GENETIC STUDIES IN THE BLUE-GREEN ALGAE

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ABSTRACT

Anacystis nidulans (Richt.) Dr. and Daily was purified by subculturing in modified liquid Cyanophycean medium. Aeration was found to be sufficient under the conditions employed without shaking the cultures. The organism grew best in medium buffered at pH 8. The optimum temperature was found to be about 40° C. Various vitamins, growth hormones and other compounds were added to liquid medium to determine whether any of these compounds were required for optimum growth. Biotin in low concentrations increased the growth rate of the organism, while all the other compounds tested inhibited growth under the conditions employed. Spontaneous mutants which were about 20 times as resistant to polymixin as wild type *A. nidulans* were selected in medium containing polymixin, and mutant cultures were established. Attempts to induce antibiotic-resistant mutants with 5-bromouracil were unsuccessful, and possible reasons are discussed. Possibilities for further genetic studies in the blue-green algae are mentioned.

INTRODUCTION

Little work has been done concerning the genetics of the blue-green algae. The blue-green algae are considered to have no chromosomes as found in other algal groups, and show no sexual recombination. Kumar (1962) reported apparent genetic recombination in *Anacystis nidulans* but did not state whether it involved sexuality or transformational effects, and in fact, whether or not bacteria-free cultures were used.

Blue-green algae have been studied so little from a physiological or genetic viewpoint because of the difficulty of obtaining pure cultures (Gerloff, Fitzgerald and Skoog, 1950). Many blue-green algae are surrounded by a gelatinous sheath (Smith, 1950) in which bacterial cells, which are difficult to remove, are often found (Gerloff, *et al.*, 1950). According to McDaniel, Middlebrook and Bowman (1962), separation of *Anacystis nidulans* (Richt.) Dr. and Daily from bacteria is particularly difficult because bacteria stick tenaciously to the mucoprotein sheath. Also, whenever living bacteria are streaked on agar along with blue-green algae, the bacteria rapidly outgrow and overgrow the algae.

Allen (1952) reviews the reports of pure cultures in the literature. She states that the earliest reports cannot be accepted

since the algae were not tested for purity on media that favor bacterial growth, and therefore may have been free only from gross contamination.

Many methods have been used for purifying blue-green algae, but none of these methods has been universally successful. Irradiation with ultraviolet light has been most commonly used. *Nostoc muscorum* Kütz., *Plectonema nostocorum* Gom., *Calothrix parietina* (Naeg.) Thuret, *Phormidium tenue* Gom., *Coccochloris peniocystis* (Kütz.) Dr. and Daily, *Aphanizomenon flos-aquae* Born. and Flah., *Diplocystis aeruginosa* (Kütz.) Trevis, *Gloeocapsa dimidiata* (Kütz.) Dr. and Daily (Gerloff, *et al.*, 1950), *Mastigocladus laminosus* Cohn (Fogg, 1951), *Chlorogloea fritschii* Mitra. (Fay and Fogg, 1962), *Nostoc entophytum* Born and Flah., *Calothrix scopulorum* (Weber and Mohr.) Ag. ex Born. and Flah. and *Oscillatoria brevis* Kütz. ex Gom. (Stewart, 1962) have been purified by this method which depends upon differences in susceptibility to ultraviolet irradiation between bacteria and the alga. Allen (1952), however, would rather purify cultures without using a possible mutagen.

According to some workers, antibiotics have been utilized successfully in the purification of blue-green algae. Stewart (1962) used antibiotics following ultraviolet irradiation to obtain his pure cultures. Provasoli and Pintner (1954) isolated

Phormidium persicinum by streaking the organism onto antibiotic medium, but stated that the rapid growth of filaments contributed greatly to successful isolation.

Not all workers consider antibiotics useful in purifying blue-green algae. Tchan and Gould (1961) found it possible to eliminate fungal contaminants with actidione and fungostatin, but had no success in freeing cultures from bacteria using either single antibiotics or combinations of antibiotics. They stated that although many of the bacteria, when tested alone, were sensitive to antibiotics, they were not affected in the presence of the algae.

Fogg (1942) purified *Anabaena cylindrica* Lemm. by treatment with dilute chlorine water but stated that this method was not useful in purifying any other species of blue-green algae.

The history of pure cultures reported by Allen (1952), as well as most of those reported here, includes only filamentous blue-green algae. The reported purification of unicellular species is much less common. Allen (1952) stated that streaked plates are of little value for purifying unicellular algae, since they do not grow as well as the accompanying bacteria. The only unicellular species of blue-green algae reported purified since those reported by Gerloff, *et al.* (1950) was *Anacystis nidulans*, which was purified by shaking with detergent and phenol, centrifuging, and plating on agar medium from which

any pure colonies present could be picked up (McDaniel, *et al.*, 1962).

None of the techniques for purification described in the literature have proved successful, according to Bunt (1961b), partly because one must rely largely on chance. He also tried techniques based on the assumption that an agent to which only the bacteria would be susceptible could be found, but these also gave disappointing results. The only technique which he found successful utilized selection of newly formed and uncontaminated trichomes. However, his technique is not applicable to species which do not form hormogonia readily, and, of course, not to unicellular species.

Another reason the blue-green algae have not been studied from a genetic viewpoint is the difficulty of culturing the organisms in the laboratory. Growth of some blue-green algae has been found to be superior in impure cultures as compared to bacteria-free cultures (Bunt, 1961a). Although the growth requirements of the class have not been thoroughly studied, many workers have contributed to this phase of study. The growth requirements reported for organisms in the class have been highly variable.

Allen and Arnon (1955a) found that although shaking is desirable for obtaining uniform cultures of *Anabaena cylindrica*, it is not strictly necessary for good growth if a large liquid-gas interface is present. They also found that although *A. cylindrica* could grow well

at high light intensity, growth was essentially the same whether 13 hours or 24 hours of light per day were supplied to the organism.

The optimum temperatures for growth of several species of blue-green algae were found to be quite variable by Kratz and Myers (1955). They found that *Anacystis nidulans*, when grown at 41° C, had the highest growth rate ever recorded for an alga, with a generation time of two hours.

It is generally assumed that blue-green algae thrive best under alkaline conditions (McLachlan and Gorham, 1961). Gerloff, *et al.* (1950) found that a pH of 8.0 to 9.5 was necessary for the successful cultivation of *Nostoc muscorum*. McLachlan and Gorham (1962) found that *Microcystis aeruginosa* Kutz. grew well at pH 7.0 to 10.0 with an optimum of pH 9.0 to 10.0, while Gerloff, *et al.* (1953), employing different methods, found that the same species grew very little from pH 6.0 to 8.0, with better growth at pH 9.0 and maximum growth at pH 10.0. Zehnder and Gorham (1960) stated that the growth of the same species was little affected throughout a pH range of 8.0 to 11.0. Kratz and Myers (1955) found that *Anacystis nidulans* had a broad optimum of pH 7.4 to 9.0, with little or no growth below pH 6.8.

The addition of organic compounds either has no effect or is toxic to some species of blue-green algae but increases the growth rate

of others. Kratz and Myers (1955) found that the addition of vitamins, soil, yeast and liver extracts did not increase growth in *Anacystis nidulans*. They also added several sugars and metabolic intermediates to the medium and obtained the same results. They concluded that *A. nidulans* appears restricted to assimilation of carbon dioxide as a carbon source. Bunt (1961a), working with an unidentified species of *Nostoc*, added yeast extract, casein hydrolysate, thiamine, biotin, vitamin B_{12} and a range of single amino acids without increasing the growth rate. He found, however, that a few drops of a 1 ppm solution of indole-3-acetic acid would effect the recovery of filaments in the colorless condition. Zehnder and Gorham (1960) added ten different vitamins to cultures of *Microcystis aeruginosa* with no effect. The addition of yeast extract was toxic to the organism.

Provasoli and Pintner (1954), on the other hand, found that *Phormidium persicinum* requires glutamate, glycine and alanine for optimum growth. This species, unlike other blue-green algae, also has a purine requirement. This requirement is satisfied by guanine. The addition of adenine, cytidilic acid and uracil also stimulates growth. They also found vitamin requirements in the organism which were satisfied by a mixture of thiamine, vitamin B_{12}, thymine and folinic acid. They stated that a thiamine requirement had been firmly established, while the determination of the activities of the

other components of the mixture was still in progress at the time of writing. Pintner and Provasoli (1958), however, reported that this thiamine requirement did not exist. Instead, vitamin B_{12} was found to be required by the organism, although all the other cobalamins, even factor B, could be substituted for intact B_{12}. They were unable to replace B_{12} with methionine or with deoxyribonucleotides as had been done in some bacterial studies.

An unusual trait of many blue-green algae is a requirement for sodium. Allen and Arnon (1955b) found that normal development of *Anabaena cylindrica* did not occur in the absence of sodium. The phycocyanin content of the cells was markedly lower, although the chlorophyll content was not affected. This requirement appears to be specific, and is not replaceable by K, Li, Rb or Cs. *Anacystis nidulans* also has been found to have a requirement for sodium (Kratz and Myers, 1955). Allen (1952) stated that 23 cultures of various Myxophyceae grow in sodium salt media without added potassium.

In order to study the genetics of an organism, mutant strains of the organism must be obtained. Mutants have rarely been found among the blue-green algae. Kumar (1962) was the first to report the establishment of mutant strains of a blue-green species. He obtained strains of *Anacystis nidulans* which were about ten times

as resistant to penicillin and polymixin as the wild type. These were derived from subcultures supplemented with gradually increasing concentrations of the respective antibiotics. He concluded that the strains seemed stable since a streptomycin-resistant strain was passed through 40 subcultures in the absence of the antibiotic, and still retained its resistance to the antibiotic. One strain which was resistant to both penicillin and streptomycin also was able to grow in chloramphenicol medium, although neither of the single mutants could do so. Kumar (1962) did not state whether or not his cultures were bacteria-free, so one cannot tell how much bacterial contaminants may have contributed to the growth of the mutant strains.

Singh and Singh (1964a), however, produced mutant strains from cultures that were reported to be pure. They used ultraviolet irradiation as a mutagenic agent and obtained several nutritional mutant strains of *Anabaena cycadeae* Reinke. One which grew only on medium supplemented with KNO_3, casein hydrolysate and glucose was found to have lost the nitrogen-fixing capacity of the wild type of this species. This strain was subjected to further irradiation, and three new strains were obtained. One was colorless, and light was not effective in supporting growth or stimulating pigment production, suggesting genetic control of pigment formation. The

second grew only in the dark. All photosynthetic pigments were present except β-carotene. The third had no change in pigments, but unlike the parental mutant strain, was unable to utilize nitrate nitrogen in the light. Singh and Singh (1964b) also obtained mutant strains from *Anabaena doliolum* Bharadwaja by using ultraviolet irradiation. One strain was characterized as a glucose-requiring, nitrogen-fixing mutant, while the other lyses after a certain period of time which is dependent on the medium. This constancy of lysis indicates genetic origin.

The present study involved work on purification, determination of optimum growth requirements and the production of mutant strains of *Anacystis nidulans*, using 5-bromouracil, for which no work has been reported yet.

MATERIALS AND METHODS

A culture of *Anacystis nidulans* (No. 625) was obtained from the Culture Collection of Algae at Indiana University. Although this was presumed to be a pure culture, it was found to contain not only bacteria, but also fungi.

Media and Culturing. The basal medium used was modified from the Cyanophycean medium described by Starr (1960). Ten per cent of the water in this medium was replaced by Sörensen's phosphate buffer (Gortner, 1949, p. 85) to buffer the medium at pH 8. Stock cultures were grown in 50 ml. of buffered liquid medium in 125 ml. Erlenmeyer flasks. Streaks and platings were made on agar medium in sterile disposable petri dishes and tissue culture flasks (Falcon Plastics).

Purification. Low speed centrifugation was employed for a few seconds to eliminate fungal mycelia, and for two minutes in attempts to eliminate many fungal spores and bacteria.

Basal medium with serial twofold dilutions of actidione ranging from 2000 μg/ml to 125 μg/ml was inoculated with a suspension of *A. nidulans*. Flasks containing these concentrations

of actidione, the basic medium to which both penicillin and bacitracin in serial concentrations ranging from 50 units/ml to 1.56 units/ml had been added, also were inoculated. The cultures of A. nidulans were allowed to grow for about two weeks, and portions of the culture were streaked onto agar medium in plastic tissue culture flasks. These cultures were grown until visible colonies were present. They were checked for the presence of bacteria by phase-contrast microscopy, and, after being transferred to liquid medium and allowed to grow, were streaked onto solidified Bacto Nutrient Broth (Difco).

A culture of A. nidulans which had been treated with actidione was obtained from Mr. Gerald Harwood, and subcultured numerous times in both liquid and solidified medium. These cultures were tested for the presence of bacteria by the same methods.

Stock cultures of A. nidulans were treated with polymixin (final concentrations: 20, 10 and 5 ppm), actidione (final concentrations: 1000, 500 and 250 μg/ml), and all possible combinations of these concentrations of actidione and polymixin. The cultures were tested for the presence of bacteria by phase-contrast microscopy after about two weeks.

Suspensions of A. nidulans containing no more than 1.5×10^6 bacterial cells/ml were diluted to concentrations of 19,000 algal cells/ml and 100 algal cells/ml, and 0.5 ml of these dilutions were

poured onto agar medium in petri dishes. These cultures were checked daily under the microscope for the presence of algal colonies which were free from bacteria. Any presumed bacteria-free colonies present were transferred to liquid medium, allowed to grow, and checked for the presence of bacteria on solidified Bacto Nutrient Broth (Difco). Aliquots of undiluted stock cultures were treated and checked by the same methods, as were cultures of mutant *A. nidulans*.

Optimum Growth Requirements. Impure cultures of *A. nidulans* were tested for aeration requirements. Flasks of algae in liquid medium were shaken while being illuminated for 16 hours per day. Similar cultures were grown under the same light and temperature conditions without shaking. Growth of these cultures was observed.

The effects of temperature on the growth of the organism were determined by placing cultures in temperatures of 23° C, room temperature (ca. 26° C) and 40 ± 5° C. Growth was determined by observation of the intensity of the green color of the cultures.

A series of flasks containing medium of varying pH was set up by incorporating 100 ml/liter of Sörensen's phosphate buffer (pH 4.5, 5.0, 6.0, 7.0 and 8.0) into Cyanophycean medium. Growth was determined by observation of the intensity of the color of the cultures.

A culture of A. nidulans which had been growing at 40 ± 5° C for nine days was analyzed with a Bausch and Lomb Spectronic 20, and was found to have a transmittance peak at 555 mμ. Older, denser stock cultures were analyzed and were found to have a transmittance peak at the same wave length. The concentration of cells in the nine-day old culture was determined with a haemocytometer. Serial two-fold dilutions then were made from the culture. The concentration of cells in each dilution was determined, as was the per cent transmittance of each dilution at 555 mμ. These results were graphed, and the concentrations of cells in the growth tests with supplemented medium were interpolated from the graph.

The effects of seven vitamins (folic acid, ascorbic acid, pyridoxine, niacin, riboflavin, inositol and thiamine) were determined by incorporating each vitamin into basal liquid medium at concentrations of 1000, 100 and 10 ppm. A cobalamine complex containing vitamin B_{12} (3000μg/g) was incorporated into basal medium at the same concentrations, yielding concentrations of 30.0, 3.0 and 0.3 ppm vitamin B_{12}. Biotin was incorporated into basal medium in concentrations of 500, 50 and 5 ppm. Ten ml of each of these media were then inoculated with a drop containing 4×10^6 algal cells. The cultures were grown at 37 ± 1° C, along with control cultures.

Growth was determined after seven days with a Bausch and Lomb Spectronic 20 at a wave length of 555 $m\mu$.

A series of test tubes containing 10 ml of liquid medium containing indole-3-acetic acid in concentrations of 1000, 100 and 10 ppm, and a series containing the same concentrations of gibberellic acid in place of IAA were inoculated with 4×10^6 algal cells. These cultures were grown along with control cultures for seven days at $37 \pm 1^\circ$ C, at which time growth was determined spectrophotometrically at a wave length of 555 $m\mu$.

Casamino acids were added to sterile disposable test tubes (Falcon Plastics) containing basal medium in concentrations of 10,000, 5000, and 2500 ppm. The casamino acids used contained 3700 ppm sodium chloride. A tube of basal medium containing 3700 ppm sodium chloride was therefore used as a control.

Basal medium containing 20,000, 10,000, 3700, 1850 and 925 ppm sodium chloride was placed into sterile disposable test tubes and each was inoculated with 4×10^6 algal cells. These cultures were tested for growth by the same method as were the previous cultures.

Mutation. Some cultures which had been treated with polymixin grew back after the cultures had apparently died. Cells from these

cultures were centrifuged, washed and added to medium containing polymixin at concentrations of 40, 20 and 10 ppm. Cells from a culture that had no previous exposure to polymixin were added to separate flasks containing these media as controls. Cells from those cultures which grew in polymixin were added to medium containing 80, 40 and 20 ppm polymixin. Controls were again established. The growth of these cultures was observed and recorded.

Basal medium containing 50, 100 and 200 ppm 5-bromouracil was inoculated with A. nidulans and allowed to grow for four days. At the end of this time, 0.5 ml from each culture was plated on agar medium to which had been added 100μg/ml streptomycin, 10 μg/ml streptomycin, 100μg/ml neomycine and 10μg/ml neomycine, respectively. Cells which had not been exposed to 5-bromouracil were plated on these antibiotic media as controls. A series of these plates was incubated at 40 $\pm$ 5° C, and another series was grown at 22 $\pm$ 1° C. Whenever a colony of algae became visible, it was transferred to liquid medium in order to increase the number of cells. Cells from these cultures then were placed in liquid medium containing the antibiotic on which it had previously grown at concentrations of 1000μg/ml and 100μg/ml, as well as on solid

medium containing 100μg/ml and 10μg/ml. Growth was observed and compared to control cultures in the same medium.

RESULTS AND CONCLUSIONS

Purification. Low speed centrifugation was found to eliminate all fungal mycelia after a few seconds. When the suspension was recentrifuged for two minutes, most of the bacteria and fungal spores remained in the supernatant. This method was not sufficient to purify an algal culture, however, as a few spores and bacterial cells were either of sufficient density to be sedimented with the algal cells, or were so closely associated with the algal cells that they were sedimented at the same time.

The amount of contamination present in stock cultures was reduced slightly by the addition of actidione in all concentrations used. However, this reduction was not sufficient for purification. Actidione in the presence of penicillin and bacitracin also reduced the amount of contamination, but the cultures still contained bacterial cells and some fungal spores.

Treatment of stock cultures with polymixin brought about the death of most of the algal cells, even in concentrations as low as 5 ppm. However, many bacterial cells remained in the cultures. Actidione had only a slight effect in removing fungal contamination, but did not affect bacteria. Combinations of polymixin and

actidione killed most of the algal cells, without eliminating contaminants from the cultures.

Only a few bacterial colonies arose from plating cultures containing 95 cells/ml, but no algae were present. Algal growth was evident on one plate which had been inoculated with 9500 algal cells. These were transferred to liquid medium, allowed to grow, and then an aliquot was plated on solidified Bacto Nutrient Broth (Difco). After two days, some bacterial growth was evident, indicating that a pure culture had not been obtained. Three mutant clones of algae growing in 40 ppm polymixin were treated by the same methods and were found to have some contamination. One stock culture which had been subcultured several times from the culture donated by Mr. Harwood was found to contain no contaminants. This culture was used for growth tests in supplemented medium.

Optimum Growth Requirements. Shaking impure cultures of *A. nidulans* allowed the contaminating bacteria present to grow at an accelerated rate, thus crowding out the algae. Those algal cultures growing under identical conditions except for shaking grew faster, indicating that shaking is not required for the growth of *A. nidulans*.

Anacystis nidulans grows at a much faster rate at $40 \pm 5^{\circ}$ C than at room temperature (ca. 26° C) or at $22 \pm 1^{\circ}$ C. Differences

between cultures grown at 22 $\pm$ 1° C and at room temperature were not observed.

Anacystis nidulans was found to grow very well at pH 8 in medium containing Sörensen's phosphate buffer, only slightly at pH 7, and no growth was observed at pH 6 or below. For this reason, all cultures were grown in medium buffered at pH 8.

Of all the supplements tested, only biotin in concentrations of 50 and 5 ppm increased the growth rate of A. nidulans. Cultures grown in medium containing 5 ppm biotin had the highest growth rate. Casamino acids, sodium chloride, and all the vitamins tested except biotin inhibited growth of A. nidulans severely (Table 1.).

Mutation. After cultures treated with polymixin had apparently died, those cells in the cultures which were resistant to polymixin grew, forming cultures resistant to the antibiotic. These resistant cultures only appeared in medium containing 5 or 10 ppm polymixin. When cells from these cultures were transferred to medium containing 10, 20 and 40 ppm polymixin, each culture transferred to 10 ppm polymixin showed visible growth at the end of two weeks. Some cultures had grown slightly in 20 and 40 ppm polymixin during this time. After two more weeks, growth had occurred in all of

these cultures, even in 40 ppm polymixin. When transferred again to 20, 40 and 80 ppm polymixin for incubation at 37 $\pm$ 1° C, growth occurred within two days in cultures containing 20 and 40 ppm polymixin. Only a small amount of growth was evident in 80 ppm polymixin after 14 days.

In order to determine the resistance of wild type *A. nidulans* to polymixin, cells were inoculated into medium containing 4.0, 2.0, 1.0, 0.5 and 0.1 ppm polymixin. All of these cultures except the one in 4.0 ppm polymixin grew as well as the control, while growth of the organism in 4 ppm polymixin was much slower than the control. Therefore, wild type *A. nidulans* appears to be resistant to 2 ppm polymixin. Since the mutant strains obtained were resistant to 40 ppm polymixin, it may be concluded that these mutant strains are about 20 times as resistant to polymixin as is wild type *A. nidulans*.

When cultures exposed to 200, 100 and 50 ppm 5-bromouracil were plated on antibiotic medium, all cultures, including the controls, grew on the lowest concentrations used of both streptomycin and neomycine at 22 $\pm$ 1° C. Among those cultures grown at 40 $\pm$ 5° C, only one plate containing 10 μg/ml neomycine and one plate containing 10 μg/ml streptomycin showed growth of algae. The colonies from each of these plates were transferred to liquid medium,

allowed to grow, and replated along with control cultures. Growth again only occurred on medium containing 10μg/ml streptomycin and 10μg/ml neomycine. It may therefore be concluded that 5-bromouracil caused no mutations which would allow A. nidulans to grow on either streptomycin or neomycine, or caused so few that all mutants would be missed in the samples that were plated.

Table 1. Growth of cultures in modified Cyanophycean medium supplemented with various compounds.

Supplement	Concn ppm	% Transmittance Samples #1	#2	#3	Ave.	Ave. No. cells/ml x 10^6
NaCl	$2x10^4$	99.0	99.5	100	99.5	0.4
	$1x10^4$	100	100	100	100	0.0
	3700	96.0	95.0	97.0	96.0	4.2
	1850	95.5	96.0	96.0	95.8	4.6
	925	96.5	97.0	96.0	96.5	3.6
Indole-3-Acetic Acid	1000	78.0	76.5	81.0	78.5	**
	100	96.0	93.0	96.0	95.0	5.2
	10	96.0	96.5	98.0	96.8	3.4
	1	96.0	90.0	96.5	94.2	6.4
	0.1	96.5	97.5	97.5	96.8	3.2
Gibberellic Acid	1000	98.5	99.0	100	99.2	0.7
	100	88.0	93.5	95.5	92.3	8.6
	10	96.0	95.5	96.0	95.8	4.6
	1	95.5	96.0	94.5	95.3	5.0
	0.1	94.0	93.5	92.5	93.3	7.2
Casamino Acids	$1x10^4$	94.5	95.0	86.5	92.0	8.8
	5000	92.0	95.0	90.0	92.3	8.6
	2500	95.0	95.5	97.5	96.0	4.2

Table 1--Continued.

Riboflavin	1000	87.0	97.0	96.0	93.3	7.2
	100	100	100	100	100	0.0
	10	94.5	93.0	94.0	93.8	6.6
Inositol	1000	93.0	94.0	93.0	93.3	7.2
	100	95.0	96.0	97.0	96.0	4.2
	10	94.5	96.5	96.0	95.7	4.7
Ascorbic Acid	1000	100	100	100	100	0.0
	100	95.5	95.0	94.5	95.0	5.2
	10	95.0	97.0	95.0	95.7	4.7
Pyridoxine	1000	100	99.5	98.5	99.3	0.6
	100	99.5	100	99.0	99.5	0.4
	10	97.5	97.0	97.5	97.3	2.8
Niacin	1000	98.0	98.5	99.0	98.5	1.3
	100	94.0	93.5	92.5	93.3	7.2
	10	96.0	96.5	96.5	96.3	4.0
Folic Acid	1000	76.0	80.5	70.0	75.5	***
	100	98.0	99.5	100	99.2	0.7
	10	97.0	95.0	94.0	95.3	5.0
Cobalamine Complex	1000	97.5	97.0	98.0	97.5	2.4
	100	97.5	97.0	94.5	96.3	4.0
	10	93.0	77.0	97.0	89.0	12.2

Table 1--Continued.

Thiamine	1000	99.0	100	99.5	·99.5	0.4
	100	99.0	96.0	97.5	97.5	2.4
	10	95.0	96.0	97.0	96.0	4.2
Biotin	500	94.0	91.5	94.5	93.3	7.2
	50	65.5	38.5	95.0	66.3	39.2
	5	24.5	33.5	97.0	51.7	57.0
Control		31.0	28.0	68.0	70.2	34.4
		98.5	95.5	100		

** Brown color observed, indicating that the IAA had been oxidized.

*** Precipitate was formed.

DISCUSSION

<u>Purification.</u> The methods employed for the purification of *A. nidulans* relied largely on chance, as previously stated by Bunt (1961b). The removal of gross contamination was relatively simple, but the elimination of small concentrations of bacteria in an algal culture was quite difficult. The difficulty encountered in the purification of *A. nidulans* did not involve removing bacteria from the sheaths of the cells, as stated by McDaniel, *et al.* (1962). When cultures were observed under the phase-contrast microscope, no bacteria were observed clinging to the algal sheath. Any bacteria observed in these cultures were freely suspended. The difficulty in eliminating bacteria from the cultures involved the differences in the growth rates of *A. nidulans* and the contaminating bacteria. When cultures were streaked on agar medium, bacterial colonies appeared much more rapidly, while algal colonies did not appear for several days, if at all. These results agree with the findings of Allen (1952) and McDaniel, *et al.* (1962), who stated that streaking is of little value for purifying unicellular blue-green algae, as the contaminating bacteria rapidly overgrow the algae. However, streaking is not completely without merit, as an occasional pure colony of the alga may by chance occur far enough away from rapidly growing

bacterial colonies that it may be transferred before becoming recontaminated. After gross contamination has been eliminated, subculturing of algal cultures also may be of value in purifying unicelluair blue-green algae. This is possible only by the chance of a given aliquot of a culture containing no bacteria, and only can be used successfully when the concentration of bacteria in the culture is very low.

The antibiotics used in attempts to purify A. nidulans were not successful. The algae were more susceptible to polymixin than the accompanying bacteria, while actidione, which did not kill the algae, also had little or no effect on the bacteria. These results are in agreement with the findings of Tchan and Gould (1961).

Ultraviolet irradiation was not used for purification of A. nidulans, even though it has been reported successful for the purification of other blue-green algae (Gerloff, et al., 1950; Fogg, 1951; Fay and Fogg, 1962; Stewart, 1962). The author is in agreement with Allen (1952), who stated that it is preferable to purify algal cultures without using possible mutagenic agents. This is particularly important where mutant strains of an organism are to be established, as the cultures used might contain a much higher proportion of mutant individuals than the stock cultures.

Optimum Growth Requirements. Shaking cultures of *Anabaena cylindrica* was found to be desirable for obtaining uniform cultures, but not necessary when a large liquid-gas interface was present (Allen and Arnon, 1955a). The results of this study indicate that shaking *A. nidulans* is not desirable when impure cultures are used. The use of 50 ml of medium in 125 ml Erlenmeyer flasks provides a sufficient liquid-gas interface for good growth of the alga, whereas shaking the cultures provides better aeration for the growth of bacterial contaminants.

The optimum temperature for rapid growth of *A. nidulans* was found to be 41° C by Kratz and Myers (1955). The present study did not involve determination of the optimum temperature within a small temperature range, as did that of Kratz and Myers (1955). However, growth was found to be much more rapid at 41° C than at room temperature or lower, thus generally agreeing with the results obtained by Kratz and Myers.

The results of this study agree with the statement of McLachlan and Gorham (1961) that blue-green algae generally thrive best under alkaline conditions. The optimum pH for growth of *A. nidulans* was found to be 8.0, which is in agreement with the findings of Kratz and Myers (1955).

Anacystis nidulans was found to require 5 ppm biotin for optimum growth. Kratz and Myers (1955) stated that the addition of vitamins gave only negative results, but did not state what vitamins were tested. All vitamins (except biotin) which were tested in this study inhibited growth of the algae. Bunt (1961a) found that the addition of a low concentration of indole-3-acetic acid (IAA) would effect the recovery of filaments of Nostoc sp. which were in a colorless condition. However, IAA was found to inhibit the growth of A. nidulans.

Requirements for sodium have been found for some filamentous blue-green algae (Allen, 1952; Allen and Arnon, 1955), as well as for A. nidulans (Kratz and Myers, 1955). The addition of sodium chloride to the basal medium in this study, however, greatly inhibited growth of A. nidulans. One possible explanation of this inhibition is that too high a concentration of sodium was present in the medium. The basal medium contained 0.47% Na_2HPO_4, which was required for buffering the medium at pH 8. The addition of more sodium, as sodium chloride, may have caused inhibition of growth.

Mutation. Only two workers have reported the establishment of mutant strains of blue-green algae. Kumar (1962) selected spontaneous antibiotic-resistant mutants of A. nidulans, while Singh and Singh obtained nutritional mutants and mutants lacking one or

more photosynthetic pigments of *Anabaena cycadeae* (1964a) and a nutritional mutant and a lysing mutant of *Anabaena doliolum* (1964b). No reports of attempts to use chemical mutagens in studies with blue-green algae have been published.

Several independent cultures of *A. nidulans* were established which were about twenty times as resistant to polymixin as is the wild type, as compared to the mutant strains established by Kumar (1962) which were ten times as resistant to antibiotics as wild type *A. nidulans*.

Specific chemical mutagens, such as 5-bromouracil, have been used with success in obtaining mutants for genetic studies of bacteriophage (Benzer and Freese, 1958; Freese, 1959a, 1959b; Benzer, 1961; Terzaghi, Streisinger and Stahl, 1962). Freese (1959b) stated that the mechanism of mutation involves incorporation of 5-bromouracil into the DNA of the bacteriophage, a pairing error during replication of DNA, and the ultimate replacement of a purine for a purine and a pyrimidine for a pyrimidine. Mutations caused by 5-bromouracil may therefore involve only a single base pair. Spontaneous mutations may involve the replacement of a purine by a pyrimidine and vice versa (Freese, 1959a), or, more likely, the insertion or deletion of a base pair (Brenner, *et al.*, 1961). The present study involved attempts to isolate mutants caused by treatments

of A. nidulans with 5-bromouracil. Since antibiotic medium was used for selection of antibiotic-resistant mutants, no other mutant types which might have been induced could be found. No mutants resistant to either streptomycin or neomycine were found, but this does not necessarily mean that no other mutant types were produced. It is possible that the 5-bromouracil was unable to penetrate the sheath or the cell wall of the cells, and therefore did not come into contact with the algal DNA, that no chance collisions occurred with the enzymes responsible for DNA replication, or that the algae were unable to incorporate the 5-bromouracil into a nucleotide. A change in a nucleotide pair will produce an observable mutation only if it causes the formation of a protein with an altered function (Freese, 1959a). Perhaps base pair substitutions occurred, but only a non-essential amino acid was changed, and no protein function was altered. These are some possible reasons why no 5-bromouracil induced mutants were obtained.

Future work with 5-bromouracil, other base analogues and other mutagenic agents may produce more mutant types with which further genetic studies can be made. Attempts should be made to isolate morphological mutant types, as well as nutritional and antibiotic-resistant strains. When several mutant strains have been isolated, work should progress toward proving the presence of sexual

recombination, or, if this should not prove possible, toward utilizing transformation in order to determine genetic mechanisms in the blue-green algae.

SUMMARY

1. *Anacystis nidulans* was apparently purified by subculturing a culture which was free from gross contamination. Other methods used were unsuccessful.

2. Rapid growth of *A. nidulans* was found to occur at a temperature of approximately 40° C.

3. *Anacystis nidulans* grows most rapidly in medium buffered at pH 8.

4. *Anacystis nidulans* requires biotin in low concentrations for optimum growth under conditions of this study. All other vitamins tested, as well as growth hormones, casamino acids and sodium chloride inhibited growth.

5. Mutant strains were selected in antibiotic medium which were about 20 times as resistant to polymixin as is wild type *A. nidulans*. These strains arose from spontaneous mutations. Attempts to induce mutation with 5-bromouracil were unsuccessful.

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