. Germany) pre-coated with Silica

gel 60F-254 (which contains a fluorescent indicator) to a thickness of 0.25 mm, in a solvent system consisting of n-butanol:acetic acid:ether:water (9:6:3:1, v/v; Piskornik and Bandurski, 1972).

e) Cellulose thin layer chromatography. Cellulose thin layer chromatography was performed on plates pre-coated with a layer of cellulose, 0.25 mm thick (Analtech, Inc., Willmington, DE), in a solvent system consisting of isopropanol:58% NH_4OH:water (9:1:1, v/v).

f) Microdistillation. Microscale vacuum distillation was performed according to R. Roper and T. S. Ma (1957) with modifications. A methanolic solution of the sample was introduced into the lower chamber of an L-shaped glass tube. After evaporation of the solvent, the end of the tube containing the sample was immersed in mineral oil which had been placed in a well of a heating block. The distillate was collected in a U-shaped tube immersed in an ethanol-dry ice bath connected to a vacuum pump. A vacuum of 30 to 40 μm of Hg was maintained during the distillation.

g) Mass spectroscopy. Mass spectroscopic studies were performed with a CH5-direct probe mass spectrometer (Varian MAT, Bremen, W. Germany) with electron impact ion source. This instrument was interfaced to a Digital

Equipment Company PDP-11/40 computer. Exact mass measurements were made by the peak matching technique (Quisenberg *et al.*, 1956), with perfluoroalkanes as a reference.

h) Nuclear magnetic resonance (NMR) spectroscopy. NMR spectra were taken with a Bruker Model WH-180S Fourier-transform spectrometer (Bruker Spectrospin, Wissenbourg, France). The samples were dissolved in [^{2}H]acetonitrile.

i) Infrared (IR) and ultraviolet spectroscopy. IR spectra were taken with a Perkin-Elmer model 621 grating infrared spectrophotometer (Perkin-Elmer Corp., Norwalk, CT). A sample-containing solution in glass-distilled acetone was mixed with KBr in a mortar by drop-wise addition of the solution to the salt. The mixture was then evacuated overnight in a desiccator. Discs were made in an evacuable KBr die (Perkin-Elmer Corp.) pressed with a Carver hydraulic press (F. S. Carver, Inc., Summit, NJ). Ultraviolet absorption spectra were taken with a DB-G spectrophotometer (Beckman Instruments, Inc., Irvine, CA).

Assay for Hydrogenase

Hydrogen uptake was measured manometrically at 30 C using a Gilson differential respirometer (Gilson Medical Electronics, Middleton, WI). One and eight tenths ml of test sample, 0.2 ml of 100 mM electron acceptor, and

0.1 ml of 5 N KOH were placed, respectively, in the side arm, main chamber, and center well of a Warburg flask filled with hydrogen.

Isolation of Heterocysts

Heterocysts were isolated by the method of Peterson and Wolk (1978b). Cells harvested from five gallon carboys of AA/8 by centrifugation at 17,300 x g (RC-2B centrifuge equipped with an SS-34 rotor and a continuous flow attachment: Dupont-Sorvall Inc., Norwalk, CT) were resuspended in approximately 30 ml of medium. The suspension, placed in a polyethylene centrifuge tube fitted with a rubber serum stopper, was twice evacuated to 50 μm of Hg and refilled to 1 atm with H_2. All subsequent operations were performed under H_2, except that the buffers were thoroughly sparged with N_2. Cells were centrifuged (500 x g, 10 min), resuspended in HP buffer containing 10 mM HEPES, 10 mM PIPES, 1 mM $MgCl_2$, and 1 mM cysteine, centrifuged again as before, and then resuspended in 40 ml of the same buffer to which had been added 10 mM Na_2EDTA and 1.0 mg lysozyme/ml. The suspension was shaken at 200 rpm in a reciprocating water bath at 30 C for 30 minutes. The suspension was then centrifuged at 500 x g for five minutes, and the pellet resuspended in 30 ml of HP buffer. The

suspension was transferred to a stoppered 125-ml Erlenmeyer flask and was cavitated in a sonic cleaning bath at 12 to 13 C for 20 to 30 minutes until the ratio of vegetative cells to heterocysts decreased to 0.02. Heterocysts were sedimented at 500 x g for five minutes, and the supernatant fluid was collected as a vegetative cell fraction. The heterocyst-containing pellet was washed twice with HP buffer. The isolated heterocysts and intact filaments were resuspended in HP buffer and were totally disrupted with a Ribi press (Dupont Sorvall Inc.) at a pressure of 20,000 psi.

Chemicals

Celite 545 was obtained from Fisher Scientific Co. (Pittsburgh, PA); Darco G-60 activated charcoal from Sargent-Welch (Skokie, IL); PMS from Calbiochem (Los Angeles, CA); calcium glucuronate from Mann Research Laboratories, Inc. (New York, NY); isopropanol, diethyl ether, formic acid, and 58% NH_4OH from Mallinkrodt (St. Louis, MO); n-butanol from Aldrich Chemicals Co. (Milwaukee, WI); and glass-distilled methanol from Burdick and Jackson Lab., Inc.(Muskegon, MI). All gases were obtained from Matheson Gas Products (East Rutherford, NJ). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Solvents were always redistilled before use.

RESULTS

PART I. Isolation and Characterization of a Substance which Stimulates the Formation of Akinetes in *C. licheniforme*

Production of factors stimulating the formation of akinetes

As is illustrated in Fig. 1, *C. licheniforme*, inoculated from a stock culture into SSM, grows actively for 12 days. Akinetes are produced during the period of from four to 12 days. After 12 days, more than 80% of the heterocysts are accompanied by akinetes. After the 14th day, the filaments fragment and the heterocysts become detached from the filaments. Portions of supernatant fluid taken every other day from the above culture were lyophilized and extracted with methanol, and the methanolic extracts dried, redissolved in SSM, and bioassayed. Figure 2 shows the percentage of heterocysts with contiguous akinetes after four days of bioassay. A stimulation of the formation of akinetes is first demonstrable using the methanolic extract of the supernatant from a two day old culture, and then increases monotonically for ten days.

Figure 1. Growth of *Cylindrospermum licheniforme* and formation of akinetes, in SSM. Growth is expressed as µg Chl/ml. Chl content (———) was determined by measurement of the optical density at 665 nm of methanolic extracts of *C. licheniforme* (Mackinney, 1941). The formation of akinetes (-----) is expressed as the percent of heterocysts with a contiguous akinete. One hundred ml of suspension were sampled from a one-liter culture every other day.

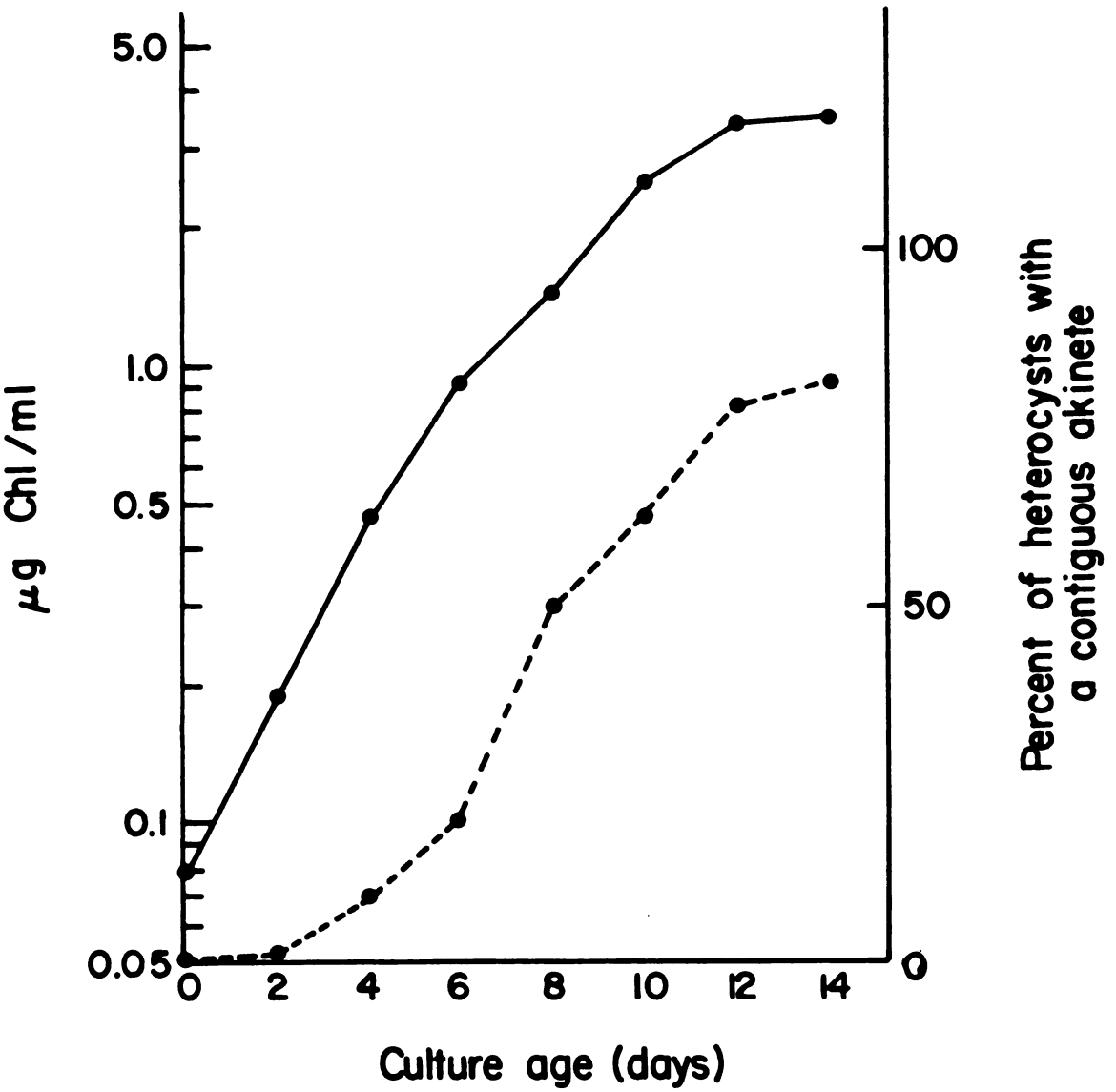

5.0
1.0
0.5
0.1
0.05
µg Chl/ml
0
2
4
6
8
10
12
14
Culture age (days)
100
50
0
Percent of heterocysts with a contiguous akinete

Figure 1

Figure 2. Time course of production of material stimulating the formation of akinetes, as measured by bioassay of its stimulatory activity. Supernatant liquids from the culture of Fig. 1 were lyophilized and extracted with methanol, and the methanolic extracts dried and redissolved in SSM. The activities are expressed as the percent of heterocysts with contiguous akinetes after four days of incubation. The concentrations of solids in the suspension used for bioassay of the culture filtrate are shown, relative to their concentration in the original filtrate: x2, x1, x½. The control value (3.5% heterocysts with a contiguous akinete) obtained with 0 x filtrate, i.e., with SSM, have been subtracted from the data as presented.

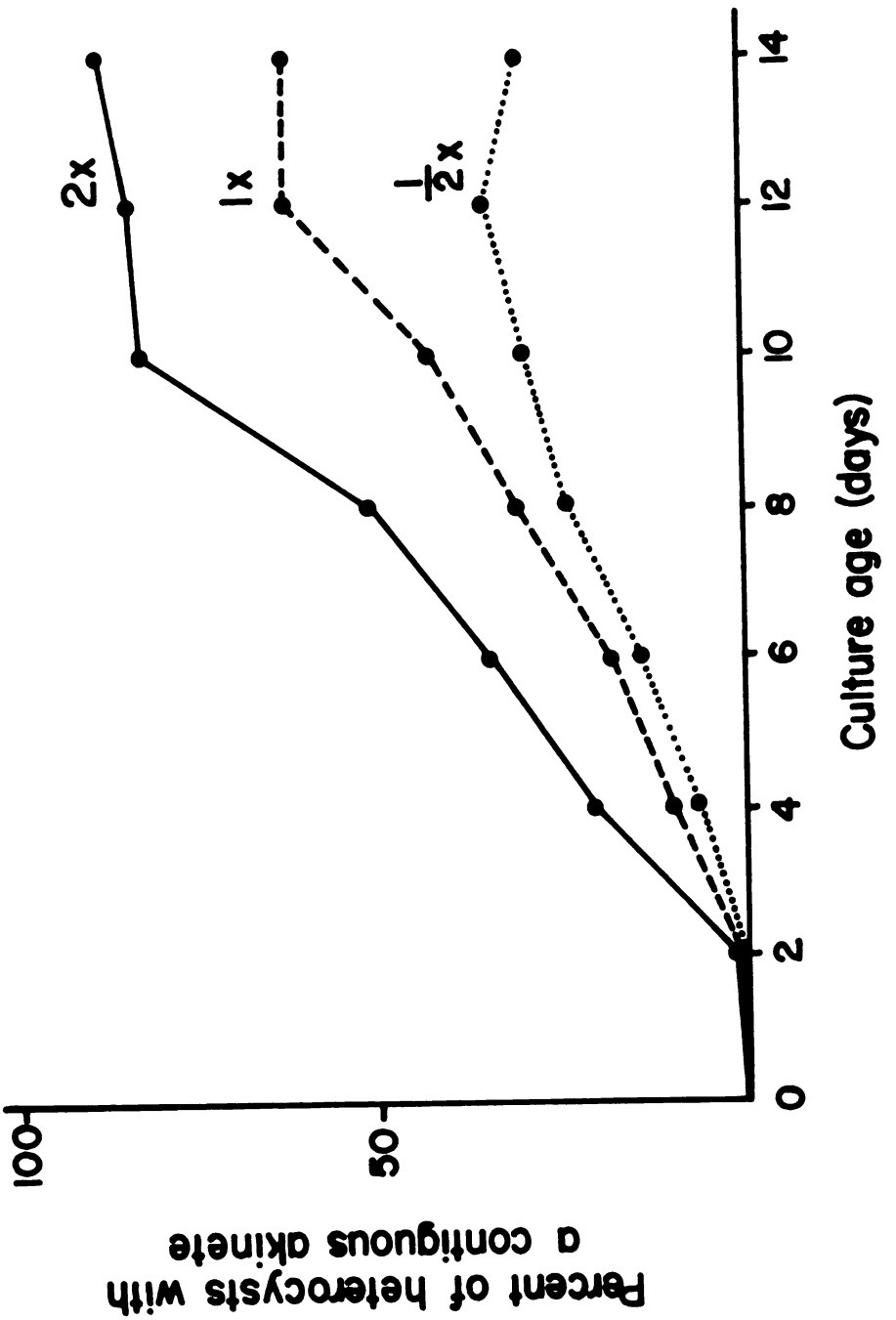

Percent of heterocysts with
a contiguous akinete
100
50
0
2
4
6
8
10
12
14
Culture age (days)
2x
1x
1/2x

Figure 2

Gel filtration

After Sephadex G-25 gel filtration of the supernatant fluid from a 12 day old culture in SSM, most of the activity (capacity to stimulate the formation of akinetes) was eluted from the column between 140 and 200 ml (Fig. 3), i.e., within the internal volume of the gel. This suggests that the molecular weight of the stimulatory factor is less than 5000 D.

Extract with methanol

Cell-free supernatant fluids from 12 day old 16-liter batch cultures in SSM were obtained by centrifugation at 17,300 x g with a Sorvall SS-34 rotor equipped with a continuous flow attachment. Because an oily substance was present in the supernatant, the supernatant could not be evaporated completely to a powder. The supernatant dired in a Büchi rotary evaporator (Flawil, Switzerland) at 55 C, was extracted three times with 800 ml of absolute methanol. The insoluble residue was removed by filtration through Whatman No. 1 filter paper and was reextracted with an additional 500 to 600 ml of absolute methanol. The residue was denoted the methanol-insoluble fraction. The methanol-soluble material was concentrated approximately five-fold in a rotary evaporator at 35 C. During this condensation a large amount of white

Figure 3. Gel filtration, on a column (2.5 cm i.d. x 40 cm long) of Sephadex G-25, of 20 ml of a culture filtrate concentrated 25-fold. Each 20 ml fraction in the eluate was diluted 25-fold, and its sporulation-stimulatory activity assayed.

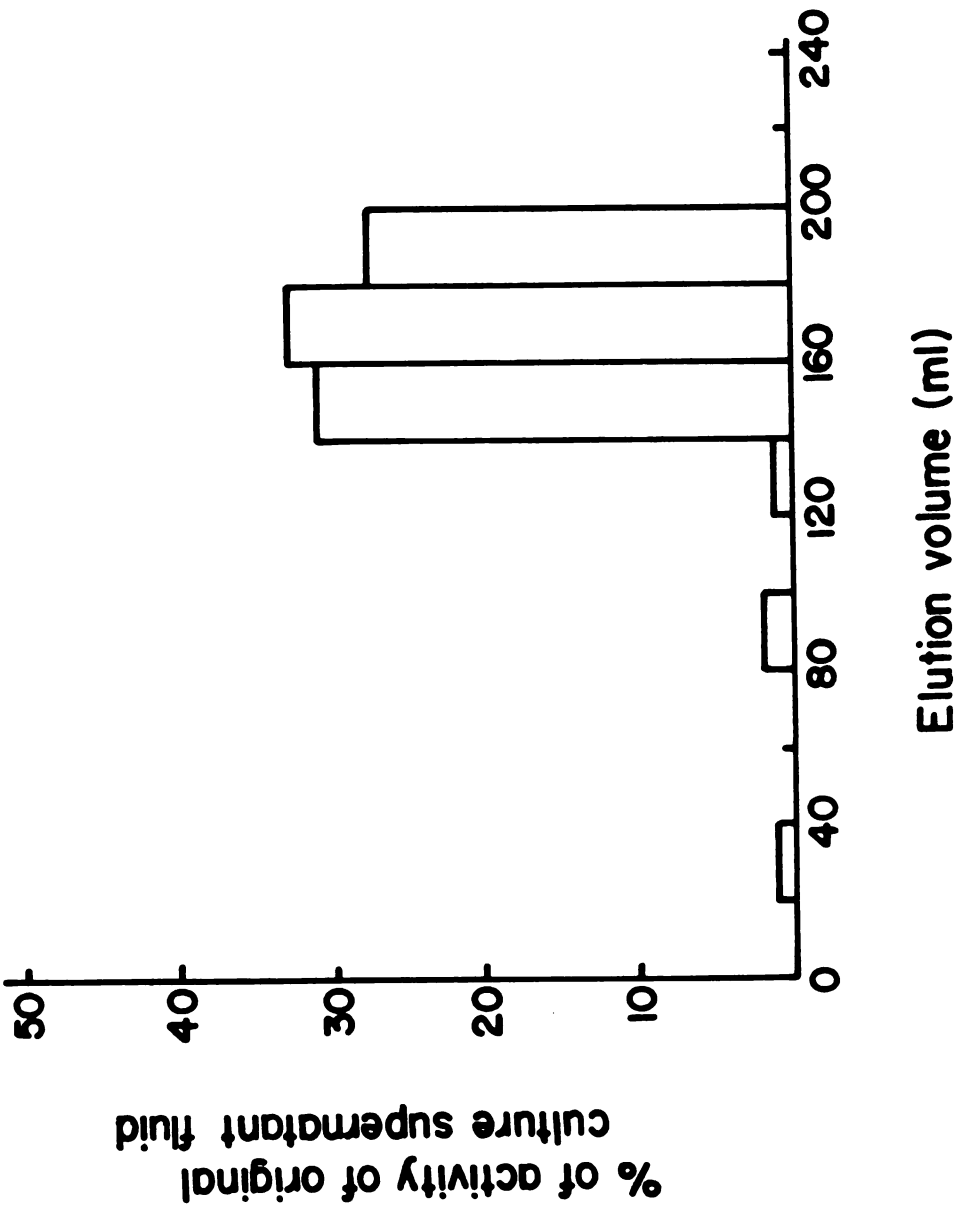

% of activity of original
culture supernatant fluid
50
40
30
20
10
0
40
80
120
160
200
240
Elution volume (ml)

Figure 3

material precipated. The precipitate was removed by centrifugation at 20,000 x g for 20 min, and the pellet was washed once with a small volume of absolute methanol. The combined supernatants were stored in the freezer (-20 C) overnight. A precipitate appeared during this cold treatment, and was removed by centrifugation at 20,000 x g for 20 min. The pellet, combined with that from the previous step and dried under a current of air, was designated the methanol-precipitate fraction. The final centrifugal supernatant fluid was evaporated to dryness at 35 C with a rotary evaporator. The dried methanol-soluble materials were redissolved in 160 ml of double distilled water and stored as necessary. This fraction was designated the methanolic extract fraction. Table I shows the results of bioassay of the above fractions. The methanol-soluble fraction shows 75 to 82% of the original sporulation-stimulatory activity of the culture supernatant. Only three to 12% of the original activity is seen either in the methanol-insoluble fraction or in the methanol-precipitate fraction (Table I).

Stability

The methanolic extract can be stored in a refrigerator at 4 C for months without loss of activity.

Table I. Biological activities of the fractions derived by extraction, with methanol, of a dried culture supernatant fluid. The methanol precipitate appeared during condensation and cold treatment of the methanolic extract. Each value presented is the percent of heterocysts with contiguous akinetes, out of 200 heterocysts surveyed.

	0x	½x[a]	1x	2x	3x
Control	3.5				
Original supernatant fluid		38.0	72.3	——[b]	——
Methanol-insoluble		7.5	5.5	3.5	3.0
Methanol-precipitate		6.0	9.0	5.5	2.0
Methanol-soluble		29.5	60.0	——	——

[a]Concentration of the active substance, relative to its concentration in the original supernatant fluid, based on the assumption that all of the substance is present in the fraction being assayed.

[b]Extensive fragmentation of filaments occurred in these flasks.

Eighty percent or more (see Fisher and Wolk, 1976) of the original activity survives autoclaving at 121 C for 20 min. Ninety-four percent of the original activity was lost upon treatment with 6 N HCl at 110 C for 24 hr.

Charcoal adsorption chromatography

A major disadvantage of charcoal chromatography is that solutes may be bound irreversibly to the charcoal. In order to minimize the loss by irreversible binding, the minimum amount of charcoal required to remove the sporulation-stimulatory substances from the culture supernatant was determined. Culture supernatant fluid concentrated 40-fold was incubated with different amounts of charcoal for 15 min, and the stimulatory activity remaining in the filtrates was assayed (Table II). With a concentration of 13 µg of charcoal per ml of the original supernatant, 82 to 85% of the original activity was removed from the supernatant. Figure 4 shows the UV absorption spectra of the same samples. A decrease in the absorption between 270 nm and 290 nm coincides with the loss of activity. The concentrated methanolic extract was diluted with distilled water to from 0.04 to 0.1 of the volume of the original culture supernatant and passed through the column at a flow rate of 100 ml/hr. It was necessary

Table II. Sporulation-stimulatory activities of a supernatant fluid (methanol-soluble fraction) following treatment of that fluid with different amounts of activated charcoal. The culture filtrate was treated with different amounts of charcoal for 15 min each, the charcoal removed by filtration, and the fluid then assayed. The activities are expressed as the percent of heterocysts with contiguous akinetes, out of 200 heterocysts surveyed.

	0x	1x	2x	3x
Control	1.0			
Original filtrate		5.5	30.5	40.0
Amount of charcoal used (µg/ml)				
2		4.5	7.0	25.0
6		2.5	6.0	10.5
9		2.5	6.0	5.5
13		1.0	5.5	8.5
39		2.0	2.0	4.0

Figure 4. UV absorption spectra between 260 and 300 nm, of charcoal-treated culture filtrates. In each of the scans, 260 nm is at the left and 300 nm at the right.

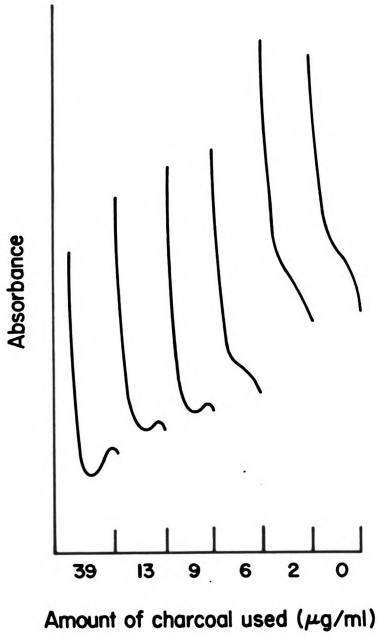


Figure 4

to dilute the methanolic extract in order to obtain complete adsorption of the sample.

Table III shows the result of adsorption of the active material to a column of charcoal and celite, and its subsequent elution from the column with 50% ethanol and then with 50% ethanol plus 2% NH_4OH. The first and second 500 ml of unadsorbed effluents from the column exhibited 10 to 14% of the activity of the original methanolic extract. However, the activities associated with the effluents did not increase with concentration. Elution of the column with 50% ethanol removed 50 to 60% of the original activity. An additional 24 to 28% of the original activity was eluted with a solution of 50% ethanol containing 2% NH_4OH. The combination of the four fractions resulted in full recovery of the original activity of the methanolic extract applied to the column. The activities in the ethanolic eluate and ethanol-ammonia eluate increased with concentration, as did the activity associated with the methanolic extract.

Paper chromatography

Chromatography papers (20 cm x 20 cm, Whatman No. 1) were washed by descending chromatography with both of two solvent systems. Solvent I consisted of isopropanol: formic acid:water (20:1.1:5,v/v), and solvent II of

Table III. Activities of the fractions from the column of charcoal and celite. One liter of a 27.5-fold concentrate of an aqueous solution of the dried methanolic extract was passed through a 0.9 cm i.d. x 4.5 cm long column of a 1:2 (w/w) mixture of charcoal and celite. The following samples of eluate were collected: two samples of unadsorbed effluent, 500 ml each; a 50% ethanolic eluate, 1 liter; and an eluate consisting of 50% ethanol with 2% NH_4OH, 1 liter. Activities are expressed as the percent of heterocysts with contiguous akinetes, out of 200 heterocysts surveyed.

	0x	½x	1x	2x
Control	3.5			
Methanolic extract		23.0	37.0	50.0
First 500 ml unadsorbed		2.5	4.0	4.0
Second 500 ml unadsorbed		6.5	5.0	6.5
Ethanol eluate		12.5	23.0	26.5
Ethanol-ammonia eluate		5.0	14.0	14.0
Combination of all four eluate fractions			37.5	

isopropanol:58% NH_4OH:water (9:1:1, v/v). The paper was dried between and after the two washings. A toxic effect upon the growth of *Cylindrospermum* exhibited by the extracts from unwashed papers was completely prevented by washing, and neither did the extracts show growth-stimulatory effects.

Figure 5 shows a drawing of a paper chromatogram of the combined ethanolic eluates from the charcoal-celite column, in solvent system II. The bands shown were observed under UV light or after spraying with a solution of ninhydrin. The brown-colored material observed at the origin in visible light could not be extracted from the chromatogram. The chromatogram was divided into three sections (Fig. 5), and each section was cut into small pieces and extracted with solvent system II. The results of bioassay of the extracts is shown in Table IV. The eluate from section #1 was inactive, that from section #2 exhibited 71 to 90% of the original activity of the ethanolic eluates, and that from the third section exhibited one to five percent of the applied activity.

Chromatography on thin layers of silica gel

All silica gel 60F-254 plates were prewashed by chromatography with solvent system III, n-butanol:acetic acid:ether:water (9:6:3:1, v/v).

Figure 5. Paper chromatography of the combined ethanolic eluates from a charcoal-celite column, developed with a solvent system of isopropanol:58% NH_4OH:water (9:1:1, v/v). The bands were located under UV light or by subsequent spraying with a 2% solution of ninhydrin in acetone acidified with 2 N formic acid. The chromatogram was cut into three sections and extracted for bioassay. The results of the bioassay are shown in Table IV.

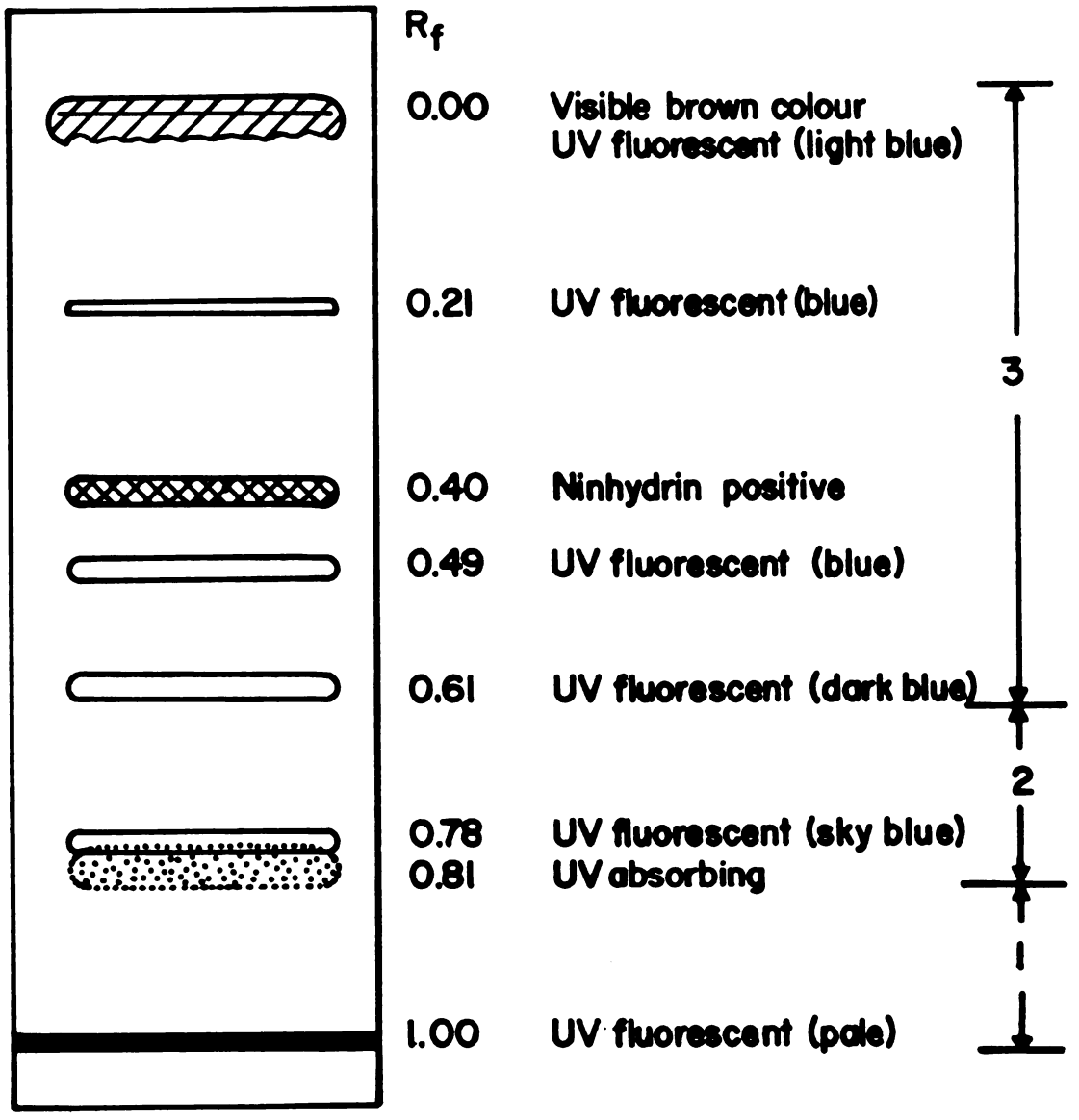

R_f
0.00
Visible brown colour
UV fluorescent (light blue)
0.21
UV fluorescent (blue)
3
0.40
Ninhydrin positive
0.49
UV fluorescent (blue)
0.61
UV fluorescent (dark blue)
2
0.78
UV fluorescent (sky blue)
0.81
UV absorbing
1
1.00
UV fluorescent (pale)

Figure 5

Table IV. Bioassay of the substances extracted from a paper chromatogram. Three sections of the paper chromatogram (see Fig. 5) were extracted with the solvent system used for chromatography. Activities are expressed as the percent of heterocysts with contiguous akinetes, out of 200 heterocysts surveyed.

	0x	1x	2x	4x	8x
Control	7.0				
Original eluate		26.0	43.0	——	——
Section #1		7.0	7.0	6.0	——
Section #2		24.3	32.5	18.3	8.0
Section #3		8.0	11.5	13.0	——

The extract from section #2 of the paper chromatogram was chromatographed on silica gel in solvent system III. Figure 6 shows bands visualized against the fluorescent background by means of UV light. The chromatogram was divided into six sections. Each section was scraped from the plate, and extracted three times with 50% ethanol. The results of bioassay of the extracts from the six sections are shown in Table V. The extract from section #1 exhibited 80 to 98% of the activity of the extract applied to the plate. The extract from section #4 exhibited 11 to 12% of the original activity. Section #1 included a band showing a mixture of UV absorbance and fluorescence. When it was dried, the extract from this section contained a large amount of crystals. These crystals were shown by a separate experiment to have come from the plate itself rather than from the extract which had been applied to the plate, and to be without effect on the formation of akinetes or on the growth of an assay culture.

Chromatography on thin layers of cellulose

All cellulosic thin layer plates were prewashed with solvent systems I and II. The active extract from section #1 of a silica gel chromatogram was further chromatographed on a prewashed cellulosic plate in

Figure 6. Silica gel thin layer chromatogram of the active extract from a previous paper chromatogram. The chromatography was performed in a solvent system of n-butanol:acetic acid: ether:water (9:6:3:1, v/v). The chromatogram was divided into six sections for bioassay. The results of the bioassay are shown in Table V.

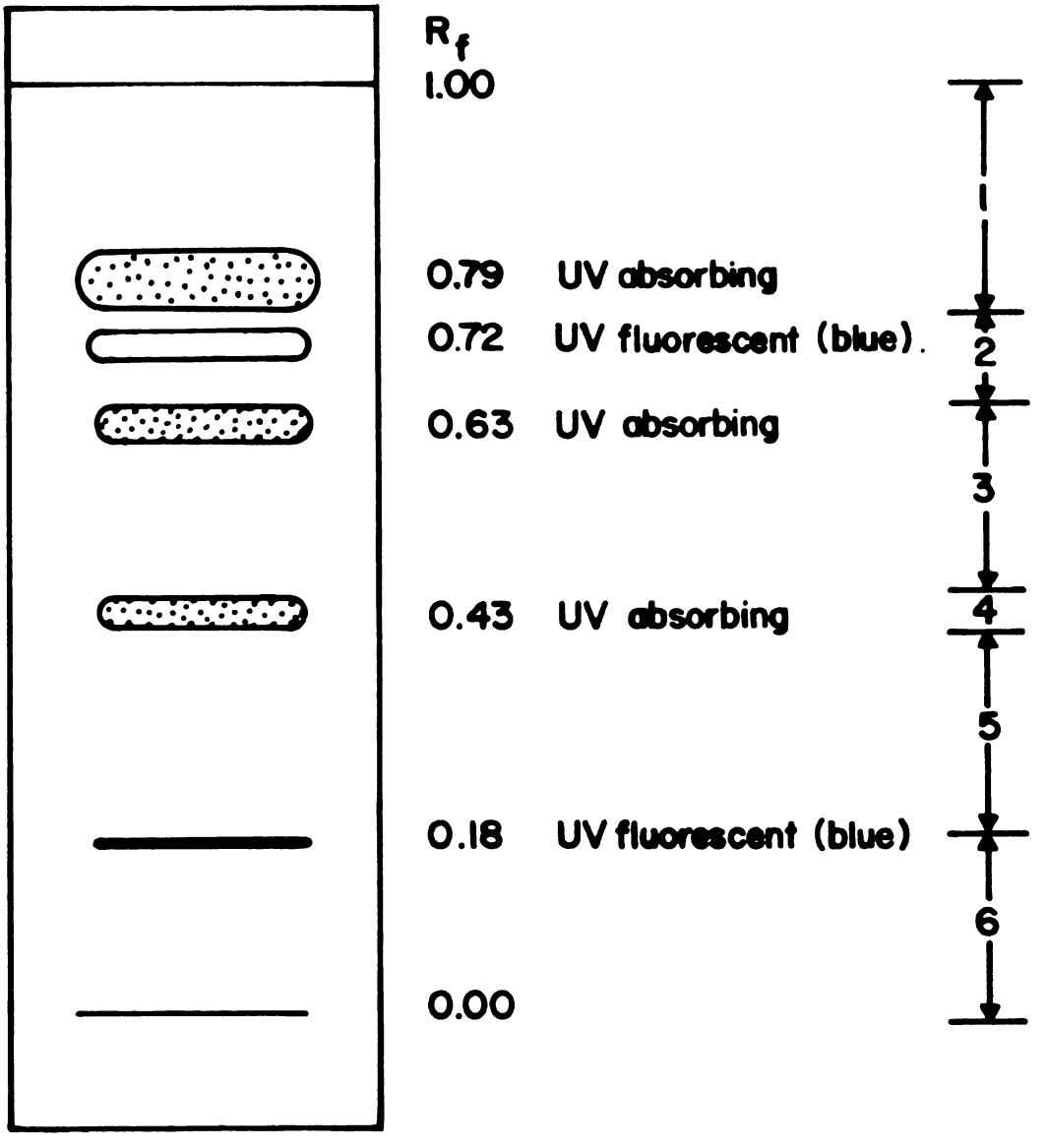

R_f
1.00
0.79 UV absorbing
0.72 UV fluorescent (blue).
0.63 UV absorbing
0.43 UV absorbing
0.18 UV fluorescent (blue)
0.00
1
2
3
4
5
6

Figure 6

Table V. Activities in extracts from sections of a silica gel thin layer chromatogram. Six sections were scraped from the plate (see Fig. 6) and extracted with 50% ethanol. Activities are expressed as the percent of heterocysts with contiguous akinetes out of 200 heterocysts surveyed.

	0x	1x	2x	4x	8x
Control	3.5				
Extract of paper chromatogram		48.0	55.0	50.0	26.0
Section #1		39.0	49.5	49.0	26.0
Section #2		5.0	4.5	5.5	5.5
Section #3		4.0	3.5	4.5	3.5
Section #4		8.5	9.5	8.5	8.5
Section #5		4.5	3.5	5.5	4.5
Section #6		3.5	4.5	4.5	3.5
Combined		40.5	47.5	40.5	30.5

solvent system II. Three bands, all of which were present in the first paper chromatogram (Fig. 5), can be seen, better separated, on the plate (Fig. 7). The chromatogram was divided into four sections; each was scraped from the plate and extracted three times with solvent system II. The UV-absorbing band with an R_f of 0.82 exhibited 76 to 91% of the activity of the extract applied to the plate (Table VI).

As will be discussed in a later section, further purification was achieved by means of vacuum distillation at 150 C.

Figure 8 illustrates the entire purification procedure.

Determination of dry weight

Samples were lyophilized in tared culture tubes (1 cm i.d. x 6 cm long), and the tubes with dried samples weighed with a S5 microbalance (Mettler, Zürich, W. Germany). Table VII shows the dry weights and biological activities of samples from successive purification steps starting with one particular culture supernatant. The loss of dry weight during paper chromatography is due to irreversible binding of brown-colored material to the paper. The increase in dry weight during chromatography on thin layers of silica gel is due to the extraction of crystalline

Figure 7. Chromatogram, on a thin layer of cellulose, of the active extract from a silica gel thin layer chromatogram. The solvent system was isopropanol:58% NH_4OH:water (9:1:1, v/v). The chromatogram was divided into four sections for bioassay. The results of the bioassay are shown in Table VI.

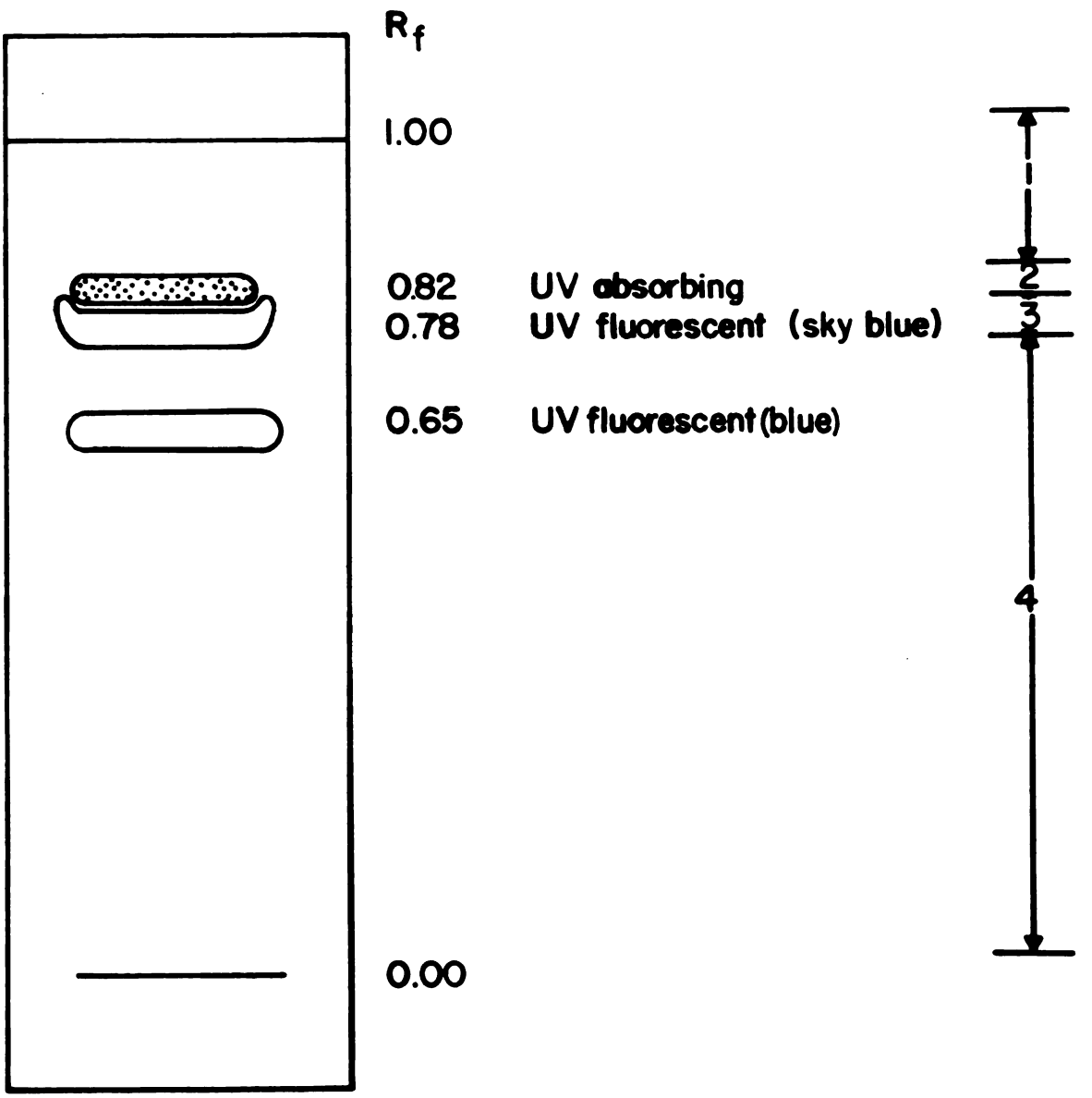

R_f
1.00
0.82
UV absorbing
0.78
UV fluorescent (sky blue)
0.65
UV fluorescent (blue)
0.00
2
3
4

Figure 7

Table VI. Activities of the extracts from the sections of a cellulosic thin layer chromatogram. Four sections scraped from the plate (see Fig. 7) were extracted with the same solvent system used for the chromatography. Activities are expressed as the percent of heterocysts with contiguous akinetes.

	0x	1x	2x	4x	8x
Control	4.0				
Silica gel extract		33.5	44.5	48.0	40.5
Section #1		4.5	5.5	7.5	4.5
Section #2		27.0	35.5	44.0	40.0
Section #3		6.0	6.5	6.6	5.5
Section #4		5.5	4.5	4.0	4.5

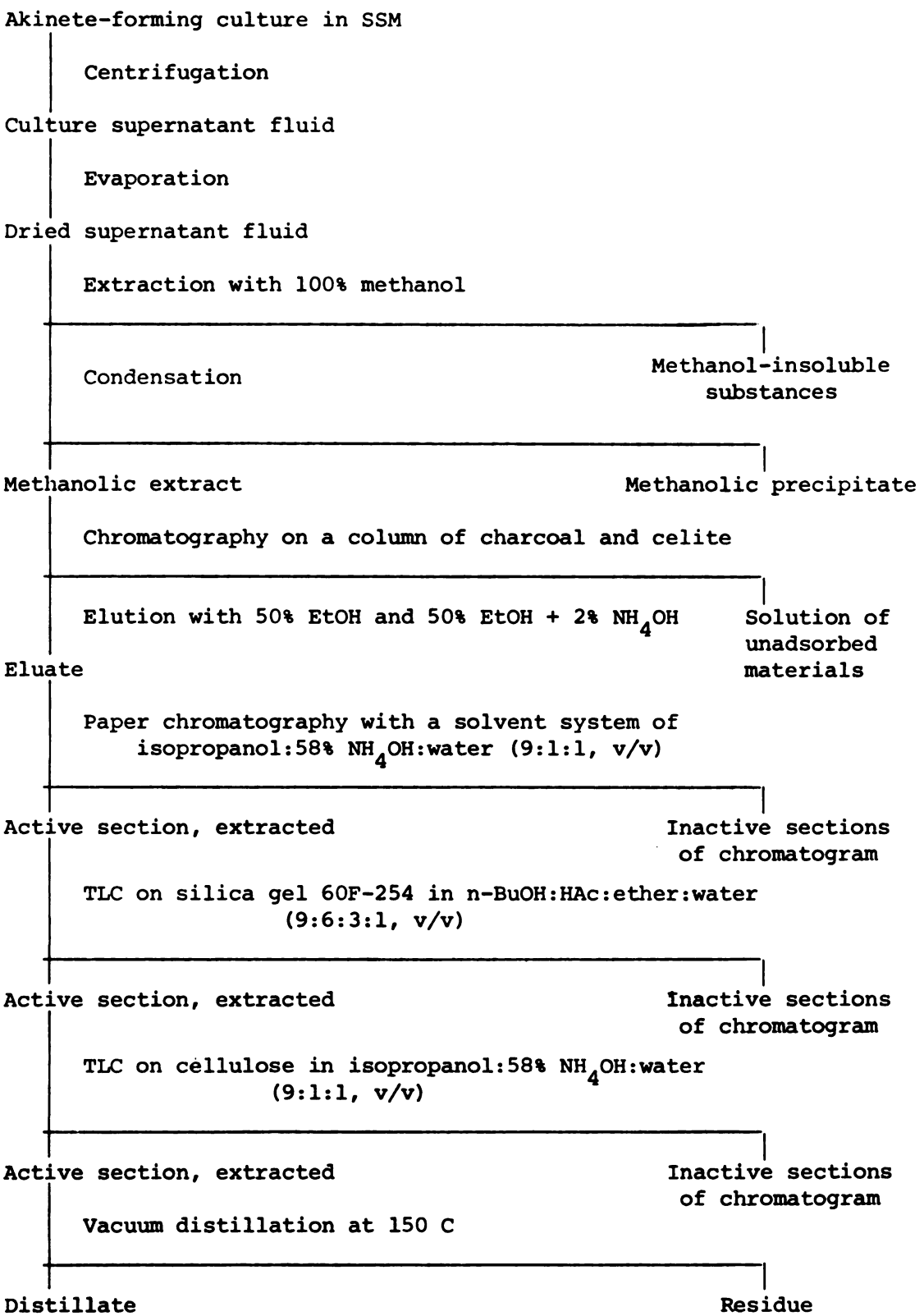


Figure 8. Purification procedure for the sporulation-stimulatory substance.

Table VII. Dry weights and biological activities of samples from different stages of purification.

	µg dry weight/ml[a]	Concentration of factor					Specific activity[c]
		0x	1x[a]	2x	4x	8x	
Control		11.5					
Culture supernatant fluid	5,580.0		54.0	——	——	——	0.00762
Methanolic extract	3,590.0		49.0	——	——	——	0.0104
Methanol-insoluble fraction	591.0		16.5	——	——	——	
Methanol precipitate	847.0		16.5	——	——	——	
Ethanolic eluate from charcoal	35.0		46.0	46.5	60.0	41.5	0.986
Charcoal pass-through	3,604.0		19.5	——	——	——	
Extracts from paper chromatogram #1	0.260		14.0	12.0	14.5	13.0	
#2	0.813		21.0	40.0	41.0	37.5	11.7
#3	9.870		9.5	8.5	10.0	6.0	
Extracts from silica gel plate #1 SF[b]	0.310		12.0	10.0	17.5	15.0	
#1 REM[b]	0.901		21.0	30.0	33.0	36.5	10.5
#2	0.597		12.0	16.0	13.5	9.5	
#3	0.301		6.0	19.0	16.0	10.0	
Extracts from cellulose plate #1 SF[b]	0.002		9.5	11.0	14.0	16.0	
#1 REM[b]	0.002		14.0	12.0	18.0	17.0	
#2	0.011		24.0	27.5	31.5	20.5	1140
#3	0.019		14.0	14.0	13.5	11.0	
#4	0.860		11.5	14.0	16.0	10.0	

a. Values based on dilution of the fraction to that portion of the volume of the original culture supernatant fluid not used for biological assays. *b*. Section #1 of Fig. 6 & 7 was subdivided into a solvent front zone (SF) and the remainder (REM) of the section. *c*. (activity of 1x concentrate - control activity) $(\mu g \text{ dry weight/ml})^{-1}$.

material from the plates. The dry weight-based specific activity of the most purified sample is 1.5 x 10^5 times higher than that of the original culture supernatant.

Isolation, from cells, of material stimulating the formation of akinetes

Cells from the same 100 liters of sporulating cultures used for the experiment whose data are tabulated in Table VII were collected by centrifugation, washed with fresh SSM, and lyophilized. The dry weight of the cells was 15.45 g. The lyophilized cells were extracted three times with 250 ml of absolute methanol. The combined extracts were evaporated to dryness and suspended in 20 ml of distilled water. Low concentrations of the extract exhibited significant sporulation -stimulatory activity (Table VIII).

Mass spectral analysis

The active, UV-absorbing band extracted from the cellulosic thin layer plate and used for mass spectral analysis was contaminated with a slight amount of material from a band of lower R_f. Figure 9a shows a mass spectral fragmentogram of seven fragments predominant (m/e = 41, 44, 56, 69, 123, 151 and 265) in the sample. Among them, only the fragment with m/e ratio of 96, 123, and 151 are both dominant in the

Table VIII. Biological activity of the methanolic extract from cells. 15.45 g of lyophilized cells from 100 liters of culture (i.e., 0.154 mg dry weight of cells per ml of original suspension), were extracted with absolute methanol, and the methanolic extract dried and redissolved.

Mg dry weight of cells extracted, per ml of assay-suspension	Percent of heterocysts with a contiguous akinete
618	cells lysed
309	
154	
77.2	
38.6	
19.3	35.0
3.86	44.0
1.93	33.0
0.386	29.0
0,193	16.5
0.000	7.0

Figure 9. Mass spectral fragmentograms of (a) the active extract from a cellulosic thin layer chromatogram and (b) an inactive extract of material with slightly lower R_f.

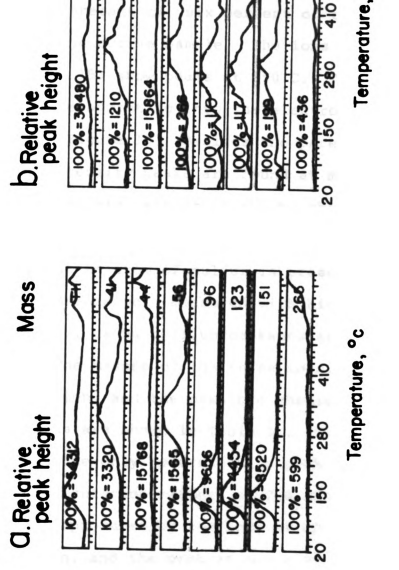

a. Relative peak height
Mass
100%=3432
100%=3320
100%=15768
100%=1565
100%=3686
100%=4454
100%=8520
100%=599
96
123
151
264
20
150
280
410
Temperature, °c
b. Relative peak height
Mass
100%=38490
100%=1210
100%=15864
100%=146
100%=417
100%=196
100%=436
56
90
123
151
265
20
150
280
410
Temperature, °c

Figure 9

sample containing the sporulation-stimulatory substance and not characteristic of the fragmentogram (Fig. 9b) of the neighboring UV-fluorescent band extracted separately from the cellulosic plate. The other fragments represent ca. six percent of the material present in the active sample. The ions with m/e = 96, 123, and 151 show peaks at 150 C. The mass spectrum of material evaporating into the electron beam at 150 C is shown in Figure 10. Besides the parent peak at m/e = 151, high intensity peaks are found at m/e = 123 and m/e = 96. The high ratios of the heights of the peaks at m/e +2 to the heights of the parent (m/e) peaks indicate that an atom of sulfur is present in the molecule. Masses were measured with high resolution by peak matching, using perfluoroalkanes as internal standard. The masses of the three ions, measured with high resolution, and the possible chemical composition of those ions are shown in Table IX. The chemical formula for the only molecular ion peak which can account for the fragmentation pattern is C_7H_5OSN. The peak at m/e = 123 is produced by the loss of CO from the molecular ion, and the peak at m/e = 96 is produced by the additional loss of HCN. The paucity of hydrogen atoms relative to carbon atoms indicates that the molecule contains unsaturated bonds.

Figure 10. Mass spectrum of the material evaporating into the electron beam at 150 C.

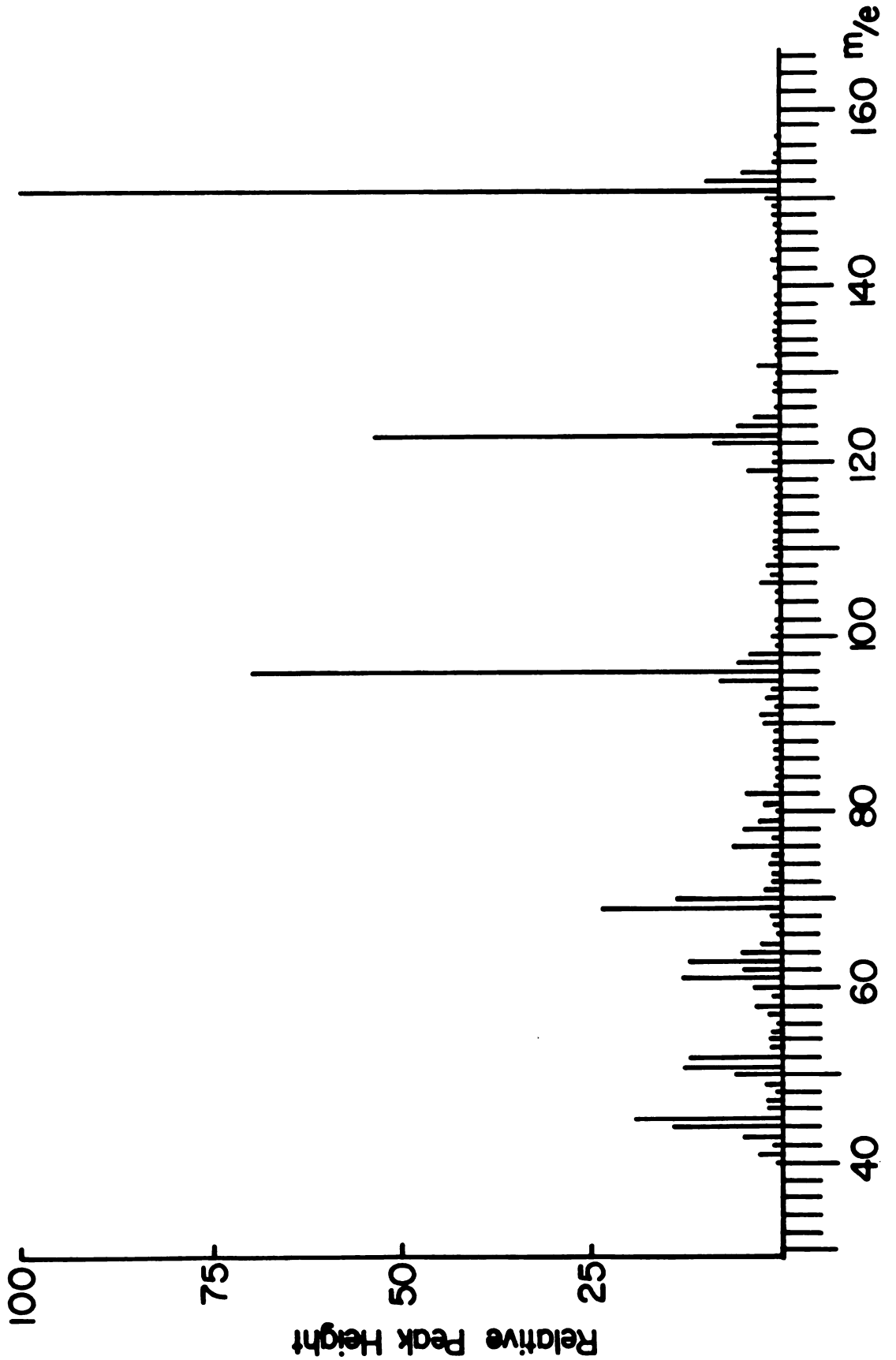

Relative Peak Height
100
75
50
25
40
60
80
100
120
140
160
m/e

Table IX. Molecular weights, measured with high resolution, and possible chemical formulae of the major ions in the mass spectrum of the active material.

Molecular weight	Possible chemical formulae [a]	Mass difference	Loss
151.00931	$C_1H_3O_5N_4$	-0.00104	
	$C_2H_7S_2N_4$	-0.00191	
	$C_4H_7O_4S_1$	0.00281	
	$C_5H_3S_1N_4$	0.00145	
	$C_7H_5O_1S_1N_1$	0.00012	
			-27.99513 (CO)
123.01418	$H_3O_4N_4$	-0.00125	
	$C_2H_5O_5N_1$	-0.00258	
	$C_3H_7O_3S_1$	0.00260	
	$C_6H_5S_1N_1$	0.00009	
			-26.98278 (HCN)
96.00314	$C_1H_4O_5$	-0.00273	
	$C_5H_4S_1$	-0.00023	

[a] Permissable tolerance of mass, relative to the measured molecular weight: $\pm$ 0.0030 amu.

A further step of purification

Mass spectroscopy indicated that the active material eluted from the cellulosic thin layer chromatogram contained predominately a substance vaporizing at approximately 150 C under high vacuum. The active extract prepared from the cellulosic thin layer plate was therefore further purified by distillation under vacuum. The distillate, which formed a single UV-absorbing band at an R_f of 0.81 on a cellulosic thin layer chromatogram developed with solvent system II, exhibited 52% of the activity of the original methanolic extract, in SSM (Table X). The distillate also was active in stimulating akinete formation in phosphate-containing AA/8.

NMR spectroscopy

A proton magnetic resonance spectrum of ca. 0.5 mg of the distillate is shown in Figure 11. Peaks at δ = 1.940, 2.165, 7.845, 7.840, 7.157, 5.84, and 5.73 ppm originate from the solvent. Peaks from the sample are confined to the region 7.19 to 7.29 ppm, and form a complex pattern. The absence of resonance peaks at a low ppm range together with the complex pattern at ca. 7.2 ppm suggest that the sample has a conjugated ring structure (Paudler, 1971).

Table X. Biological activity, in SSM and AA/8[a], of the vacuum-distillate of the sporulation-stimulatory material purified by the entire procedure of Figure 8. Activity is expressed as the percent of heterocysts with contiguous akinetes.

	0x		1x		2x		4x		8x	
	SSM	AA/8	SSM	AA/8	SSM	AA/8	SSM	AA/8	SSM	AA/8
Control	7.0	0.0								
Methanolic extract of original (dried) culture supernatant fluid			39.5	26.0	45.5	37.5				
Vacuum-distillate			24.0	14.5	25.5	17.5	44.0	34.5	27.5	19.5
Residue from vacuum distillation			8.0	8.0	8.5	8.5	9.0	9.5	7.0	7.0

[a]Akinetes which formed in AA/8 were able to complete morphological maturation in that medium despite the fact that it contains all of the elements required for vegetative growth.

Figure 11. Proton magnetic resonance spectrum of the vacuum-distilled sporulation-stimulatory substance.

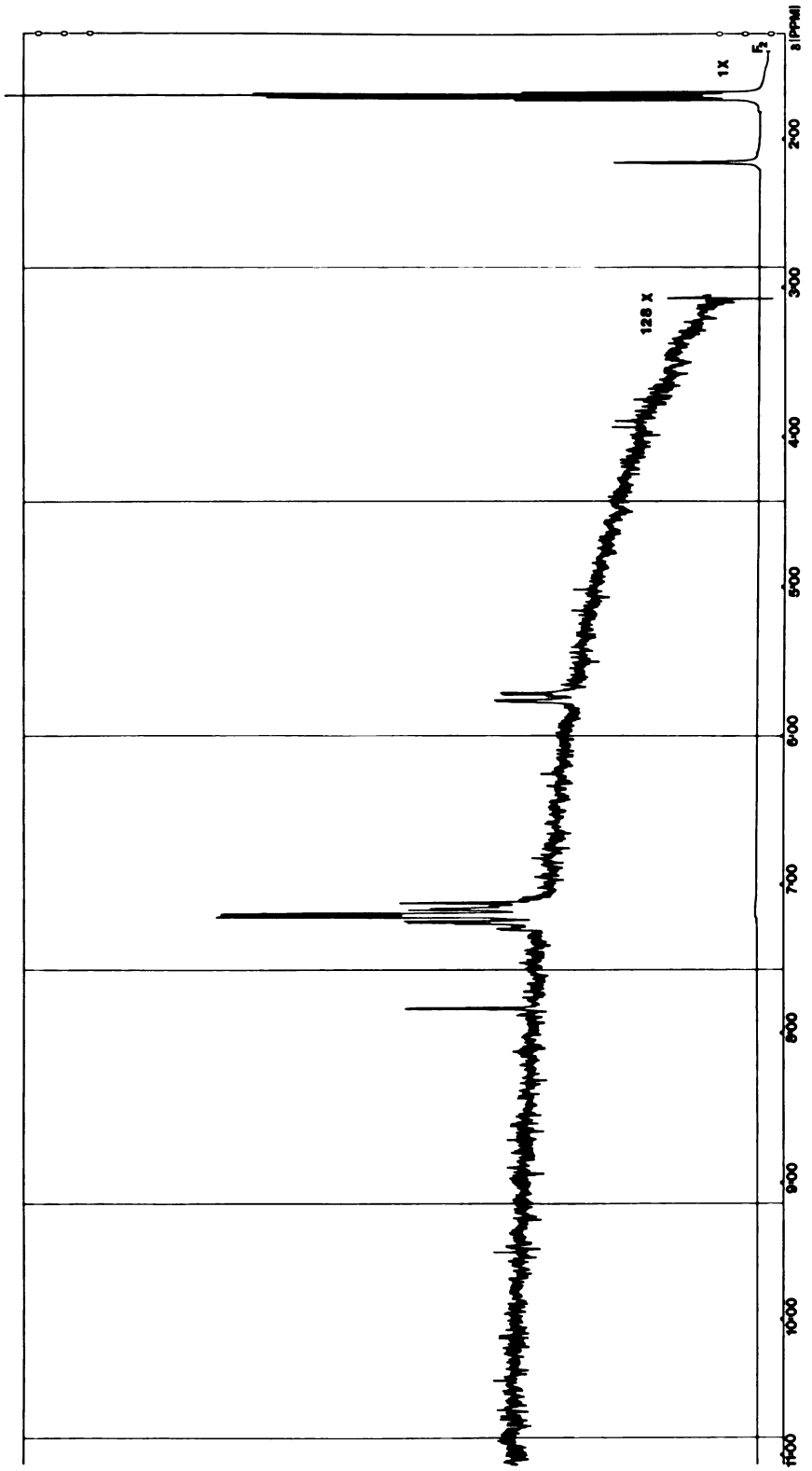


Figure 11.

IR spectroscopy

Infrared spectra were taken of ca. 0.5 mg of the vacuum-distilled sporulation-stimulatory material in a disc of KBr (Fig. 12). A peak at 3429 cm^{-1} is referred to an -NH or -OH stretch. Although in some cases KBr itself shows a peak at this frequency, perhaps due to water absorbed by the salt, the peak in the spectrum of the sporulation-stimulatory material is attributable to the sample because a control spectrum of a disc containing only the same amount of KBr as in the sample disc shows no peak at that frequency. Peaks were observed consistently at 800, 1010, 1085, 1257, 1627, 1725, 2855, 2930, 2988, and 3429 cm^{-1} (Fig. 12a).

UV absorption spectroscopy

An ultraviolet absorption spectrum was taken of the sporulation stimulatory material in acetonitrile (Fig. 13). Peaks were observed at 242, 247, 253, 263, and 268 nm.

Figure 12. Infrared spectra of (a) the vacuum-distilled sporulation-stimulatory substance in a KBr pellet and (b) a KBr pellet without additional material.

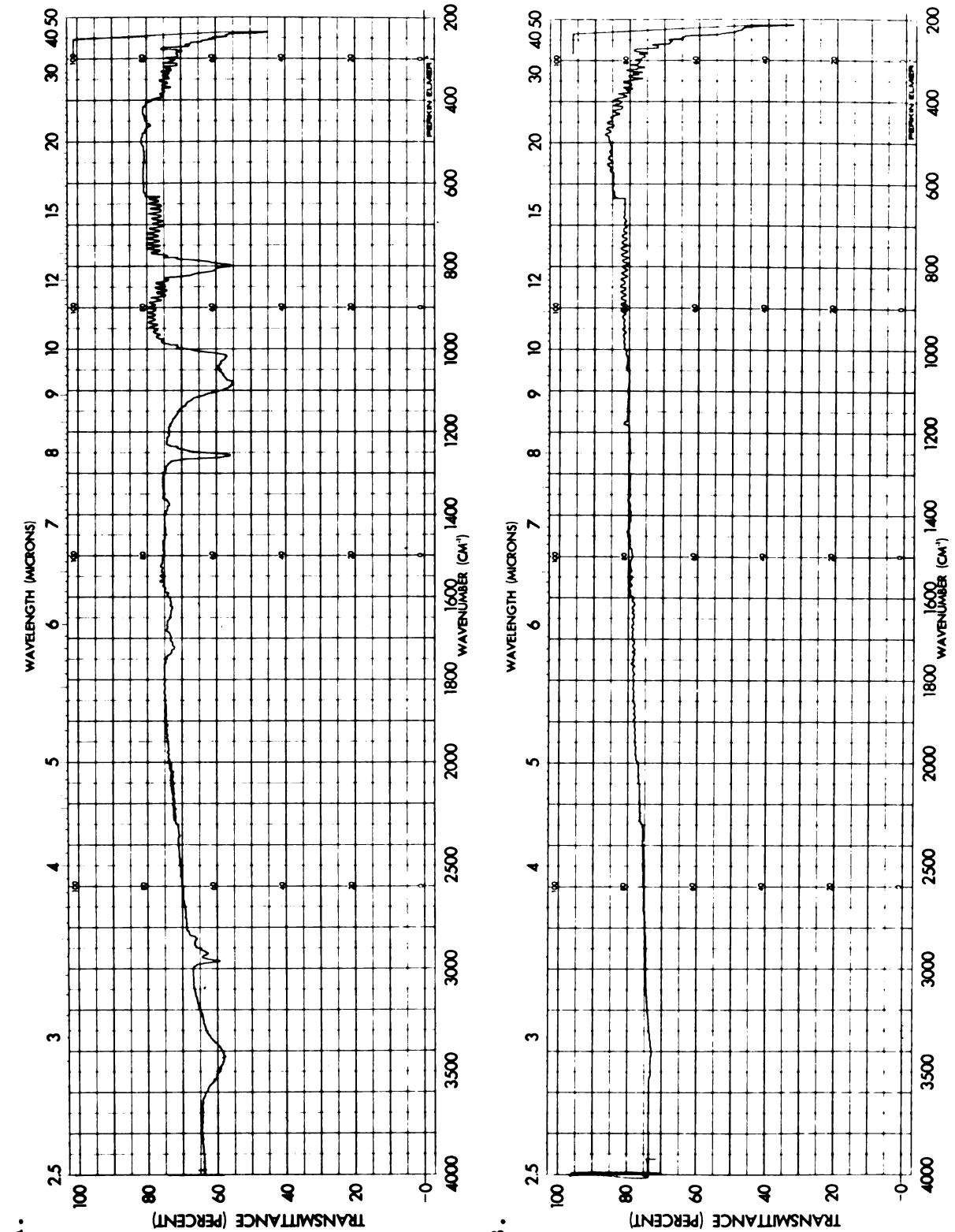

A.
B.
WAVELENGTH (MICRONS)
WAVENUMBER (CM⁻¹)
TRANSMITTANCE (PERCENT)
PERKIN ELMER

Figure 13. UV absorption spectrum of the sporulation-stimulatory substance dissolbed in 0.5 ml of acetonitrile.

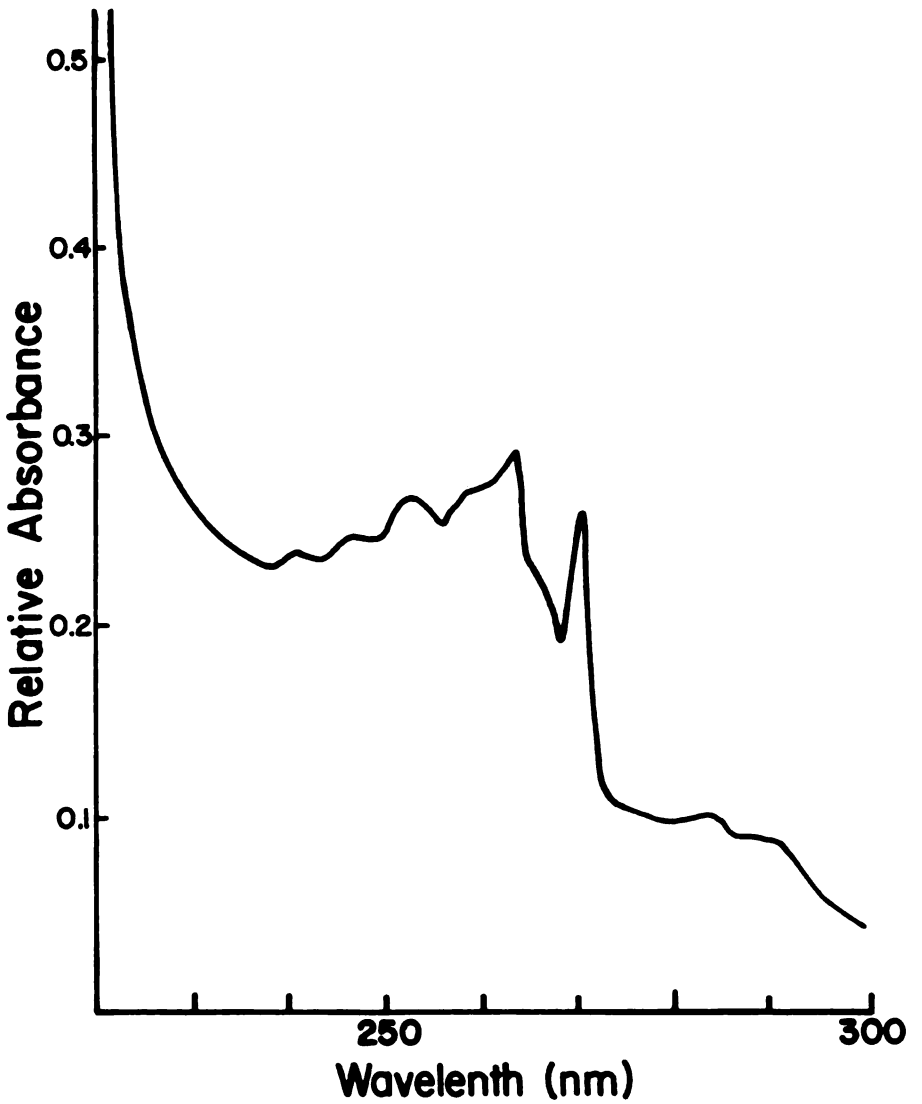


Figure 13

PART II. Effects of Various Environmental Factors on the Formation of Akinetes in *C. licheniforme*

Effect of hydrogen on the formation of akinetes

As shown in Table XI, gassing of a suspension of filaments in SSM with a mixture of 12.5% hydrogen, 87.5% air stimulates the formation of akinetes in SSM 2.5-fold relative to a control gassed with air. Growth is not affected. Table XI also shows that the addition of hydrogen to a gas mixture consisting of 19.9% O_2, 0.1% CO_2, balance Ar has the same stimulatory effect on the formation of akinetes. Hydrogen has no significant effect upon the reduction of acetylene by *C. licheniforme* (Table XII).

Uptake hydrogenase and its localization

Peterson and Wolk (1978a) showed that uptake hydrogenase activity in aerobically grown *Anabaena* strain 7120 is localized solely in heterocysts. Ferricyanide, which served as electron acceptor in a

Table XI. Effect of hydrogen gas upon the formation of akinetes. The gas mixtures in the flasks used for the assays were replaced every 12 hr.

% Hydrogen	Remaining gas phase	
	Air	19.9% O_2, 0.1% CO_2, 79.9% Ar
0	13.5[a]	13.0
5.0	26.5	16.5
10.0	26.0	18.0
12.5	34.0	18.5
15.0	25.5	27.5
20.0	25.5	28.0

[a]Sporulation-stimulatory activity, expressed as the percent of heterocysts with contiguous akinetes.

Table XII. Effect of hydrogen gas upon acetylene reduction in air. Each 5-ml assay vial contained a gas phase of air, 0.3 ml C_2H_2, and hydrogen as shown, plus 2 ml of a suspension of *C. licheniforme* in SSM. The reactions were stopped after 30 min, by addition of 1.0 ml of 20% TCA.

% Hydrogen	µmoles C_2H_4 produced (mg Chl)$^{-1}h^{-1}$
0	12.3
5	11.2
10	13.1
12.5	12.1
15.0	11.2

cell-free preparation of *Anabaena* 7120, could not do so in a cell-free preparation of *C. licheniforme*. Of several alternative electron acceptors tried (Table XIII), only 10 mM phenazine methosulfate (PMS) was found to be active as an acceptor. Fujita *et al.* (1964), working with the uptake hydrogenase from *A. cylindrica*, reported that the most effective electron acceptor tried was PMS, and that methylene blue, toluidine blue, and DCPIP were less than 25% as effective. One method for localizing uptake hydrogenase in filaments is presented in Table XIV; on a per heterocyst basis and as measured by *in vitro* assays, 84 $\pm$ 2% (two experiments) of the hydrogenase activity in whole filaments was recovered in isolated heterocysts, whereas no activity was detected in a fraction derived from vegetative cells.

The effects, upon the formation of akinetes, of various substances of low molecular weight

Table XV shows the effects of the 20 usual amino acids found in proteins on the formation of akinetes. One mM L-tryptophan shows particularly high stimulatory activity. Aspartic acid and pheylalanine, at a concentration of 16 mM, also show high activity. Lower, but significant, stimulatory activity was produced by 16 mM proline and 15 mM isoleucine. Although ammonium inhibits

Table XIII. Uptake of hydrogen by a cell-free suspension, measured manometrically, with various electron acceptors at a concentration of 10 mM.

Acceptor	E'_o (mv)	Activity μmole H_2 (mg Chl)$^{-1}h^{-1}$
Ferricyanide	+429	0
DCPIP	+217	0
PMS	+80	5.8
Methylene blue	+11	0
Methyl viologen	-550	0

Table XIV. Localization of hydrogenase. Hydrogen uptake by cell free suspensions derived from whole filaments, from heterocysts, and from vegetative cells was measured manometrically. The Warburg flasks used contained 1.8 ml of sample suspension and 0.2 ml of 100 mM PMS.

	$\mu l\ H_2$ taken up·$(10^9$ heterocysts$)^{-1} min^{-1}$
Whole filaments	3.03
Heterocysts	2.48
Vegetative cells	<0.01[a]

[a] This figure is calculated on the basis of the heterocysts in the filaments from which the vegetative extract was derived.

Table XV. Effects of amino acids on the formation of akinetes. Values shown are the percent of heterocysts with contiguous akinetes.

Amino acid concentration, mM	16	10	5	1	0.5	0.1	0
Control							4.0
Glu	8.0	10.0	7.5	6.0			
Gln	10.0	10.0	10.0	1.5			
Pro	31.5	10.0	9.0	6.0			
Arg	20.5	8.0	6.0	5.0			
Asp	47.0	25.5	26.5	22.5			
Asn	—[a]	—	6.0	18.5			
Lys	1.5[b]	3.0[b]	0.0	0.0			
Thr	—	—	—	4.0[b]	3.5[b]	0.0	
Met	—	—	—	—	—	—	
Tyr	—	—	—	—	—	0.0	
Trp	—	—	—	60.5	42.0	36.0	
Phe	68.5	38.0	20.5	19.0			
His	0.0[b]	0.0	0.0	3.0			
Ser	—	—	12.5	13.5			
Gly	—	—	0.0	4.5			
Cys	—	—	14.0	8.5			
Ala	—	—	8.0	5.0			
Val	—	—	0.0	0.0			
Leu	2.5[b]	0.0[b]	0.0	0.0			
Ile	—	—	35.0	17.0			

[a] — : cells lysed

[b] Inhibitory to growth

the formation of akinetes in *Anabaena doliolum* (Singh and Srivastava, 1967; Tyagi, 1974), it does not do so in *C. licheniforme* (Table XVI) except at growth-inhibitory concentrations. The cyclic nucleotides, dibutyryl c-AMP, dibutyryl c-GMP, and c-AMP showed little or no sporulation-stimulatory activity by themselves, and did not substantially enhance the stimulatory effect of culture supernatant fluid (Table XVII). As shown in Table XVIII, the formation of akinetes is completely inhibited by 1.0% C_2H_2 or 0.5% C_2H_4.

Mann calcium glucuronate, reported by Wolk (1965) to stimulate the formation of chains of akinetes in *A. cylindrica*, did not elicit the differentiation of chains of akinetes in *C. licheniforme*. However, after four days of culture in SSM supplemented with 25 mM Mann calcium glucuronate, an akinete was present adjacent to every heterocyst. Growth was not affected. In control cultures in SSM, ca. 5% of the heterocysts had contiguous akinetes.

Cytochemistry with a redox dye

Fay and Kulasooriya (1972) reported that upon treatment of filaments of *Anabaena cylindrica* under air with nitro blue tetrazolium chloride (NBT), a gradient of the amount of formazan produced by reduction of the

Table XVI. Effect of ammonium upon, added to SSM or to sporulation-stimulatory supernatant fluid, the formation of akinetes. Activities are expressed as the percent of heterocysts with contiguous akinetes.

NH_4Cl (mM)	SSM	Sporulation-stimulatory supernatant fluid
5.00	5.0	28.5[a]
2.50	1.5	40.0[a]
1.00	2.0	23.5[a]
0.50	2.0	58.0
0.10	2.5	71.5
0.02	2.0	70.5
0.00	6.0	64.0

[a] Growth was inhibited.

Table XVII. Effects of cyclic nucleotides upon the formation of akinetes. Activities are expressed as the percent of heterocysts with contiguous akinetes.

	SSM	Methanolic extract of dried culture supernatant fluid assayed at 1x concentration
Control	1.0	30.5
Dibut-c-AMP 5 mM	5.0	49.5
Dibut-c-AMP 2.5 mM	2.5	30.5
Dibut-c-GMP 2.5 mM	1.5	
Dibut-c-GMP 0.5 mM	5.0	28.5
c-AMP 2.5 mM	7.5	34.0
c-AMP 0.5 mM	2.0	31.0
Dibut-c-AMP 2.5 mM + Dibut-c-GMP 0.5 mM	7.0	

Table XVIII. Effects of C_2H_2 and C_2H_4 upon the formation of akinetes. The mixture of gases was replaced every 12 hr. The extent of sporulation is expressed as the percent of heterocysts with contiguous akinetes. The numbers inside the parentheses express the growth of the culture during four days, as μg Chl/ml at the end of the four days.

Percent C_2H_2 or C_2H_4	0%	0.001%	0.01%	0.1%	0.5%	1%	5%	10%
Control	7.0[a] (0.51)							
C_2H_4		6.5 (0.50)	2.0 (0.46)	1.0 (0.50)	0 (0.46)	0 (0.46)		
C_2H_2					6.0 (0.48)	0 (0.46)	0 (0.48)	0 (0.51)

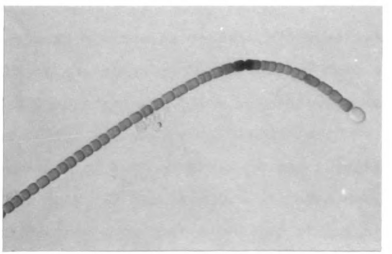
[a]Sporulation.

NBT was observed, with the most formazan present in vegetative cells next to heterocysts and with the amount of formazan present per cell gradually diminishing with increasing distance from heterocysts. A very similar result was obtained with *C. licheniforme* (Fig. 14). However, as shown in Figure 14, such a gradient can form from any point of the filament which is bent mechanically or is nicked by a fine glass needle. Therefore, the gradient originally shown may be the consequence of localized penetration of NBT at the connection between heterocysts and vegetative cells rather than of a gradient of reducing activity.

Figure 14. Reduction of nitro blue tetrazolium chloride by (a) intact, (b) bent, and (c) cut filaments.

(a)

(b)



(c)

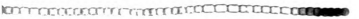


Figure 14

DISCUSSION

The substance inducing the formation of akinetes

A substance which is capable of stimulating the formation of akinetes has been purified from the centrifugal supernatant fluids of akinete-forming cultures of *Cylindrospermum licheniforme*. Evidence of the occurrence of such a substance was reported by Fisher and Wolk (1976). Because only the most active fraction or fractions from each step of the purification procedure were processed in the subsequent steps, on the order of 70% of the total activity (i.e., activity/volume, times volume) is lost during the purification. Significantly, the purified compound alone, even at high concentration, does not stimulate the formation of akinetes as strongly as does the original culture supernatant fluid. Thus, other substances, which may be active by themselves or may only potentiate the sporulation-stimulatory activity of the substance which has been isolated, contribute to the original activity.

However, the purified substance, the specific activity of which is 1.5 x 10^5-fold greater than is the specific activity of the (dried) original supernatant fluid, appears to be the major single substance responsible for the activity.

It is not easy to distinguish whether a compound is released by the breakdown of cells or is released by healthy cells (compare Fogg, 1962, 1966). Because the sporulation-stimulatory activity in culture supernatant fluids increases during the early part of the growth period (Fig. 1, 2), i.e., at a time when few cells are moribund or dead, it appears that hte stimulatory substance is released by healthy cells. The interpretation that the active substance is not a product of decomposition is further supported by the observation that significant activity may be extracted from the cells themselves (Table VIII). Others have observed that the formation of akinetes is inhibited by the presence of phosphate in the culture medium, i.e., that phosphate is inhibitory to the formation of akinetes (Glade, 1914; Wolk, 1965; Gentile and Maloney, 1969). Because the substance which I have isolated stimulates sporulation in phosphate-containing growth medium (AA/8: see Table X), this substance is not merely a promoter of a developmental process which occurs in response to a deficiency of phosphate, but instead itself initiates

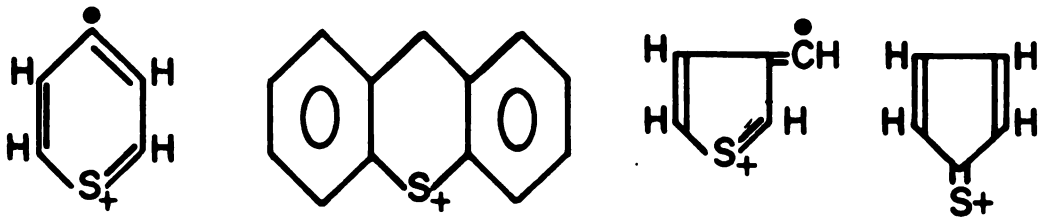
the differentiation of akinetes. Although a variety of substances is known to be released by cyanobacteria (Fogg, 1962, 1966; Stewart, 1963; Pattnaik, 1966; Walsby, 1974a, b; Walsby and Fogg, 1975), none of those substances is known to affect processes of differentiation (see especially Whitton, 1965). The substance reported here is maximally active at a concentration of 44 µg/l (4x value of active band from final step in Table VII). It is the first example of an organic compound, produced by a cyanobacterium, which specifically affects the differentiation process in the cyanobacterium.

Spectral analysis of the purified substance yielded the following information about its composition and structure:

i) Its molecular weight (Table IX) corresponds to the chemical formula C_7H_5OSN.

ii) The two major ions resulting from its fragmentation have the chemical formulae C_6H_5SN and C_5H_4S, respectively. The first fragment is produced by a loss of CO from the molecular ion. The loss of CO cannot be attributed to the presence of an aldehyde group in the molecule, because the absence of aldehydic hydrogen is clearly indicated by the lack of proton magnetic resonance in the region of δ = 9 ppm to δ = 10 ppm (Paudler, 1971), and by the absence of absorption in the IR at ca. 2720 and 2820 cm^{-1} (Conley, 1972). The composition of the second fragment is consistent with the idea that the fragment

is produced by the loss of HCN from the first fragment. The high intensity of the peak at m/e = 96 in the mass spectrum and the absence of other peaks of comparably high intensity at lower values of m/e indicate that the fragment with m/e = 96 has a stable ring structure; three such structures are consistent with the chemical formula (C_5H_4S) of that ion. Of these, a six-membered ring (a) may be excluded because it is structurally



(a) (b) (c) (d)

so strained that benzene rings at both sides of the ring, as in (b), are required to stabilize the structure, and because the formula of the fragment would require two unligated bonds. A second possible structure (c), a thiophene ring with a methylene substituent, may also be excluded because no fragment was observed which corresponded to the thiophene ring itself, and because the formula of the substituted thiophene would also require two unligated bonds. The third possibility, a five-membered ring with a thioketone group (d), is consistent with (i) the presence of a strong absorption, attributable to C=S, at 1085 cm^{-1}, and (ii) the absence of absorptions, attributable to C-S and and S-H, at 590 to 700 and at 2500 to 2600 cm^{-1}, in the IR spectrum. Peaks attributable to C=C, C=C-H, C-C, and methylene C-H bonds

are observed at 1627, 2988, 800, and 2855 plus 2930 cm^{-1}, whereas peaks attributable to -N=C=O and C≡C are not observed.

iii) The proton NMR spectrum shows a complex of peaks in the region of δ = 7.19 to δ = 7.29 ppm, implying that the substance has a highly conjugated ring structure.

iv) A peak at 3429 cm^{-1} in the infrared absorption spectrum may be assigned to the N-H stretch of a secondary amine or amide. The alternative possibility that that peak corresponds to an O-H stretching vibration was eliminated because no absorption attributable to an O-H bending vibration was observed in the region of 1300 to 1500 cm^{-1} or at ca. 650 cm^{-1}. Because only one peak is detected in the region of 3429 cm^{-1}, the nitrogen cannot be present as a primary amine ($-NH_2$). Lack of absorption in the region of 1550 cm^{-1} to 1530 cm^{-1}, which would have been attributable to N-H bending vibration, suggests that the N-H group is a constituent of a cyclic amide. Absorption at 1725 cm^{-1} supports the possibility that the oxygen atom in the molecule is present as a cyclic C=O, to which that absorption may be attributed provided that the carbonyl group is both conjugated and part of an amide group in a five-member ring (Conley, 1972; see pp. 168, 180). A major peak at 1257 cm^{-1} is attributable to the amide III band (C-N stretch) of a secondary amide. Taken together, these results provide strong support for the idea

that the nitrogen and oxygen in the molecule are present as a secondary amide group in a five-membered ring. This interpretation accords closely with the mass spectrum provided that the nitrogen atom is not adjacent to group (d) above, so that NCH will be lost as a group after (or together with) loss of CO. The structure of the substance is therefore approximately as shown in Figure 15, although the precise positions of the C=S and of the C=C double bonds cannot yet be assigned.

v) The ultraviolet absorption spectrum shows a multiplicity of peaks over the range, but these have not helped to elucidate the structure of the molecule.

Other Factors which Control the Formation of Akinetes

Hydrogen gas is active in stimulating sporulation adjacent to heterocysts, although less active than is a culture supernatant fluid. A mixture of 12.5% H_2 in air stimulates the formation of akinetes next to heterocysts 2.5-fold. Peterson and work (1978a), working with *Anabaena* strain 7120, reported that the kinetics of solubilization of hydrogenase, during cavitation, closely paralleled the kinetics of destruction of heterocysts, and that heterocysts isolated from the cyanobacterium accounted for 86% of the cell-free hydrogenase activity derivable from whole filaments. In my experiments, heterocysts isolated from *Cylindrospermum* accounted for 84% of the uptake hydrogenase present in

Figure 15. Approximate structure of the substance which stimulates the formation of akinetes in *C. licheniforme*. The positions of the thioketone group within the left-hand ring, and of the C=C double bonds, are not determined by the available data.

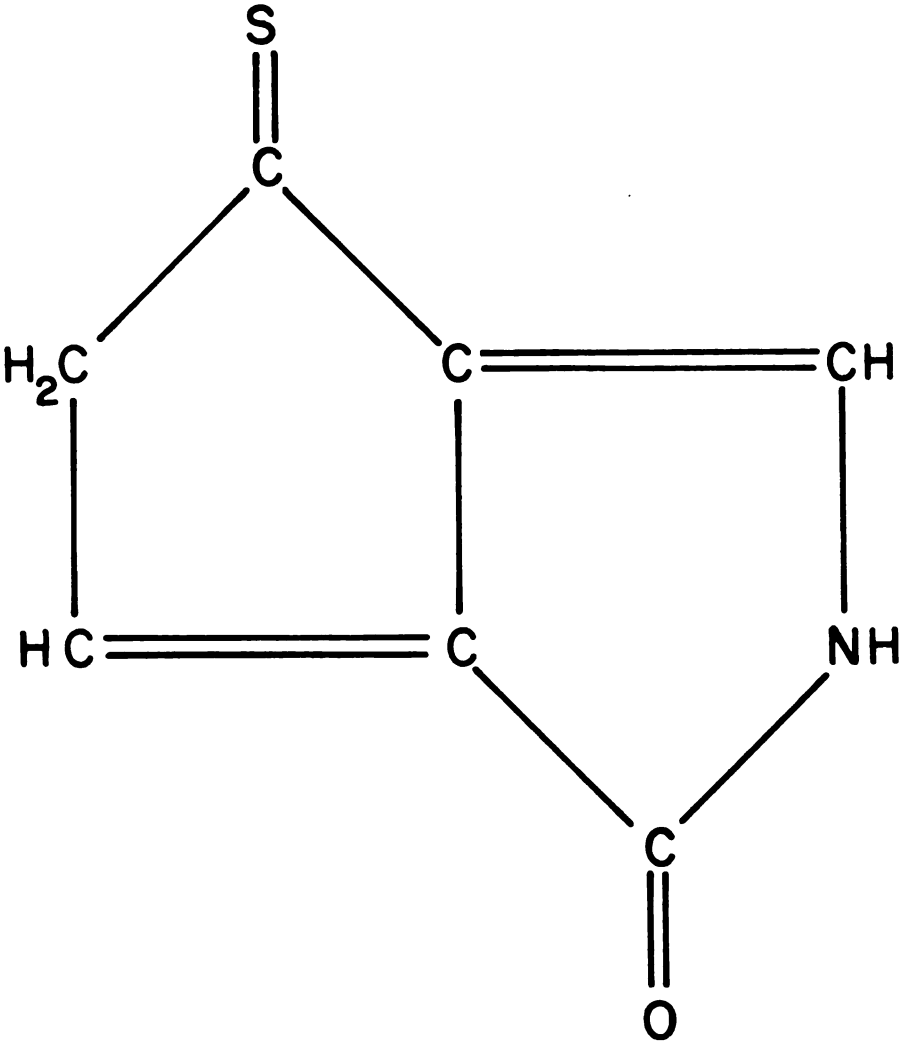


Figure 15

filaments of that organism, as assayed *in vitro*. It has been reported that hydrogen can serve as electron donor to nitrogenase (Wolk and Wojciuch, 1971; Bothe *et al.*, 1977; Tel-Or *et al.*, 1977). However, H_2 did not significantly stimulate the reduction of C_2H_2 by *Cylindrospermum* under air, and H_2 stimulated sporulation under $Ar/CO_2/O_2$. The stimulation by H_2 cannot, therefore, be mediated by an effect upon the fixation of nitrogen.

What is the role of the stimulatory substance and of H_2 in the formation of the pattern consisting of akinetes contiguous with heterocysts? If the substance is synthesized solely in heterocysts and is transported to nearby vegetative cells, a concentration gradient of this compound could be formed, so that the vegetative cells adjacent to heterocysts would be the first to differentiate into akinetes. It might be argued, however, that exogenous supply of the stimulatory substance should provide the substance equally to all vegetative cells, and so should change the pattern into one in which all vegetative cells differentiate simultaneously into akinetes. No such change of the pattern is observed. It is possible, however, that the substance can enter the filaments only at the connections between heterocysts and vegetative cells. That such a localized penetration is possible is suggested by the results of experiments with nitro blue tetrazolium chloride (Fig. 14). In those experiments,

the NBT was reduced in cells contiguous with heterocysts and in any other cell which was bent or nicked. Thus, vegetative cells contiguous with heterocysts may contain loci, such as — perhaps — the junctions to heterocysts, which are more permeable than are other sites along the filaments. If the stimulatory substance can enter the filaments only through the vegetative cells contiguous with heterocysts, the site of formation of a concentration gradient could be unchanged, as would be the pattern of formation of akinetes. Autoradiography with radioactively labeled sporulation-stimulatory substance may be able to establish whether it enters filaments only at the location of the cells which sporulate.

If heterocysts are not the sole site of the synthesis of the stimulatory substance or if the exogenously supplied stimulatory substance permeates into all vegetative cells, what can control the pattern? The stimulation of the formation of akinetes by H_2 suggests that the pattern may be controlled by some substance which is reduced by the uptake hydrogenase in heterocysts, and then moves into neighboring vegetative cells. As discussed in the Introduction, reducing conditions may well characterize the interior of heterocysts, which might therefore provide an excess of some reductant to vegetative cells. The pattern, akinetes forming contiguous with heterocysts, could be explained

if the onset of sporulation requires (i) a stimulatory substance that might enter all of the cells of the filament, and (ii) a reductant provided by heterocysts.

Both C_2H_2 and the product of its reduction by nitrogenase, C_2H_4, inhibit the formation of akinetes. From the rate of reduction of C_2H_2, 12.3 μmoles (mg Chl)$^{-1}h^{-1}$ (Table XII), and the concentration of chlorophyll (9.6 μg Chl/flack) at the end of the period of assaying the effect of gases upon the formation of akinetes, the amount of C_2H_4 produced during the final 12 hr of that period may be calculated to be 12.3 (reduction rate) x 9.6 x 10^{-3} (mg Chl) x 12 (hr) x 22.4 (μl/μmole) x $\frac{298}{273}$ (correction to 25°) = 34 μl, or 0.012% of the 280-ml effective volume of the flask. The inhibition of sporulation by C_2H_2 is therefore too great to be attributed solely to the C_2H_4 produced from the C_2H_2, but may be ascribed to a combination of that C_2H_4 and to the C_2H_2 itself. This is the first report of the effect of a low concentration of C_2H_4 on a prokaryote.

The fact that low concentrations of tryptophan stimulate sporulation gains interest from the fact that among amino acid analogues tried by Mitchison and Wilcox (1973), only 7-azatryptophan was found to alter the pattern of spacing of heterocysts. Pheylalanine, another aromatic amino

acid, also strongly stimulated sporulation although at a 16-fold greater concentration. It is possible that these aromatic amino acids are normally involved in the control of the differentiation of akinetes.

REFERENCES

REFERENCES

Allen, M. B. and Arnon, D. I. (1955) Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by *Anabaena cylindrica* Lemm. Plant Physiol. 30:366-372.

Apte, S. K., Rowell, P. and Stewart, W. D. P. (1978) Electron donation to ferredoxin in heterocysts of the N_2-fixing alga *Anabaena cylindrica*. Proc. Roy. Soc. Lond. B 200:1-25.

Bharadwaja, Y. (1933) Contributions to our knowledge of the Myxophyceae of India. Ann. Bot. 47:117-143.

Bothe, H. (1970) Photosynthetische Stickstoffixierung mit einem zellfreien Extrakt aus der Blaualge *Anabaena cylindrica*. Ber. dt. bot. Ges. 83:421-432.

Bothe, H., Tennigkeit, J., Eisbrenner, G. and Yates, M.G. (1977) The hydrogenase-nitrogenase relationship in the blue-green algae *Anabaena cylindrica*. Planta 133:237-242.

Bradley, S. and Carr, N. G. (1971) The absence of a functional photosystem II in the heterocysts of *Anabaena cylindrica*. J. gen. Microbiol. 68:xiii-xiv.

Canabaeus, L. (1929) Über die Heterocysten und Gasvakuolen der Blaualgen und ihre Beziehung zueinander. Pflanzenforschung 13:1-48.

Cardemil, L. and Wolk, C. P. (1976) The polysaccharides from heterocyst and spore envelopes of a blue-green alga. Methylation analysis and structure of the backbones. J. Biol. Chem. 251:2967-2975.

Carter, H. J. (1856) Notes on the freshwater infusoria of the island of Bombay. No. 1. Organization. Ann. and Magaz. Nat. Hist. Ser. II 18:115-132, 221-249.

Clark, R. L. and Jensen, T. E. (1969) Ultrastructure of akinete development in a blue-green alga, *Cylindrospermum* sp. Cytologia 34: 439-448.

Conley, R. T. (1972) Infrared spectroscopy. 2nd ed. Allyn and Bacon, Inc., Boston, MA.

Dharmawardene, M. W. N., Haystead, A. and Stewart, W. D. P. (1973) Glutamine synthetase of the nitrogen-fixing alga *Anabaena cylindrica*. Arch. Mikrobiol. 90:281-295.

Donze, M., Haveman, J. and Schiereck, P. (1972) Absence of Photosystem 2 in heterocysts of the blue-green alga *Anabaena*. Biochim. Biophys. Acta 256:157-161.

Drawert, H. and Tischer, I. (1956) Über Redox-Vorgänge bei Cyanophyceen unter besonderer Berücksichtigung der Heterocysten. Naturwissenschaften 43:132.

Drews, G. (1955) Zur Frage der TTC-Reduktion durch Cyanophyceen. Naturwissenschaften 42:646.

Dunn, J. H. and Wolk, C. P. (1970) Composition of the cellular envelopes of *Anabaena cylindrica*. J. Bacteriol. 103:153-158.

Fay, P. (1969a) Metabolic activities of isolated spores of *Anabaena cylindrica*. J. Exp. Bot. 20:100-109.

Fay, P. (1969b) Cell differentiation and pigment composition in *Anabaena cylindrica*. Arch. Mikrobiol. 67:62-70.

Fay, P. and Kulasooriya, S. A. (1972) Tetrazolium reduction and nitrogenase activity in heterocystous blue-green algae. Arch. Mikrobiol. 87:341-352.

Fay, P., Stewart, W. D. P., Walsby, A. E. and Fogg, G. E. (1968) Is the heterocyst the site of nitrogen fixation in blue-green algae? Nature, Lond. 220: 810-812.

Fay, P. and Walsby, A. E. (1966) Metabolic activities of isolated heterocysts of the blue-green alga *Anabaena cylindrica*. Nature, Lond. 209:94-95.

Fisher, R. W. and Wolk, C. P. (1976) Substance stimulating the differentiation of spores of the blue-green alga *Cylindrospermum licheniforme*. Nature, Lond. 259:394-395.

Fogg, G. E. (1952) The production of extracellular nitrogenous substances by a blue-green alga. Proc. Roy. Soc. Lond. B 139:372-397.

Fogg, G. E. (1962) Extracellular products. In: Lewin, R. A. (Ed.) Physiology and Biochemistry of Algae. Pp. 475-489. Academic Press, New York.

Fogg, G. E. (1966) The extracellular products of algae. Oceanogr. Mar. Biol. Rev. 4:195-212.

Fritsch, F. E. (1904) Studies on Cyanophyceae. III. Some points in the reproduction of *Anabaena*. New Phytol. 3:216-228.

Fritsch, F. E. (1951) The heterocyst: a botanical enigma. Proc. Linn. Soc. Lond. 162:194-211.

Fujita, Y., Ohama, H. and Hattori, A. (1964) Hydrogenase activity of cell-free preparation obtained from the blue-green alga, *Anabaena cylindrica*. Plant & Cell Physiol. 5:305-314.

Geitler, L. (1932) Cyanophyceae, Aufl. 2, Akademische Verlagesellshaft, Leipzig.

Gentile, J. H. and Maloney, T. E. (1969) Toxicity and environmental requirements of a strain of *Aphanizomenon flos-aquae* (L.) Ralfs. Can. J. Microbiol. 15:165-173.

Glade, R. (1914) Zur Kenntnis der Gattung *Cylindrospermum*. Beitr. Biol. Pfl. 12:295-346.

Gorham, P. R. (1964) Toxic algae. In: Jackson, D. F. (Ed.) Algae and Man. Pp. 307-336. Plenum Press, NY.

Harder, R. (1917) Ernährungsphysiologische Untersuchungen an Cyanophyceen, hauptsächlich dem endophytischen *Nostoc punctiforme*. Z. Bot. 9:145-242.

Hartman, R. T. (1960) Algae and metabolites of natural waters. In: Tyron, C. A., Jr. and Hartman, R. T. (Eds.) The Ecology of Algae. Pp. 38-55. Spec. Publ. No. 2, Pymatuning Laboratory of Field Biology, University of Pittsburgh.

Jackim, E. and Gentile, J. (1968) Toxins of a blue-green alga: similarity to saxitoxin. Science 162: 915-916.

Jakob, H. (1961) Compatibilités, antagonismes et antibioses entre quelques algues du sol. Rev. gén. Bot. 68:5-72.

Jensen, T. E. and Clark, R. L. (1969) Cell wall and coat of the developing akinete of a *Cylindrospermum* species. J. Bacteriol. 97:1494-1495.

Jüttner, F. and Carr, N. G. (1976) The movement of organic molecules from vegetative cells into the heterocysts of *Anabaena cylindrica*. In: Codd, G. A. and Stewart, W. D. P. (Eds.) Proc. Second Internat. Symp. on Photosynthetic Prokaryotes. Pp. 121-123. FEMS, Dundee.

Leak, L. V. and Wilson, G. B. (1965) Electron microscopic observations on a blue-green alga, *Anabaena* sp. Can. J. Genet. Cytol. 7:237-249.

Lex, M. and Carr, N. G. (1974) The metabolism of glucose by heterocysts and vegetative cells of *Anabaena cylindrica*. Arch. Microbiol. 101:161-167.

Lockau, W., Peterson, R. B., Wolk, C. P. and Burris, R. H. (1978) Modes of reduction of nitrogenase in heterocysts isolated from *Anabaena* species. Biochim. Biophys. Acta 502:298-308.

Mackinney, G. (1944) Absorption of light by chlorophyll solutions. J. Biol. Chem. 140:315-322.

Meeks, J. C., Wolk, C. P., Thomas, J., Lockau, W., Shaffer, P. W., Austin, J. M., Chien, W.-S. and Galonsky, A. (1977) The pathways of assimilation of $^{13}NH_4^+$ by the cyanobacterium, *Anabaena cylindrica*. J. Biol. Chem. 252:7894-7900.

Meeks, J. C., Wolk, C. P., Lockau, W., Shilling, N., Shaffer, P. W. and Chien, W.-S. (1978) Pathways of assimilation of $[^{13}N]N_2$ and $^{13}NH_4^+$ by cyanobacteria with and without heterocysts. J. Bacteriol. 134: 125-130.

Miller, M. M. and Lang, N. J. (1968) the fine structure of akinete formation and germination in *Cylindrospermum*. Arch. Mikrobiol. 60:303-313.

Mitchison, G. J. and Wilcox, M. (1973) Alteration in heterocyst pattern of *Anabaena* produced by 7-azatryptophan. Nature New Biol. 246:229-233.

Pattnaik, H. (1966) Studies on nitrogen fixation by *Westiellopsis prolifica* Janet. Ann. Bot. 30:231-238.

Paudler, W. W. (1971) Nuclear Magnetic Resonance. Allyn and Bacon, Inc., Boston, MA.

Peterson, R. B. and Burris, R. H. (1976) Properties of heterocysts isolated with colloidal silica. Arch. Microbiol. 108:35-40.

Peterson, R. B. and Wolk, C. P. (1978a) Localization of and uptake hydrogenase in *Anabaena*. Plant Physiol. 61:688-691.

Peterson, R. B. and Wolk, C. P. (1978b) Localization and activity of nitrogenase in heterocysts isolated from *Anabaena variabilis* by a new procedure. Abst. 3rd Int. Symp. N_2 Fixation, Madison, WI.

Piskornik, Z. and Bandurski, R. S. (1972) Purification and partial characterization of a glucan containing indole-3-acetic acid. Plant Physiol. 50:176-182.

Prescott, G. W. (1960) Biological disturbances resulting from algal populations in standing waters. In: Tryon, C. A., Jr. and Hartman, R. T. (Eds.) The Ecology of Algae. Pp. 22-37. Spec. Publ. No. 2, Pymatuning Laboratory of Field Biology, University of Pittsburgh.

Quisenberg, K. S., Scolman, T. T. and Nier, A. O. (1956) Atomic mass of H^1, D^2, C^{12}, and S^{32}. Phys. Rev. 102:1071-1075.

Rippka, R., Neilson, A., Kunisawa, R. and Cohen-Bazire, G. (1971) Nitrogen fixation by unicellular blue-green algae. Arch. Nikrobiol. 76:341-348.

Rippka, R. and Stanier, R. Y. (1978) The effects of anaerobiosis on nitrogenase synthesis and heterocyst development in nostocacean cyanobacteria. J. Gen. Microbiol. 105:83-94.

Roper, R. and MA, T. S. (1957) A new microdistillation technique. Microchem. J. 1:248-251.

Simon, R. D. (1971) Cyanophycin granules from the blue-green alga *Anabaena cylindrica*: a reserve material consisting of copolymers of aspartic acid and arginine. Proc. Nat. Acad. Sci., U.S.A. 68:265-267.

Simon, R. D. (1977a) Macromolecular composition of spores from the filamentous cyanobacterium *Anabaena cylindrica*. J. Bacteriol. 129:1154-1155.

Simon, R. D. (1977b) Sporulation in the filamentous cyanobacterium *Anabaena cylindrica*. The course of spore formation. Arch. Microbiol. 111:283-288.

Simpson, F. B. and Neilands, J. B. (1976) Siderochromes in Cyanophyceae: Isolation and characterization of schizokinen from *Anabaena* sp. J. Phycol. 12:44-48.

Singh, H. N. (1967) Genetic control of sporulation in the blue-green alga *Anabaena doliolum* Bharadwaja. Planta 75:33-38.

Singh, H. N. and Srivastava, B. S. (1968) Studies on morphogenesis in a blue-green alga. I. Effect of inorganic nitrogen sources on developmental morphology of *Anabaena doliolum*. Can. J. Microbiol. 14:1341-1346.

Singh, R. N. and Sinha, R. (1965) Genetic recombination in a blue-green alga, *Cylindrospermum majus* Kuetz. Nature, Lond. 207:782-783.

Smith, R. V., Noy, R. J. and Evans, M. C. W. (1971) Physiological electron donor systems to the nitrogenase of the blue-green alga *Anabaena cylindrica*. Biochim. Biophys. Acta 253:104-109.

Stewart, W. D. P. and Codd, G. A. (1975) Polyhedral bodies (carboxysomes) of nitrogen-fixing blue-green algae. Br. phycol. J. 10:273-278.

Stewart, W. D. P., Haystead, A. and Pearson, H. W. (1969) Nitrogenase activity in heterocysts of blue-green algae. Nature, Lond. 224:226-228.

Stewart, W. D. P. and Lex, M. (1970) Nitrogenase activity in the blue-green alga *Plectonema boryanum* strain 594. Arch. Mikrobiol. 73:250-260.

Talpasayi, E. R. S. (1963) Polyphosphate containing particles of blue-green algae. Cytologia 28:76-80.

Talpasayi, E. R. S. and Bahal, M. R. (1967) Cellular differentiation in *Anabaena cylindrica*. Z. Pflanzenphysiol. 56:100-101.

Tel-Or, E., Luijk, L. W. and Packer, L. (1977) An inducible hydrogenase in cyanobacteria enhances N_2 fixation. FEBS Letters 78:49-52.

Tel-Or, E. and Stewart, W. D. P. (1975) Manganese and photosynthetic oxygen evolution by algae. Nature, Lond. 258:715-716.

Tel-Or, E. and Stewart, W. D. P. (1977) Photosynthetic components and activities of nitrogen-fixing isolated heterocysts of *Anabaena cylindrica*. Proc. Roy. Soc. Lond. B 198:61-86.

Thomas, J. (1970) Absence of the pigments of photosystem II of photosynthesis in heterocysts of a blue-green alga. Nature, Lond. 228:181-183.

Thomas, J., Meeks, J. C., Wolk, C. P., Shaffer, P. W., Austin, S. M. and Chien, W.-S. (1977) Formation of glutamine from [^{13}N]ammonia, [^{13}N]dinitrogen, and [^{14}C]glutamate by heterocysts isolated from *Anabaena cylindrica*. J. Bacteriol. 129:1545-1555.

Tyagi, V. V. S. (1974) Some observations on the pattern of sporulation in a blue-green alga, *Anabaena doliolum*. Ann. Bot. 38:1107-1111.

Ueda, K. (1971) Die quantitative Bestimmung des DNS-Gehalts in den Zellen von Cyanophyceen durch Fluorochromierung mit Coriphosphin. Biochem. Physiol. Pflanzen 162:439-449.

Ueda, K. and Sawada, M. (1972a) A cytochemical study of DNA in cells of Cyanophyceae. Proc. Seventh Internat. Seaweed Symp., pp. 201-204, John Wiley & Sons, NY.

Ueda, K. and Sawada, M. (1972b) DNA-content of akinete-cells in *Cylindrospermum*. Cytologia 37:519-523.

Walsby, A. E. (1974a) The extracellular products of *Anabaena cylindrica* Lemm. I. Isolation of a macromolecular pigment-peptide complex and other components. Br. phycol. J. 9:371-381.

Walsby, A. E. (1974b) The extracellular products of *Anabaena cylindrica* Lemm. II. Fluorescent substances containing serine and threonine, and their role in extracellular pigment formation. Br. phycol. J. 9:383-391.

Walsby, A. E. and Fogg, G. E. (1975) The extracellular products of *Anabaena cylindrica* Lemm. III. Excretion and uptake of fixed nitrogen. Br. phycol. J. 10:339-345.

Whitton, B. A. (1965) Extracellular products of blue-green algae. J. Gen. Microbiol. 40:1-11.

Whitton, B. A. (1967) Studies on the toxicity of polymyxin B to blue-green algae. Can. J. Microbiol. 13:987-993.

Wildon, D. C. and Mercer, F. V. (1963) The ultrastructure of the heterocyst and akinete of the blue-green algae. Arch. Mikrobiol. 47:19-31.

Winkenbach, F. and Wolk, C. P. (1973) Activities of enzymes of the oxidative and the reductive pentose phosphate pathways in heterocysts of a blue-green alga. Plant Physiol. 52:480-483.

Wolk, C. P. (1964) Experimental studies on the development of a blue-green alga. Ph.D. Thesis, Rockefeller Institute, NY.

Wolk, C. P. (1965) Control of sporulation in a blue-green alga. Develop. Biol. 12:15-35.

Wolk, C. P. (1966) Evidence of a role of heterocysts in the sporulation of a blue-green alga. Am. J. Bot. 53:260-262.

Wolk, C. P. (1968) Movement of carbon from vegetative cells to heterocysts in *Anabaena cylindrica*. J. Bacteriol. 96:2138-2143.

Wolk, C. P. (1979) Heterocysts, ^{13}N, and N_2-fixing plants. Proc. 3rd. Int. Symp. N_2 Fixation, Madison, WI. In press.

Wolk, C. P., Austin, S. M., Bortins, J. and Galonsky, A. (1974) Autoradiographic localization of ^{13}N after fixation of ^{13}N-labeled nitrogen gas by a heterocyst-forming blue-green alga. J. Cell Biol. 61:440-453.

Wolk, C. P. and Simon, R. D. (1969) Pigments and lipids of heterocysts. Planta 86:92-97.

Wolk, C. P., Thomas, J., Shaffer, P. W., Austin, S. M. and Galonsky, A. (1976) Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium, *Anabaena cylindrica*. J. Biol. Chem. 251:5027-5034.

Wolk, C. P. and Wojciuch, E. (1971) Photoreduction of acetylene by heterocysts. Planta 97:126-134.

Yamamoto, Y. (1972) The fatty acid composition of akinetes, heterocysts and vegetative cells in *Anabaena cylindrica*. Plant & Cell Physiol. 13: 913-915.

Yamamoto, Y. (1975) Effect of desiccation on the germination of akinetes of *Anabaena cylindrica*. Plant & Cell Physiol. 16:749-752.

Zastrow, E. M. von (1953) Uber die Organisation der Cyanophyceenzelle. Arch. Mikrobiol. 19:174-205.