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The major nutrient requirements of the blue-green alga Anabaena spiroides Klebahn were investigated. It was hoped that the results would allow the development of a balanced culture medium in which the alga would exhibit rapid growth and retain typical morphological characteristics.

The alga was isolated from Upper Klamath Lake, Oregon, and was introduced to culture in Gerloff's modification of Chu's number 10 medium. This culture solution was modified by addition of Tris (hydroxymethyl) aminomethane (Tris) and the substitution of hydrogen ferric ethylenediamine di-o-hydroxyphenylacetate (EDDHA) for ferric citrate-citric acid.

Attempts to obtain bacteria-free cultures by use of antibiotics, pasteurization and ultraviolet irradiation were unsuccessful, probably because of the extremely thick gelatinous matrix surrounding the filaments.

Addition of nitrogen to the culture medium was found to be unnecessary for growth. This finding strongly suggests Anabaena spiroides is capable of nitrogen-fixation but this cannot be proven until demonstrated in bacteria-free culture. Phosphorous, although required in relatively high concentrations for optimum growth, is apparently accumulated within the cells allowing the organism to survive long periods of growth in phosphorous-deficient medium. It appears that many of the major elements are either required in very minute concentrations or that their accumulation is possible. The provision of iron as the alkaline stable, chelate complex EDDHA stimulated growth and reduced the concentration of iron required in the medium. There was a definite, high requirement for calcium. The micro-nutrient requirements were not studied.

The physiologically balanced medium developed provided enhanced growth of the alga so long as a nitrogen source was included. When nitrogen was eliminated growth was identical to that obtained in the original unbalanced medium.

The alga requires a highly alkaline medium and is inhibited by high light intensities.

MINERAL REQUIREMENTS FOR THE GROWTH OF *ANABAENA SPIROIDES* IN VITRO

by

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MINERAL REQUIREMENTS FOR THE GROWTH OF *ANABAENA SPIROIDES IN VITRO*

INTRODUCTION

Blue-green algae are very intriguing organisms. They are morphologically simple, bearing closer resemblance to bacteria than to other green plants; they are ubiquitous, extending their range from the arctic and alpine snow melt-water to thermal springs, from desert soils to plankton communities of eutrophic lakes and they are capable of living an independent existence as well as living symbiotically with higher plants and fungi. Although unique in many ways, they are among the least studied of plants. This situation is changing, however, as the blue-greens make their presence known by encroaching on the lakes, rivers, and streams made suitable for their colonization by pollution.

The occurrence of blue-green algae as the dominant forms in eutrophic aquatic habitats is so well established that they are often used as indicators of pollution. It is well known that they contribute to a decrease in the value and utility of water supplies both by the unesthetic and toxic changes that they cause and by the contribution they make to the process of eutrophication. Not only do they greatly increase the amount of organic material when present in "bloom" proportions but they may also fix nitrogen resulting in marked

enrichment of the habitat.

Klamath Lake, a large eutrophic lake in southern Oregon, is an example of a body of water which has been made unsuitable for many human uses by massive cyanophycean blooms. The algal populations inhabiting this lake, and its influent and effluent rivers, have become of immediate concern following the suggestion to divert the water from the Klamath Drainage Basin into drainage systems in central California.

The problem has become one of determining whether conditions existing in the lakes, rivers and streams into which this water would be diverted, would be made suitable for the establishment of the dominant cyanophycean organisms. In order to pursue such a study the organisms must be isolated and established in culture. Once in culture environmental and physiological conditions may be systematically controlled and the responses of the organisms studied.

This thesis reports an investigation of Anabaena spiroides Klebahn using cultural methods. Here are summarized the methods used to introduce the alga into culture, the purification procedures attempted, and the study of nutrition leading to the development of a medium supporting vigorous growth.

MATERIALS AND METHODS

Materials

Experimental Algal Organism

The organism used in this study, Anabaena spiroides, is a co-dominant in a blue-green algal bloom appearing annually in Upper Klamath Lake, latitude 42. 20 N, longitude 121. 30 W, Klamath County, Oregon. It occurs associated with Anacystis aeruginosa, Anabaena circinalis and Gloeotrichia echinulata. Aphanizomenon flos-aquae, also abundant in the lake, usually occurs in bloom proportions prior to the establishment of the Anacystis-Anabaena bloom. Collections of Anabaena spiroides,used as inoculum in establishment of cultures, were made off Modoc Point at various times during the summers of 1963 and 1964.

Water, Salts and Glassware

Distilled water was used in the preparation of all media but to ensure greater purity for nutritional studies, all glass redistilled water was utilized.

All salts used were of reagent grade.

Borosilicate glassware was used in this study. All glass containers were washed with Dreft detergent or RBS 5 Concentrated

Cleaning Solution (Fisher) followed by seven rinses in tap water and seven in distilled water. Glassware utilized in nutritional studies involving phosphate, iron, potassium and magnesium was rinsed with concentrated HCl followed by rinsing in copious amounts of glass distilled water.

Antibiotics and Other Organic Compounds

Indole-3-acetic acid, used to test the effect of auxin on Anabaena, was obtained from Eastman Organic Chemicals, New York.

Catalase (Calbiochem) was used in the crude form.

USP anhydrous caffeine (1, 3, 7-trimethyl-2, 6 dioxypurine) was obtained from Calbiochem.

Antibiotic screening was performed with Difco Bacto-Sensitivity Disks. The effects on algal and bacterial growth were studied with the following antibiotics: Intraveneous Terramycin hydrochloride (Chas. Pfizer & Co., Inc.). Intraveneous Aureomycin hydrochloride (Lederle Laboratory, Division of American Cyanimid Company), USP Neomycin sulfate, grade B (Calbiochem).

Methods

Collection

The alga was collected with the aid of a 20 mesh plankton net. The concentrated material was placed in 1) lake water, 2) millipore

filtered lake water, 3) ASMT medium or 4) Gerloff's modification of Chu No. 10 and transported to the laboratory. On one occasion Anabaena filaments were removed from samples of lake water with a micropipette and passed through three washings of sterile medium prior to transportation.

In the laboratory algal filaments were transferred by micropipette to culture flasks where they were maintained as stock inoculum.

Sterilization

All media used in the cultural and nutritional studies were autoclaved at 20 lbs pressure for 15 minutes. All micropipettes and other equipment used in making transfers were also autoclaved.

Transfer Techniques

Initially, transfer of the alga to fresh medium was accomplished by micropipettes which were drawn into extremely fine capillary tips. It was possible with these instruments to pick a single filament from the medium. These filaments were transferred through 10 consecutive washings in sterile medium before inoculation into large culture flasks. The concentration of algal filaments in the final flask was not regulated.

After obtaining unialgal cultures, inoculation of new cultures was performed simply by the transfer of determined volumes of stock

culture to fresh medium. The volume of inoculum was regulated by the volume of the sub-culture, 3 ml of algal suspension into 30 ml of medium and 25 ml into 250 ml of culture solution.

Culture Conditions

Cultures were maintained initially in Erlenmeyer flasks containing 30 ml of medium. In order to provide a larger number of cells having identical culture history, stocks were increased to 250 ml of medium contained in liter flasks.

Stocks were grown in daylight in a north facing window with deluxe, warm white, fluorescent tubes providing continuous supplementary light. The light intensity in this case varied from day to day and ranged from approximately 100 foot candles during the night to 400 foot candles on bright sunny afternoons.

In order to minimize the variability of quality and intensity of light, an upright food freezer was converted to a controlled environment growth chamber. Three tube, circular fluorescent units were mounted in the top and in the bottom of the cabinet to provide light intensities of approximately 100 foot candles. It was found that to obtain good growth in this chamber blue wavelengths emanating from the tubes had to be reduced. This was done by interposing several layers of red cellophane, which also reduced the intensity to 80-85 foot candles.

In addition to the cabinet a large trough was constructed in which flasks were immersed in a water bath at 15°C. Light was provided by deluxe, cool white, fluorescent tubes giving intensities ranging from 50 to 70 foot candles. Outside light was excluded by use of heavy black drapes.

Studies of mineral nutrition were conducted in a constant temperature room provided with deluxe, cool white, fluorescent illumination. The light intensity over the culture area varied from 85 to 96 foot candles. The flasks, in this case, were placed randomly so as to minimize this variability.

The temperature of the cultures in all of the above situations was held between 13 and 15°C. In the window and trough this was accomplished by immersion of the flasks in a cooled water bath. The temperature of the bath was maintained by water from a refrigerated cooler and circulated in copper coils placed in the bottom of the trough.

Media Used

The media used in initial attempts to introduce Anabaena spiroides to culture were McLachlans's (1961) modification of Provasoli's artificial sea water medium, called "ASM", and Gerloff's (1950) modification of Chu's number ten medium designated "G-10". Both ASM and G-10 were amended by the addition of 0.48 mg Tris

Table 1. Concentration of the major nutrient minerals in the basic media (mg/l)

Element	Compound	Medium		
		ASM	Gerloff's	Link River
S	$MgSO_4$	49.26	-	50.0
S	Na_2SO_4	-	14.6	-
Mg	$MgCl_2$	40.65	20.9	-
Mg	$MgSO_4$	-	-	see above
Ca	$CaCl_2 \cdot 2H_2O$	14.71	35.9	-
N	$NaNO_3$	85.08	41.3	1.04
N	NH_4Cl	-	-	0.89
K	K_2HPO_4	17.42	-	-
K	KCl	-	8.6	21.0
P	K_2HPO_4	see above	-	-
P	$NaH_2PO_4 \cdot H_2O$	-	8.2	0.67
Si	$NaSiO_3 \cdot 9H_2O$	-	58.2	50.0
C	Na_2CO_3	-	20.0	-
C	$NaHCO_3$	-	-	50.0
Na	NaCl	-	-	16.7

Table 2. Concentration of the minor nutrient minerals in the basic media (mg/l)

Element	Compound	Medium		
		ASM	Gerloff's [1]	Link River
Fe	$FeCl_3$	0.54	-	0.90
Fe	Ferric Citrate	-	3.0	-
	Citric Acid	-	3.0	-
B	H_3BO_3	0.62	-	0.04
Mn	$MnCl_2$	1.40	-	0.43
Zn	$ZnCl_2$	0.11	-	0.80
Co	$CoCl_2$	0.005	-	-
Cu	$CuCl_2$	0.00003	-	0.15
F	NaF	-	-	0.30
Al	$AlCl_3$	-	-	0.60
Pb	$PbCl_2$	-	-	0.08
Cr	$CrCl_3$	-	-	0.02

[1] The minor elements are contained in Hoagland's A-Z solution which is added as one ml of a 1 to 25 dilution/liter of medium.

(tris-hydroxymethyl aminomethane) per liter. In addition, a medium was prepared to simulate the average ionic concentrations found between July 1959 and December 1963 in the Link River at the Fremont Bridge in Klamath Falls (Tables 1 and 2).

A medium containing chelated iron was devised to prevent the precipitation of iron in studies of mineral nutrition involving high concentrations of minerals at high pH. This medium is referred to as the RGT medium (Table 3). This was a 2X concentration of the G-10 medium from which sodium silicate was omitted and the iron source was changed to EDDHA (hyrogen ferric ethylenediamine di-o-hydroxyphenylacetate).

Table 3. RGT medium used for growth and nutritional experiments.

Compound	Amount mg/l	ppm Essential Element
$NaNO_3$	80.0	N 6.6
$NaHPO_4$	16.0	P 1.8
KCl	18.0	K 4.7
$MgCl_2$	40.0	Mg 2.4
Na_2SO_4	30.0	S 3.4
$CaCl_2$	70.0	Ca 9.6
Na_2CO_3	40.0	
EDDHA	2.0 ppm Fe	
Tris	8.0 mM	
Hoagland's A-Z	2.0 ml 1/25 dilution	

Physical Culture Conditions

pH. The effect of varying the pH on growth of Anabaena was studied using cultures of 250 ml of RGT medium contained in liter flasks. The pH was initially adjusted with 1 N HCl or 1 N NaOH to the range pH 5-12. Thirty ml of a suspension of Anabaena were inoculated into each flask and the flasks placed under constant illumination of 70 foot candles at 15°C. The H-ion concentration was adjusted daily back to the original pH for a period of 12 days. The cultures were then harvested and the dry weights and chlorophyll contents determined. No attempt was made to maintain sterility of these cultures.

Temperature. No extensive study of the effect of variation in temperature was made. Anabaena was grown at room temperatures (20-25°C) and at 15°C with no apparent difference in growth. Since Anabaena grew well at 15°C, and this temperature was required for the growth of Aphanizomenon in the same culture space, this temperature was used in all work.

Aeration. The effects of aeration were studied using two methods. In the first, the cultures were shaken using the variable speed shaker described by Reese (1966). The temperature was maintained at 15°C and the slowest shaking rate employed so as to minimize splashing of the medium. The second method consisted of

placing a capillary tube, drawn to an extremely fine tip, near the bottom of the flask. Air was forced, by a small aquarium pump, through an in-line millipore filter and into the capillary. The finest stream of bubbles obtainable by this method still caused a great deal of turbulence in the medium. Air stones were also used in an attempt to reduce the turbulence still further.

Light. The effect of variations in light intensity on growth was studied by placing cultures under constant illumination of 90, 100, 110, 120, 130 and 140 foot candles. Two flasks were harvested from each intensity every four days for a period of 12 days. Dry weight and chlorophyll determinations were made on each flask.

Inoculation and Harvest of Nutrition Studies

Cultures grown in 250 ml Erlenmeyer flasks containing 100 ml of medium were utilized in nutritional studies. Ten ml of inoculum was added in all cases to bring the total volume to 110 ml.

Table 4 indicates how the tests were conducted. Where possible the stock cultures used for inoculum were cultured in a medium deficient in the element to be tested. The time in the deficient medium was not constant for each element since the alga differed in the length of time it could tolerate the absence of specific major nutrient ions. In the comparison of growth with ferric citrate and

EDDHA, the inoculum was so dilute that the growth period was tripled in order to obtain significant results.

Table 4. Summary of procedures in nutritional experiment.

Element	Growth in Deficient Medium (days)	Replicates	Harvest Times (days)	Total Period of Growth (days)
P	27	3/harvest	3	12
Fe				
Versene-Fe^3	6	3/harvest	4	12
Fe^3 Citrate	5	3/harvest	7	28
EDDHA	5	3/harvest	7	28
Mg	11	8	10	10
N	90	8	10	10
Ca	-	8	10	10
K	10	8	10	10
S	10	8	10	10
Na_2CO_3	-	5	10	10
Hoagland's	-	5	10	10

Cultures used to determine the requirements for iron and phosphorous were harvested periodically throughout the experiment in order to determine the trend of the growth curve. Since growth in culture is subject to many short term fluctuations, harvesting cultures every three or four days accentuated these small variations. This dictated either increasing the number of flasks harvested each period, to average out variations in individual flasks, or the harvesting of all cultures at the end of the experiment test period. Although

the first modification would have been desirable, space and time did not permit additional replicates, so all cultures were harvested at the end of 10 days of growth.

At the end of the growth period the flasks were harvested. The medium was stirred to randomly distribute the algal cells which tended to accumulate on the surface. A fifty milliliter sample was withdrawn for determination of dry weight. The sample was filtered through a weighed, fiber glass, prefilter using a Millipore pressure filter. The filter pads were air dried for 12 hours, then transferred to a desicator for several days before weighing. In tests using variations of phosphate, magnesium, iron, or calcium, the filtrate was collected and analyzed for the respective ion.

Two 25 ml samples were also removed. One was used to determine the concentration of chlorophyll, the other analyzed for organic nitrogen.

Determination of the Concentration of Chlorophyll

The samples used for determination of the concentration of chlorophyll were placed in small plastic vials and frozen for periods of three to four days. Freezing, it was discovered, aided in sedimenting the planktonic cells during centrifugation. This treatment did not seem to rupture the cells since there was no evidence of the blue, water-soluble phycocyanin pigment in the medium on thawing.

After three days, the vials were removed from the freezer and allowed to thaw either by standing at room temperature for several hours or by immersion in warm water. The sample was centrifuged at 14, 000 x g for five minutes. The supernatant was decanted and 1. 75 ml of reagent grade acetone added to the pellet. The cells were dispersed in the acetone by vigorous agitation and the tubes were tightly stoppered and placed in the dark at 4°C for 18 to 24 hours. After this time the tubes were vigorously shaken and centrifuged as before. The supernatant was decanted, made up to a volume of 2 ml, and the optical density measured on a Beckman DB Spectrophotometer at wavelengths of 663 and 645 nm.

The amount of chlorophyll a present was calculated by the formula (Holden, 1965):

Chlorophyll a (mg/l) = (12. 7 D_{663} - 2. 69 D_{645}) (0. 08) (0. 08 is the dilution factor used in these experiments).

This extraction represents a combination of the procedures of Richards and Thompson (1952) and Arnon (1949).

Determination of Organic Nitrogen

Samples to be analyzed for organic nitrogen were placed in 50-ml Erlenmeyer flasks and evaporated to dryness at 80°C.

Digestion of the samples was carried out with 5N H_2SO_4 and 150 mg $CuSeO_4$ per liter (Umbreit, Burris and Stauffer, 1957).

The microKjeldahl method (Association Official Agricultural Chemists, 1965) was used for determination of the amount of organic nitrogen present. The only deviation from this procedure was the use of standardized 0.004 N rather than 0.02 N HCl in titrating the samples. The normality was decreased to attain greater accuracy.

Colorimetric Analyses

In order to correlate uptake of specific ions with growth, colorimetric analyses were performed on the filtered media in which responses to variations in phosphorous, magnesium, iron and calcium had been determined. Analyses for the other ions were not feasible due to insensitivity of the available analyses.

All glassware used in these tests was soaked in concentrated HCl followed by rinsing with large amounts of glass distilled water.

The molybdenum blue method of Fiske and Subbarow was used for determinations of phosphorous (Chapman and Pratt, 1961).

Magnesium was determined by the brilliant yellow method (American Public Health Association, 1965) in which Clayton Yellow (Direct Yellow 9, C.I. 19540, Matheson Coleman and Bell) was substituted for brilliant yellow and Methocel HG (Dow Chemical Company) was used as the stabilizing compound.

The ortho-phenanthroline method (American Public Health Association, 1965) was used to determine concentrations of iron and

the EDTA Titrimetric Method (American Public Health Association, 1965) was used in analysis of calcium.

All colorimetric measurements were made using a Zeiss Elko II Colorimeter.

PROCEDURES USED IN PURIFICATION

The physiological study of an aquatic organism is greatly complicated when the subject must be isolated directly from nature. A single drop of water may contain myriad small organisms, both plant and animal. In order for the study to be meaningful the organism of interest must be isolated from all others and maintained separately so that its physiology alone may be studied.

The original cultures isolated from Klamath Lake contained numerous green and blue-green algae, amoebae, ciliates and bacteria. The task of extricating Anabaena spiroides from this assemblage proved to be formidable. A short discussion describing the purification procedures attempted in this study and the results obtained are presented here.

Single Filament Isolations

The first attempts at purification consisted of the mechanical isolation of single filaments. This was accomplished under a dissecting microscope using capillary tubing drawn out to extremely fine tips. By touching the surface of the medium a small amount of fluid was rapidly drawn into the tubing carrying the desired filament with it. The droplet was then blown into a test tube containing 5 ml of fresh, sterile medium and placed in the light at 15°C. It was soon

evident that, although only a minute amount of the original culture was transferred, a large variety of contaminants had been included. These organisms flourished while after two days there was no trace of Anabaena.

In order to further reduce the contaminant population filaments were washed by a minimum of ten transfers to sterile medium before inoculation. This tended to greatly decrease, but by no means eliminate, the contaminants. It was again noted that the alga failed to develop. This failure to grow could have been due to an inability to compete with the contaminants for nutrient materials or to a requirement for a certain minimum concentration of Anabaena cells before conditions were adequate for growth to occur. To test this hypothesis, a determined number of algal filaments were washed and inoculated as indicated previously. It was found that growth would not take place in cultures possessing less than 30 filaments for every 5 ml of medium. This observation tended to rule out the aspect of competition since the number of contaminants present would increase concomitantly with an increase in inoculum.

Van Baalen (1965) has suggested that when transferring blue-greens to fresh medium there appears to be a definite minimum concentration of organisms required as inoculum. He proposes that the blue-green algae produce hydrogen peroxide which becomes toxic or autoinhibitory unless it is immediately broken down. The enzyme

catalase, which decomposes hydrogen peroxide, is also produced by the algal cell. The formation of hydrogen peroxide during the early phases of growth may exceed the amount broken down by the catalase. A large number of cells may, therefore, be required which, through their combined catalase activity, are able to keep the peroxide concentration at a subinhibitory level, allowing increase in cell numbers.

In order to determine whether added catalase would stimulate growth in a culture inoculated with a suboptimal amount of Anabaena, twenty-five filaments were introduced into 10 ml of 2XG-10 medium containing 0.0, 0.001, 0.01, 0.1, and 1.0 ppm catalase. The cultures were examined frequently but at the end of one month there was no detectable difference between the cultures containing catalase and the control. In all cases only single cells and occasionally short filaments could be located by microscopic examination.

Pratt (1938) reported that indole-3-acetic acid promoted cell multiplication in Chlorella; and Bunt (1961) showed growth of Nostoc to be enhanced by the addition of minute amounts of IAA to the culture medium. It is quite possible that the need for a certain sized inoculum is a requirement for a growth stimulating factor produced either by the Anabaena cells themselves or by the contaminant organisms. In experiments with cultures inoculated with suboptimal quantities of Anabaena and containing 0.0, 0.001, 0.01, 0.1 and 1.0 ppm of indole-3-acetic acid, the results were similar to those obtained using catalase.

Since all attempts to grow isolated filaments met with failure, repeated washings followed by inoculation of at least thirty filaments for every 5 ml of medium was employed initially to maintain cultures and dilute out contaminants.

Elimination of Chlorophycean Contaminants

Chlamydomonas posed the biggest problem insofar as green algal contaminants were concerned. Through the dilution procedure described above the other members of the Chlorophyceae present, Scenedesmus and Ankistrodesmus, were rapidly eliminated from the cultures. The difficulty encountered with Chlamydomonas was undoubtedly due to its small size, rapid rate of multiplication and ability to form palmelloid stages attached to the sheaths of the Anabaena.

The attack on this organism was initiated with the antibiotic acti-dione. This compound has been reported to inhibit the growth of Chlorophyceae, Xanthophyceae and Bacillariophyceae (Whiffen, 1948; Zehnder and Hughes, 1958; Tchan and Gould, 1961). Concentrations of 50 ppm or less completely inhibit these forms while much higher concentrations have been shown to have no effect on the development of Myxophyceae.

Table 5 shows the concentrations of acti-dione utilized in an attempt to control Chlamydomonas in Anabaena cultures. Concentrations as low as 3 ppm are seen to have a definite inhibitory effect on

the green alga. This effect was evinced first by a loss of motility followed by conversion of the normal green, oval cell to an irregularly shaped, dark brown form. At concentrations exceeding 20 ppm large clear areas appeared at the anterior end of the cell. Many such cells had obviously lysed but many others were capable of rapid recovery upon transfer to acti-dione-free medium. Those concentrations of acti-dione which gave the most drastic reduction in green contaminants (100, 150 and 300 ppm) also affected Anabaena preventing cell division upon transfer.

Table 5. Effect of acti-dione on Chlamydomonas and Anabaena

Concentration ppm	Effect on Anabaena	Effect on Chlamydomonas
1	-	-
3	-	≠
5	-	≠
15	-	≠
20	-	≠
50	-	≠
60	-	≠
100	≠	≠
150	≠	≠
300	≠	≠

- no effect
≠ noticeable effect but some cells still viable
≠ cells killed

Cultures treated with 50 and 60 ppm acti-dione were subcultured, the filaments were individually selected and passed through

10 washings of sterile medium before inoculation. This procedure succeeded in diluting the viable Chlamydomonas to such an extent that a culture was finally obtained free of chlamydomonad contamination.

Control of Protozoa

Ciliates, flagellates and amoebae were extremely abundant in the initial isolations from Klamath Lake and shortly after introduction of Anabaena spiroides into culture they attained phenomenal concentrations in the cultures. It was noted that the procedures used to dilute out chlamydomonad contaminants also served to greatly decrease the concentrations of protozoa present. By repeated subculturing, allowing only a week for growth before transfer, it was possible to obtain cultures in which only a few protozoa were present.

The greatest problem organisms of this group were amoebae. Cultures which appeared to be entirely free of these organisms would suddenly develop dense populations. Because of this, several compounds known to exert toxic effects on protozoa were employed in an attempt to speed up the elimination of these organisms.

Two compounds well known for their inhibitory action on microorganisms are malachite green and crystal violet. These compounds, members of the group of supra-vital dyes, are actively absorbed by the organism and accumulate within the cell. As accumulation

proceeds, a toxic level is reached in which the metabolic processes of the organism are inhibited and death ensues. The dyes are not specific for protozoa but are taken up by the algal cells as well. It was hoped in this case that the more rapid metabolic rate of the protozoa would cause accumulation and death before a toxic level had been reached in the algal cells. This did not appear to occur, however. As indicated in Table 6 protozoa were still present after 24 hours in those concentrations of dye in which algal growth had been totally inhibited.

In 1964 Brown reported that caffeine in concentrations from 0.01 - 0.03 M were effective in killing fungi and non-photosynthetic protozoa. Caffeine did not have this effect in Anabaena cultures. The 2XG-10 medium supplemented with 0.02 M caffeine caused lysis of all Anabaena cells within a period of ten days while the protozoan population fluorished.

Since the above mentioned compounds tended to inhibit the growth of the alga at concentrations below those required to inhibit the contaminant protozoa, their use was not further studied and elimination of the protozoa was finally obtained by repeated dilution.

Table 6. Effect of dyes on growth of protozoa and Anabaena

Dye	Concentration ppm	Effect[1] on Protozoa	Effect[1] on Anabaena
Malachite Green	0.5	-	/
	1.0	/	/
	2.5	/	/
	5.0	/	/
	10.0	/	/
Crystal Violet	1.0	-	/
	2.0	/	/
	3.0	/	/
	5.0	/	/
	10.0	/	/

[1]Effect determined by growth after one week in a subculture prepared by inoculation of 2 ml of test solution into 10 ml of fresh medium.

\- no effect

/ cells killed

Attempts to Eliminate Bacteria

The contaminant bacteria were the most difficult to eliminate of the unwanted organisms. All transfers of the cultures were performed aseptically and all the media and utensils used were autoclaved at 20 lbs pressure for 15 minutes. It is assumed, therefore, that the bacteria present were those in association with the alga in nature and were not introduced after isolation.

Observation with the microscope showed the bacteria in floating masses surrounding dead algal material, and as isolated cells embedded within the sheaths of apparently healthy algal filaments.

Some of the bacteria found in the sheath material appeared to be motile since they were seen to oscillate as though trying to free themselves. Motile bacteria may have become embedded by the force of their momentum when colliding with the soft gelatinous material composing the sheath. It is very likely that although trapped, the bacterial cells would be capable of maintaining their existence by absorbing carbohydrate material from this matrix. Other bacteria were seen to be attached to the periphery of the sheath. In either case, whether embedded within or glued to the surface, it would be impossible to eliminate the bacteria by isolating single filaments or by dilution procedures since they would be transferred together with the algal filament. The floating masses of bacteria were eliminated by the prescribed transfer techniques but reappeared in new cultures as filaments died and increased availability of nutrients stimulated bacterial growth.

Since bacterial contamination has been the most persistent problem for the algal culturist, many methods of purification have been attempted both with and without success. The methods used in this study will be presented. Although most of two years was spent in an attempt to achieve a pure culture of Anabaena spiroides, no method was found which would remove bacteria without simultaneously inhibiting or destroying the algal material. This is not too surprising when the morphological and metabolic similarity between the

blue-green algae and the bacteria is considered. A compound that adequately inhibits bacterial cells will probably inhibit treated algal cells. A concentration must be found which will be toxic to bacterial cells but will allow survival of at least some of the algal cells.

Antibiotics

When mechanical techniques failed to eliminate all of the bacteria the next most logical step appeared to be the use of known antibiotics. The employment of these compounds has a definite disadvantage in that many possess mutagenic action. It is quite likely, therefore, that a culture purified by antibiotic treatments would contain algal forms differing from the wild type. Although the development of a mutant strain was a distinct possibility, the advantage of a pure culture for the study of mineral requirements outweighed the disadvantage and antibiotic screening was begun.

The bacteria present were isolated from the algal cultures by plating 0.1 ml of culture solution on nutrient agar. The plates were incubated at room temperature for 48 hours and the resulting colonies replated. No attempt was made at this time to separate the different forms and the bacterial culture was maintained in the "mixed" condition. Later separation showed the contaminants to consist of two forms: short gram-negative motile rods with lemon yellow chromogenesis and gram-negative filaments forming salmon pink

colonial masses.

The "mixed" bacteria were streaked on nutrient agar and Bacto-sensitivity disks placed aseptically on the surface. The plates were incubated at room temperature and observed after 48 hours (Table 7). Aureomycin and terramycin alone were effective in inhibiting bacterial growth at the concentrations present in the disks. Tests were begun to determine the effectiveness of these two compounds on the bacterial-algal association.

Table 7. Effect of antibiotic sensitivity disks on bacteria

Antibiotic	Concentration	Diameter Zone of Inhibition
Aureomycin	5 mcg	13 mm
Chloromycetin	5 mcg	0
Dehydrostreptomycin	2 mcg	0
Erythromycin	2 mcg	0
Neomycin	5 mcg	0
Penicillin	2 units	0
Polymyxin B	50 units	0
Streptomycin	2 mcg	0
Terramycin	5 mcg	11
Viomycin	2 mcg	0

Terramycin (Table 8) produces a deep amber solution and has a tendency to precipitate or accumulate on the sheaths of the filaments. This propensity increases the concentration of the antibiotic in the immediate vicinity of the cells. It also lengthens the time the filaments are in contact with the antibiotic solution since the

precipitated particles are transferred together with the alga to fresh medium. For these reasons terramycin was found unsuitable and was not studied further.

Table 8. Effect of terramycin on the control of bacteria in Anabaena cultures

Concentration ppm	Effect on Alga	Effect on Bacteria
1.0	-	-
2.0	-	-
5.0	-	-
7.5	/	-
10.0	/	-

- no inhibition of growth
/ inhibition of growth

Aureomycin (Table 9) was first tested by inoculating 0.5 ml of an actively growing stock culture into 10 ml of 2XG-10 amended with aureomycin. These cultures were incubated at 15°C under approximately 400 foot candle illumination intensity for 19 hours. Three-tenths ml of each culture was then plated out on nutrient agar and incubated for 24 hours at room temperature. The remaining algal filaments were rinsed with sterile medium and transferred to fresh, non-antibiotic, culture solution.

In all cases, the algal colonies appeared normal and healthy, differing insignificantly from the control. The plate counts showed a very slight decrease in bacteria at 5 ppm but this was only a

subjective estimate.

Table 9. Effect of aureomycin on the control of bacteria in Anabaena cultures

Concentration ppm	Effect on Anabaena	Bacteria Plate Count 24 hr
0.5	-	1000
1.0	-	1000
2.0	-	1000
3.0	-	1000
4.0	-	1000
5.0	-	1000

- no inhibition of growth

The concentration of aureomycin used in the second test was increased to a maximum of 20 ppm. In this case 0.1 ml of algal suspension was inoculated into 5 ml of test medium and incubated for 24 hours after which time plates were prepared using 0.5 ml of the culture as inoculum. The rest of the culture was rinsed and placed in fresh medium. Six ppm aureomycin (Table 10) caused a significantly decreased count not exceeded until a concentration of 16 ppm is reached. Eighteen ppm aureomycin appeared to completely inhibit bacterial growth, although one colony did appear on the plate after

60 hours. At the end of one week plates for counting were again prepared from the subcultures that had been treated with 16, 18 and 20 ppm aureomycin. These counts showed the bacterial population had increased phenomenally, possibly surpassing that present in the original pretreated culture. It was obvious that to free a culture of bacteria they all must be destroyed simultaneously. Any inhibition of one form before another, eliminates competition and causes a burst of growth resulting in larger populations after purification than before.

Table 10. Effect of aureomycin on bacteria

Concentration ppm	Plate Counts 24 hour	60 hour	1 week
0	>1000		
4	200		
6	28		
8	14		
10	28		
12	19		
14	120		
16	2		>1000
18	0	1	>1000
20	5	8	>1000

Since bacteria are transferred together with the alga the amount of inoculum would influence the effectiveness with which an antibiotic would work. The fewer bacteria present the more likely that all of them will be inhibited. The inoculum cannot be too small, however, since it must exceed the minimum cell density discussed

earlier.

To test the effect of aureomycin on various sized inocula 0. 1, 0. 5, and 1. 0 ml from a rapidly growing Anabaena culture was inoculated into 5 ml of medium containing 0, 5, 10 and 20 ppm of the antibiotic. The tests were incubated for 22 hours at room temperature. After this time 0. 2 ml of each culture was plated on nutrient agar and one-half of the remaining culture transferred to fresh culture solution. At the end of 44 hours of incubation the same procedure was repeated on the remainder of the original culture. The bacterial content of the cultures increased as the size of the inoculum was increased (Table 11). Even in the culture grown from the smallest inoculum a large number of bacteria were still present at the end of 44 hours. It should be noted that the 44-hour plates produced a much greater bacterial population than did the 22-hour plates, and that the plates from the highest aureomycin concentration (20 ppm) contained the largest count of the treated samples. This may be explained by the observation that after a 44-hour exposure to aureomycin Anabaena failed to grow upon transfer. When killed prior to transfer, the algal cells provided additional nutriment for bacterial growth. Since higher concentrations of antibiotic would inhibit the algal cells more readily than lower concentrations, the resulting longer growth period would allow for the production of larger populations.

In a very few cases Anabaena continued to grow after treatment with the antibiotic but most of the time the alga failed to recover and could not be detected after transfer.

Table 11. Effect of variation in volume of inoculum on ability of aureomycin to inhibit bacteria

Concentration ppm	Amt. Inoc. ml	22 hr Exposure Plate Count 24 hr	22 hr Exposure Plate Count 48 hr	44 hr Exposure Plate Count 24 hr
0	0.1	>1000	>1000	>1000
	0.5	>1000	>1000	>1000
	1.0	>1000	>1000	>1000
5	0.1	0	40	4
	0.5	170	704	>1000
	1.0	680	>1000	>1000
10	0.1	0	80	20
	0.5	10	66	546
	1.0	370	640	>1000
20	0.1	0	8	800
	0.5	0	21	206
	1.0	0	61	546

It appears that as the bacterial content was reduced the ability of the algal cells to survive antibiotic treatment decreases also. This, of course, may have been the result of simultaneous inhibition but it also is possible that the bacteria produced some material required by the alga for growth. This is known to be the case with many marine algae which require vitamins produced by bacteria inhabiting shore-line vegetation. If this is the case it was hoped that

yeast extract might provide the missing material. The addition of yeast extract to the medium produced very little stimulation of growth in cultures inoculated from untreated stocks. More importantly, there did not appear to be any adverse effects resulting from the addition of this undefined compound.

The duration of contact with the bacteria largely determines the antibiotic's effectiveness. The effect of variation in the length of the incubation period was tested using 10-ml lots of media containing 50 and 200 ppm aureomycin. These test concentrations were each inoculated with 0. 2 ml of a heavy suspension of Anabaena. Filaments were removed by micropipettes from each concentration of aureomycin after 1, 1. 5, 4. 5, 7. 5 and 24 hours and inoculated into fresh G-10 medium amended with 1 ppm yeast extract. Nutrient agar plates were inoculated with 0. 3 ml of the test suspension at the same time. In 200 ppm aureomycin a precipitate appeared in the sheath around the cells after 4. 5 hours. The cells which were surrounded by this flocculent precipitate appeared to have lysed. In no case did Anabaena survive this treatment (Table 12).

Ten ml of Anabaena were inoculated into 100 ml of medium containing aureomycin. After incubation for 5, 10, 20, 40 and 80 minutes, 1 ml of the test solution was transferred into 2XG-10 medium containing 50 ppm yeast extract. Nutrient agar plates were also inoculated at these times. Plate counts after 48 hours showed complete inhibition of the bacteria but Anabaena did not survive the

treatment.

Table 12. Effect of variation in concentration and length of exposure to aureomycin on bacterial inhibition

Incubation Time (hrs)	50 ppm Aureomycin Plate Count 48 hr	72 hr	Alga Recovery	200 ppm Aureomycin Plate Count 48 hr	Alga Recovery
0.0	>1000	>1000	+	>1000	+
1.0	1	1	-	0	-
1.5	0	0	-	0	-
4.5	0	0	-	0	-
7.5	0	0	-		
24.0	0	0	-		

The same procedure was carried out using 50 ppm aureomycin for incubation times of 5, 10, 20, 40, and 80 minutes (Table 13). After one week a few algal filaments could be observed although they were pale in color and often surrounded by bacterial masses. After 3 weeks the alga was no longer detectable.

Table 13. Effect of variation in incubation time on control of bacteria by 50 ppm aureomycin

Incubation Time (minutes)	5	10	20	40	80
Plate Count					
48 hr	1.0	4.3	1.7	2.0	0.3
96 hr	>1000	>1000	>1000	>1000	>1000

Inoculum - 10 ml
Plate counts represent an average of three replications.

Although increasing the amount of inoculum would result in a larger initial bacterial population as well as more filaments of Anabaena, it might enhance the probability of survival of some algal filaments.

From the above tests it did not appear that bacteria could be satisfactorily removed using aureomycin. This eliminated those antibiotics the screening had shown to have inhibitory power over the contaminant bacteria. The effectiveness of the sensitivity disk lies in the ability of the antibiotic to diffuse into the agar medium on which it is placed. A broad-spectrum antibiotic which had been thought to be the most likely to inhibit the contaminant organisms was neomycin. This compound is relatively insoluble and for this reason possibly unable to show inhibition on agar. It was decided as a last resort to test neomycin in liquid culture.

Neomycin had very little effect on bacteria (Table 14). The decrease noted in experiments 1 and 2 was encouraging but plate counts performed on subcultures from these tests showed large numbers of bacteria present. Since the bacterial population did not appear to be effected by neomycin or a neomycin-aureomycin combination attempts at purification with antibiotics were abandoned.

Sheath Elimination

Since antibiotics did not appear to be the answer for obtaining

Table 14. Effect of neomycin on bacteria in Anabaena cultures

Experiment Number	Concentration Neomycin in ppm	Inoculum ml	Incubation Time	Plate Count	Algal Survival	Transfer Medium
1	25	1	0	>1000	+	2XG10 + 1000 ppm
		4	4	40	+	Yeast Extract
			5	32	+	
			6	29	+	
			7	29	+	
2	15	10	9	1	±	2XG10 + 500 ppm
			11	1	±	Yeast Extract
			13	8	±	
			15	2	±	
	25		9	10	±	
			11	5	±	
			13	--		
			15	1	±	
3	5	5	12	>1000	±	2XG10
			24	>1000	±	
	10		12	>1000	±	
			24	500	±	
	20		12	500	±	
			24	500	±	
4	20	100	0.5	>1000	±	2XG10 + 50 ppm
	40		1.5	at all		Yeast Extract
	80		2	conc.		
			3.5			
			4			
			6			
			8			
			24			
			48			
5	300		1.5	>1000	±	
	500		4	at all		
			7	conc.		
			24			
6	500 ppm neomycin		0.5	500	-	
	100 ppm aureomycin		1	20	-	
			4	25	-	

+ good survival
± some survival
- no survival

pure cultures of Anabaena other methods were attempted. A large majority of the bacteria are present within the sheath material of the alga and are undoubtedly protected to some extent from antibiotics in the medium. If the sheath, at least the outer portions of it, could be removed the elimination of the bacteria would be greatly facilitated.

Foerster (1964) reported that the presence of "hardness ions" (i. e., calcium and magnesium ions) regulated the formation and thickness of the sheath in the blue-green Oscillatoria. The effect of the presence of calcium and magnesium ions on the sheath of Anabaena was investigated by inoculating cultures in a medium containing no calcium and only one-half the normal concentration of magnesium. After one weeks growth in this medium no difference in the thickness of the sheaths of filaments from control cultures could be measured using an eyepiece micrometer. After serial subculture in the minus-calcium medium for one month the sheaths appeared unaffected. Some abnormal cell formations were observed, however. This approach was then abandoned as being unproductive.

Pasteurization

Anabaena spiroides produces asexual spores called akinetes. These structures are extremely resistant and serve to perennate the alga in nature. As pasteurization is often utilized to rid material of

bacteria, it was hoped that pasteurization of an algal culture would destroy the bacteria while the akinetes would remain viable.

A heavy suspension of the alga was heated on a water bath held at 80°C. Ten ml of the suspension was withdrawn after 5, 20, 40, 60, 90, 120, 240, 480 minutes and placed in 50 ml flasks containing 30 ml of medium. These flasks were placed in a black box at 4°C for 2 months. At the end of this time a considerable amount of Anabaena was found to be present. This was quite surprising and might be explained either by the heat treatment not being sufficient to kill the vegetative cells or that akinetes present germinated during the intervening dark period. Nutrient agar plates were inoculated from each treatment and large numbers of bacteria were found to have survived. Pasteurization was repeated with temperatures as high as 100°C for periods of one hour following which both alga and bacteria were found to survive, if pasteurization was followed by a dark period at 4°C. If cultures were placed in the light immediately after pasteurization, bacteria developed but the alga did not survive. This strongly suggested that germination of the akinete requires a prior period of low temperature. It appeared likely that at least one of the bacterial forms present produced endospores. If this was the case single pasteurization would not produce 100% kill but under adequate conditions the endospores would germinate repopulating the culture with bacteria again. Two pasteurizations one week apart

was found to eliminate all algal filaments. Apparently this treatment killed ungerminated akinetes or the newly formed filaments did not have sufficient time to produce akinetes, leaving no resistant stage capable of surviving pasteurization. Bacteria were present in the culture after three days indicating that one week may be sufficient for germination and re-formation of endospores, if these structures are indeed present.

Ultra-violet Irradiation

Probably the technique most commonly used to successfully eliminate bacteria from algal cultures is irradiation with ultra-violet light. This procedure, however, is even more likely to produce mutant algal forms than antibiotic treatments. For this reason, initally irradiation was avoided and finally attempted only as a last resort.

Irradiation was carried out in a chamber formed from a cardboard box in which all material to be used during treatment and transfer were subjected to ultra-violet irradiation for at least one hour. The light source was a two-tube, 15 watt, germicidal lamp placed 12 inches above a fingerbowl containing 250 ml of a month old suspension of Anabaena. The fingerbowl was immersed in a water bath at 10^{o}C and the algal suspension stirred by a magnetic stirrer during irradiation. Approximately 1 ml of the suspension was

removed at the times indicated in Table 15 and inoculated into 25 ml of 2XG-10 amended with 50 ppm yeast extract. Irradiation times greater than 30 minutes completely inhibited algal growth while the bacteria were still abundant.

Table 15. Effect of ultra-violet irradiation on concentrated culture of Anabaena

Exposure Time	Plate Count	Alga Survival
15 sec	>1000	+
30	>1000	+
60	>1000	+
2 min	>1000	+
4	>1000	+
8	>1000	+
16	>1000	±
30	>1000	-
1 hr	7	-
2	1	-
4	40	-

Irradiation was attempted again using a Sedgewick Rafter Counting Chamber. Two ml of Anabaena culture were placed in the chamber and stirred with a wire loop during irradiation. The lamp was placed three inches above the suspension and irradiation continued for the time indicated in Table 16. After irradiation the sample was washed into 25 ml of 2XG-10 amended with 50 ppm yeast extract and placed in the light. Algal cells failed to survive 5 seconds of irradiation while bacteria were not appreciably decreased in less than 8 minutes.

The fact that subcultures of treated samples were immediately placed in the light explains the failure of Anabaena to survive even 5 seconds of irradiation. Ultra-violet irradiation acts to inhibit the photosynthetic processes of this organism. If placed in the dark for a short time the damage, if not too extensive, may be repaired and the cells are then capable of normal photosynthetic function when illuminated. If placed in the light immediately the cells are forced to use damaged machinery and become irreversibly bleached. The importance of the dark requirement was not appreciated when these experiments were performed but provide an explanation of the results obtained.

Table 16. Effect of ultra-violet irradiation on bacterial contaminants of Anabaena culture

Exposure Time	Plate Count	Alga Survival
5 sec	1000	-
10	1000	-
20	1000	-
40	1000	-
60	1000	-
2 min	1000	-
4	1000	-
8	325	-
15	0	-

plate counts average of three replicas
- no survival

Since the Anabaena cultures grew very slowly there was a possibility that the cells were not in optimum condition. In this

case the chances of survival after application of a bacteriostat would be lessened further. In order to obtain cultures in which the algal cells were in an optimal state, a study of the mineral requirements was initiated to allow development of a medium which might provide a more vigorous algal material for further study.

EXPERIMENTAL RESULTS

Preliminary Investigations of Culture Conditions

The medium originally found to be most suitable for the growth of Anabaena spiroides was 2XG-10 modified by substitution of 2 ppm chelated iron as EDDHA, in place of ferric citrate, and addition of 8 mM Tris buffer.

Aeration of the cultures was found to be a definite disadvantage. In all cases, aeration caused the algal filaments to turn yellow and eventually lyse. The detrimental effect may have been the result of the turbulence caused by air bubbles rising through the medium. Although as fine a stream of air as possible was utilized, the disturbance in the liquid was sufficient to cause the filaments to break. So long as breakage occurred at the heterocysts there was probably no damage to the cells. If the turbulence was sufficient to cause intercalary cells not attached to heterocysts to break apart injury was likely. Filaments were observed that had broken in this fashion in cultures where most of the filaments were only 3 or 4 cells in length and there were numerous solitary cells. When filaments were reduced to such a state, vegetative cell division appears to be significantly reduced.

Because of the detrimental effects, aeration was discarded and

the cultures were maintained in a static condition, shaken by hand once a day to prevent the "zone of deficiency" which develops around each cell from inhibiting growth.

When Anabaena was grown at different hydrogen-ion concentrations, maximum growth occurred at pH 10 (Figure 1). Dry weight was significantly diminished at pH 11 and negligible at pH 12. Although some growth was obtained in an acid medium, Anabaena spiroides appeared to prefer relatively high pH.

There was an increase in dry weight with an increase in light intensity up to the eighth day of the test (Figure 2A). After this time, at high light intensities (120, 130, 140 foot candles), increase in cell numbers may have been controlled by factors other than intensity. Since the cultures were grown in a static condition in those flasks where growth was most rapid the nutrients and dissolved gases may have been depleted in the upper layer of the medium. Such deficiency would inhibit further division and, in time, cause the cells to lyse. Lysis may have caused the decline obtained on day 12 at 140 foot candles. A leveling off of the chlorophyll concentration occurred in high light intensities (Figure 2B). This tendency followed that of the dry weight data closely except at 100 foot candles where the concentration of chlorophyll increased after dry weight had stabilized. At 140 foot candles, chlorophyll reached a maximum concentration by day 12 and, although the dry weight

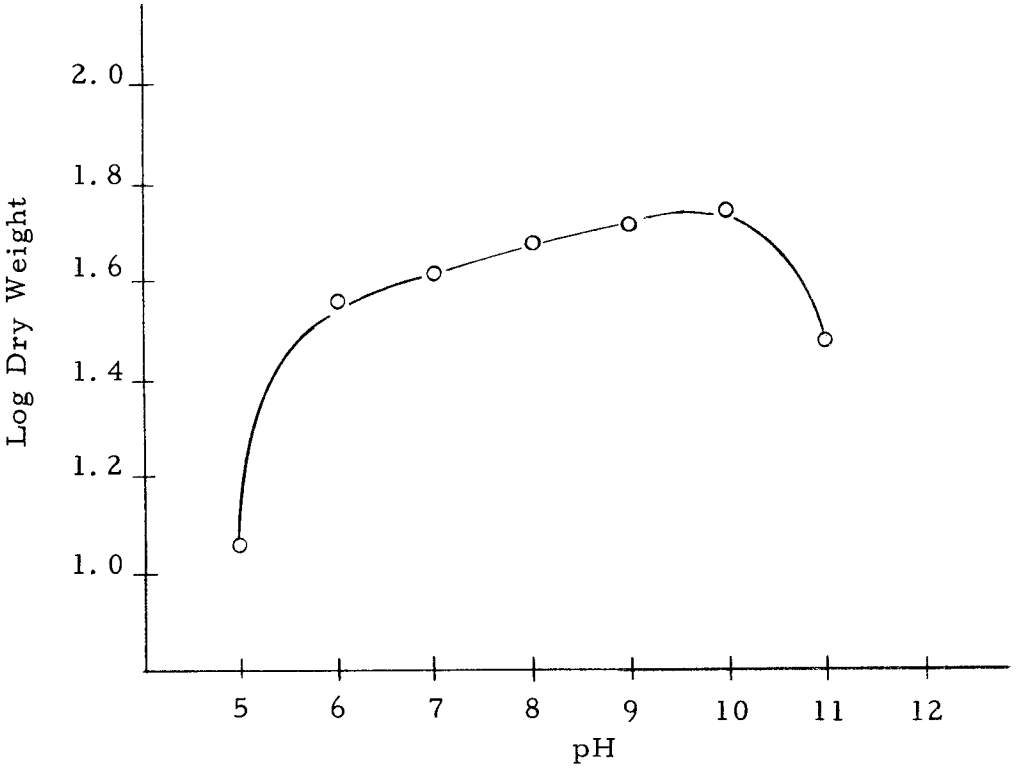

Figure 1. Growth of Anabaena in relation to pH

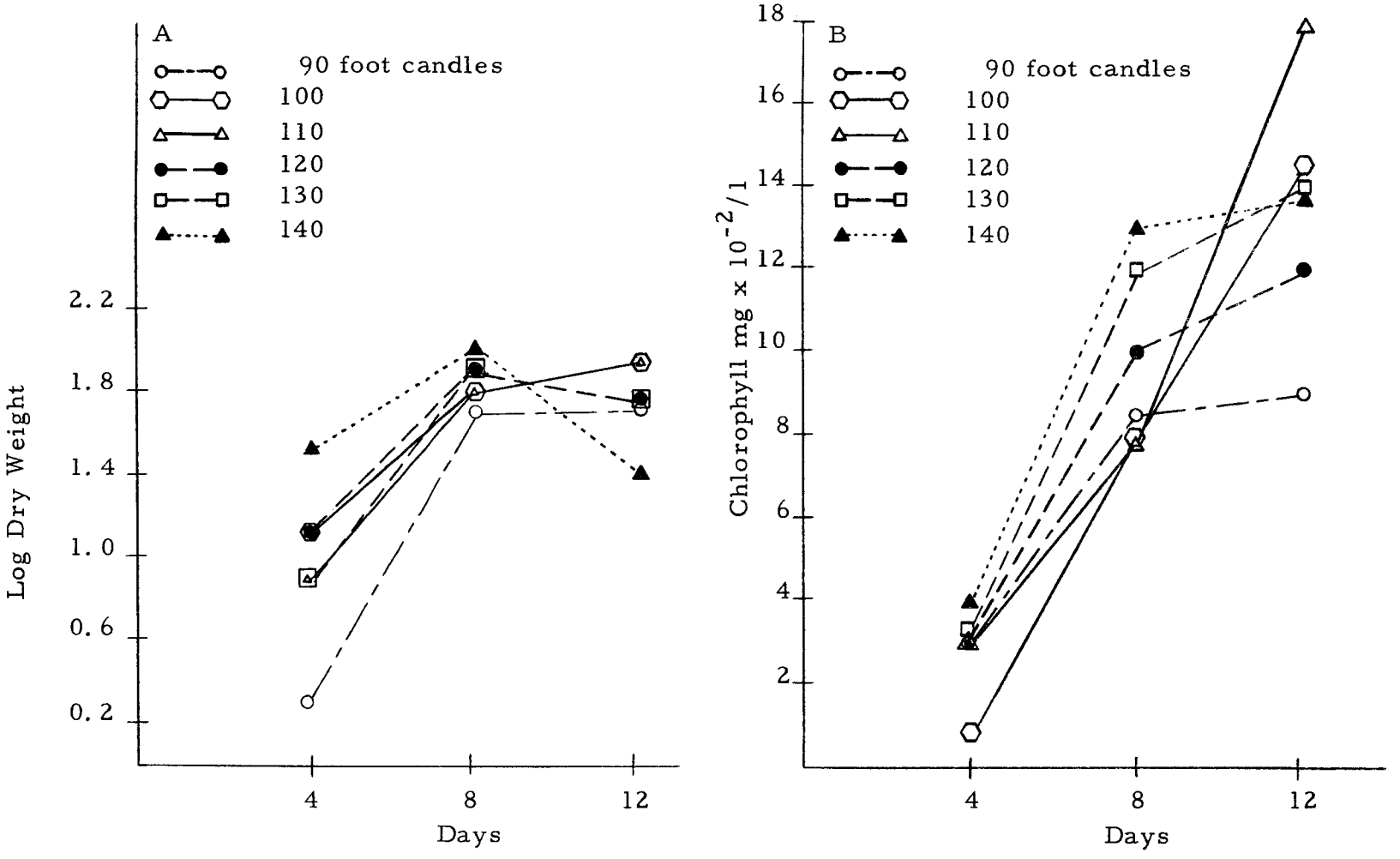

Figure 2. Effect of light intensity on the growth of Anabaena. (A) dry weight (B) chlorophyll

decreased due to cell lysis, it is possible that as many cells were present on day 12 as on day 8.

In order to determine the behavior of Anabaena in culture, cultures were harvested periodically and a growth curve plotted (Figure 3). A slight lag phase was noted during which time the alga was assumed to be adjusting to the medium and preparing for the rapid growth exhibited in the logarithmic phase. The phase of logrithmic growth is initiated on day 6 and maintained until day 15 when the culture entered the maximum stationary phase. All data not based on a growth curve sampling procedure were obtained by harvesting during the log phase between days 10-12.

Growth of Anabaena as Related to the Major Nutrients

Iron

Ferric citrate was the iron souce used in the normal G-10 medium, to which citric acid was added to act as a weak chelating agent. This iron source was used for the initial isolation medium and for maintenance of stock cultures up to the time of the nutritional studies.

To test the effect of varying concentrations of ferric citrate-citric acid on the growth of Anabaena, media containing 0.0, 0.075, 0.15, 0.3, 0.6, 1.2, 2.4, and 5 ppm iron were tested. The flasks were harvested in triplicate every 7 days over a period of 27 days (Figure 4). Abundant precipitate formed in the medium preventing

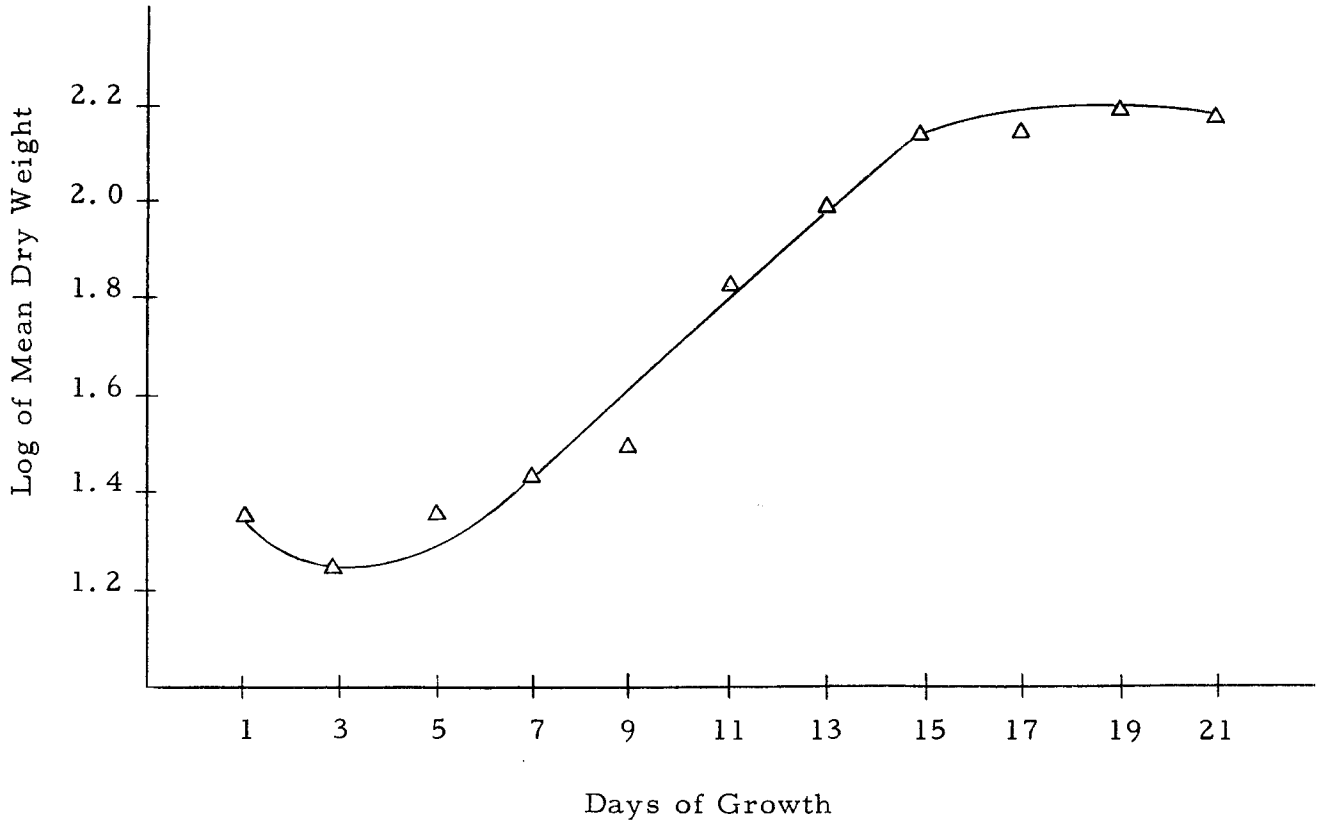

Figure 3. Growth of Anabaena in 2XG-10 medium

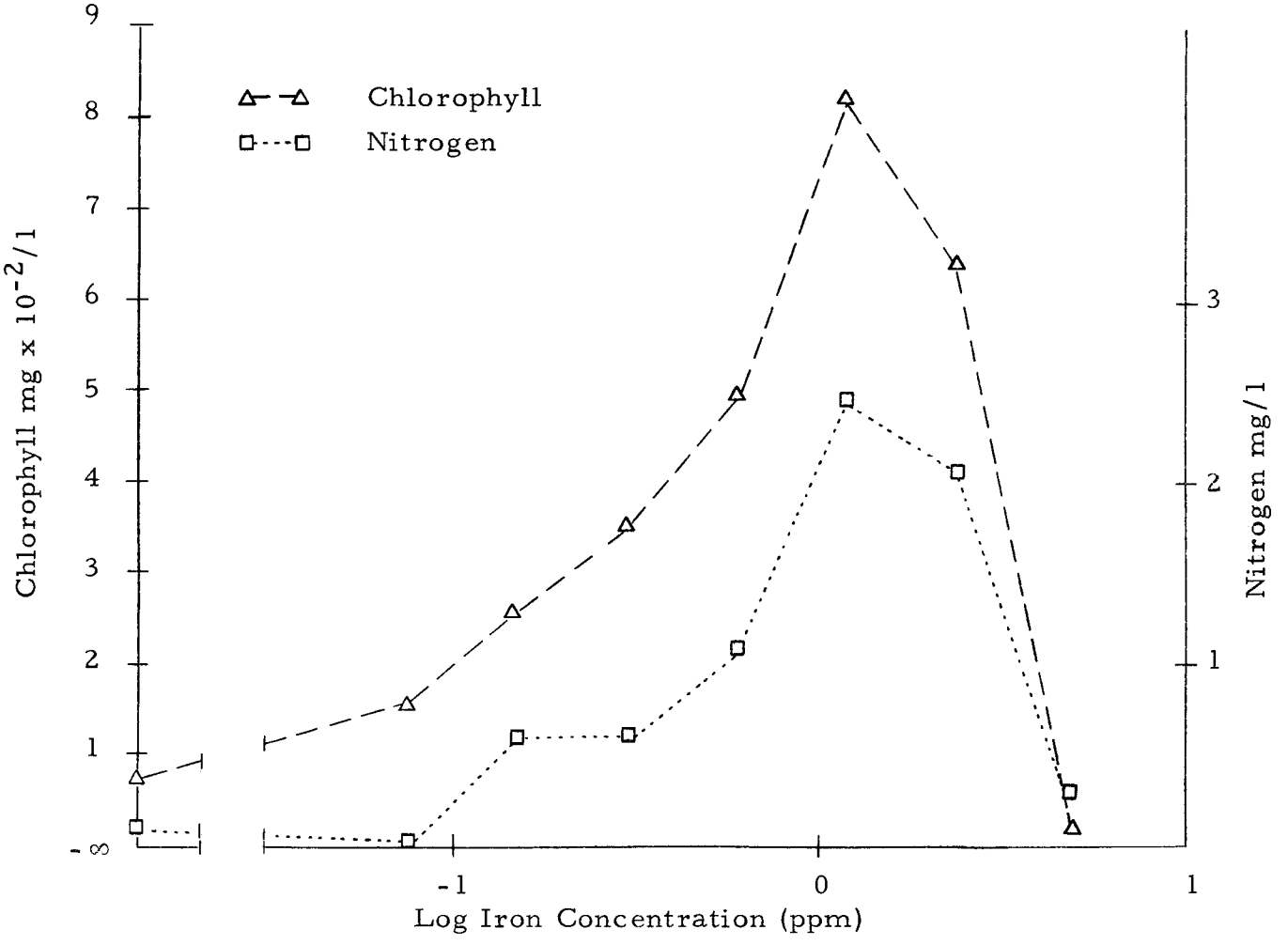

Figure 4. Effect of the concentration of iron supplied as ferric citrate on growth of Anabaena.

accurate determination of dry weights. Chlorophyll, nitrogen and presumably dry weight increased with increase in the concentration of iron up to 1. 2 ppm. In higher concentrations a plateau was reached followed by a sharp decline. Five ppm iron appeared to be detrimental to growth of the alga. Since a large proportion of iron precipitated out of the medium, as evidenced by the yellow precipitate, the ortho-penanthroline method was used to determine the amount of soluble iron remaining, available to the cells. The soluble iron content ranged from 0. 02 to 0. 06 ppm. This is a very crude estimate, however, since the colorimetric test is quite insensitive at these low concentrations.

Chelating compounds were tested for their ability to prevent the precipitation of the iron and hence to provide iron in a more stable and available form. Two such compounds, widely used in nutritional studies of higher plants, are Versene-Fe^3 (ferric ethylenediaminetetraacetic acid) and EDDHA (hydrogen ferric ethylenediamine di-o-hydroxyphenylacetate).

Versene-Fe^3 was tested in concentrations ranging from 0. 009 to 5. 0 ppm iron, each concentration being double the preceding one. In this test sufficient inoculum was employed so that harvests could be made in triplicate every four days for a period of 12 days. Growth was very irregular, as indicated by dry weight (Figure 5), but there was a distinct tendency for weight to increase with an increase in the concentration of iron. This trend was followed also by

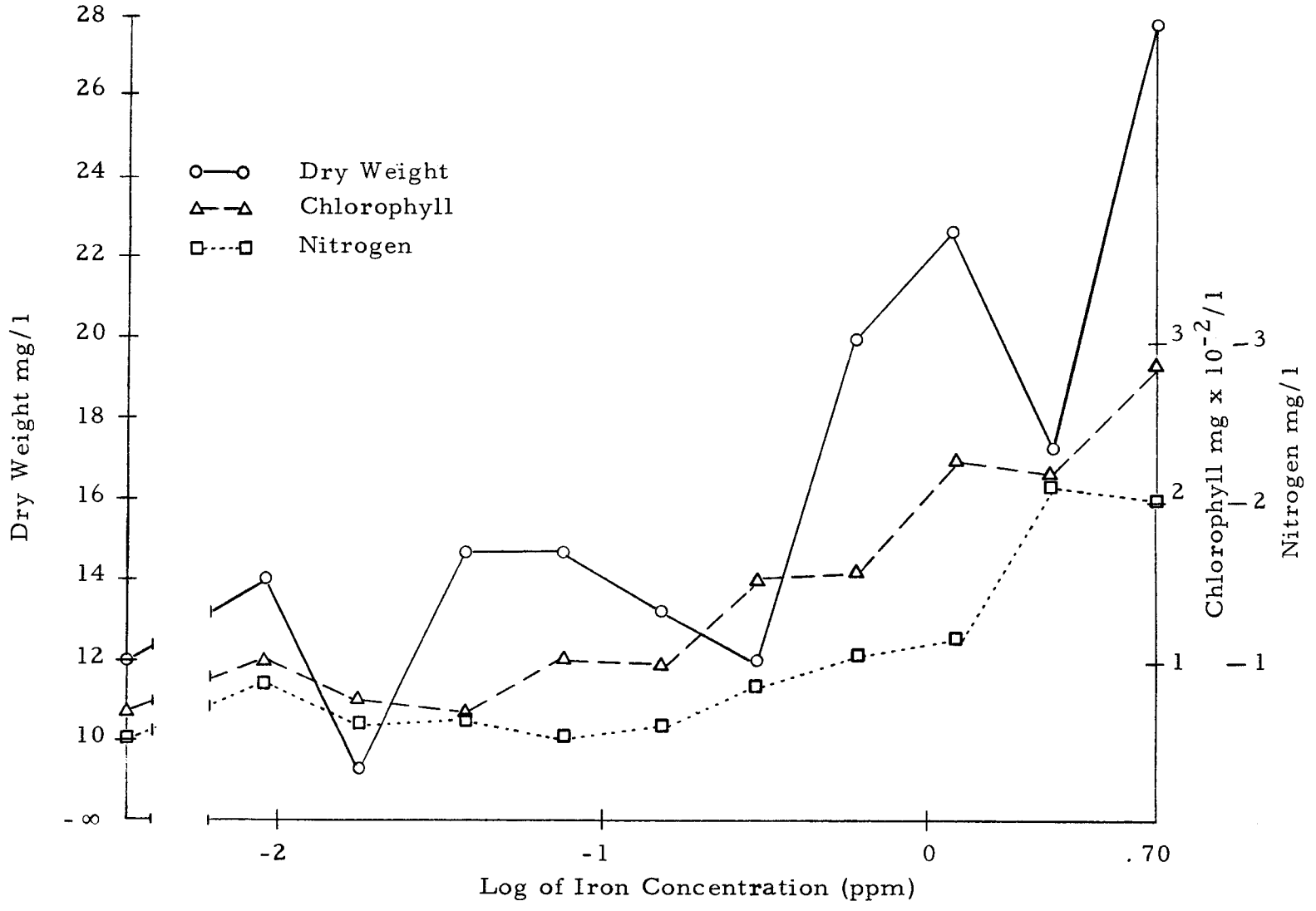

Figure 5. Effect of the concentration of iron supplied as Versene-Fe^3 on growth of Anabaena.

content of chlorophyll and organic nitrogen. Versene did not produce a plateau nor did it appear to inhibit growth at high concentrations. Colorimetric analyses showed only detectable quantities of soluble iron present in the uninoculated medium after autoclaving. It was later learned that Versene complexes are unstable under alkaline conditions and tend to lose bonded metal. This compound was even less satisfactory as an iron source than the ferric citrate complex.

Iron chelated EDDHA was tested using the same concentrations of iron and the same amounts of inoculum as in the ferric citrate tests. The increase in growth with increasing iron was much more rapid than with citrate (Figure 6), the maximum dry weight and chlorophyll content occurring at 0.3 ppm as compared with citrate at 1.2 ppm. The nitrogen peak at 0.6 ppm laged behind the dry weight-chlorophyll peak. The plateau, where increasing concentration of iron had but little effect on growth, extended from 0.3 ppm to 1.2 ppm. Inhibition occurred at concentrations of iron above 1.2 ppm. Colorimetric analysis showed soluble iron present in the media after 27 days of growth. No precipitate was formed after autoclaving even in the highest concentrations. This iron source was adopted, and was utilized in the media of all subsequent experiments.

Phosphorous

The effect of varying the concentration of phosphorous on the

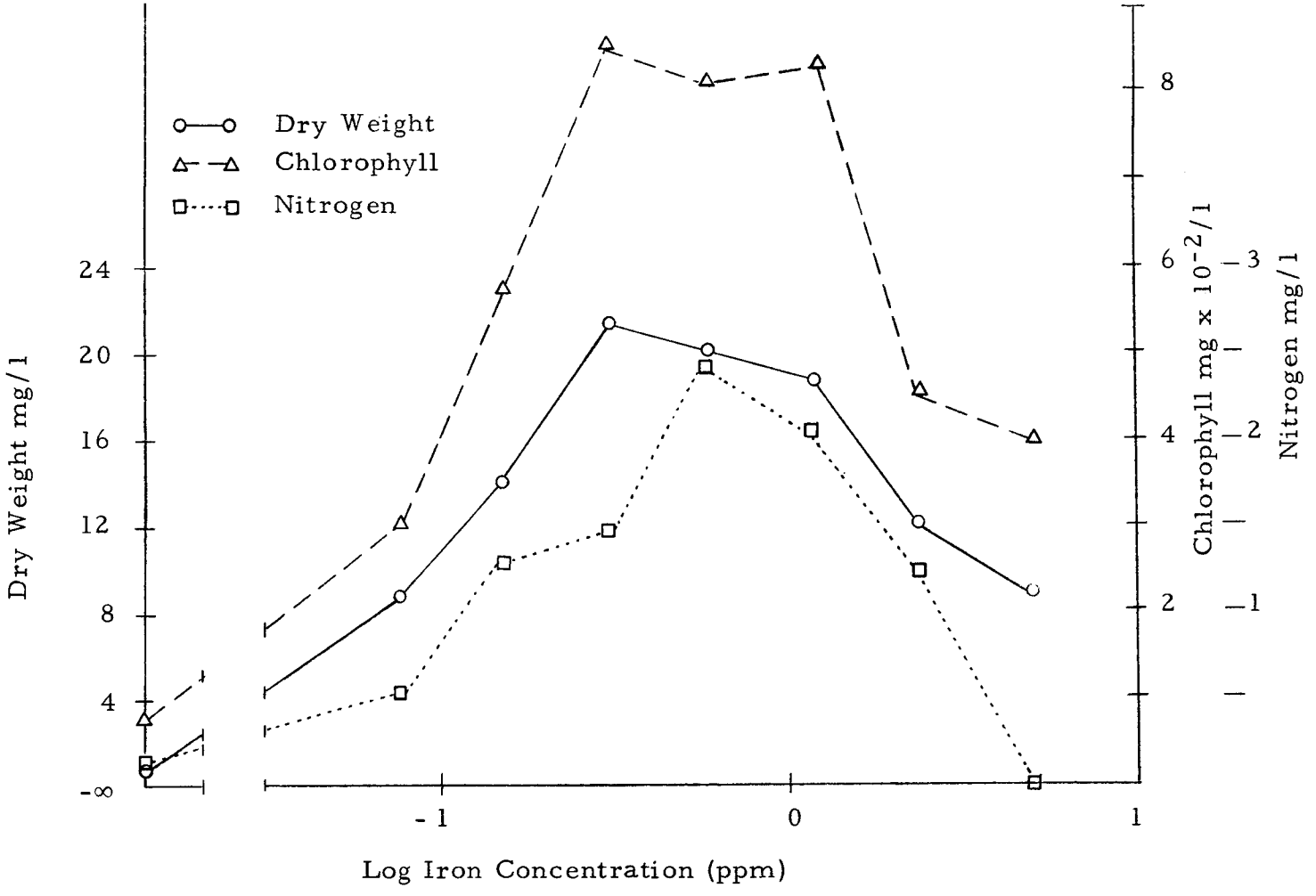

Figure 6. Effect of the concentrations of iron supplied as EDDHA on growth of Anabaena.

growth of Anabaena was tested by adding increasing concentrations of sodium monophosphate (NaH_2PO_4) to the basal medium. A range of concentrations from 0. 013 to 5. 2 ppm phosphorous was established, each concentration being twice that of the preceding one.

The alga was grown at a constant temperature of 15°C and harvested every three days over a period of 12 days. The results obtained for day 12 are presented (Figure 7), as are typical curves obtained for the growth curve analysis of dry weight, chlorophyll and nitrogen (Figures 8, 9 and 10). These curves are included to provide an indication as to the relationship these three analyses bear to the growth of Anabaena. Since the curves become confusing when growth rates at all concentrations are plotted, only representative samples, depicting the general trends, are shown.

Quite substantial growth was obtained when phosphorous was completely eliminated from the medium. This indicated that the alga was capable of sustained growth for fairly long periods of time while utilizing accumulated supplies of this element.

In general, increase in growth occurred with increasing phosphorous concentration up to 0. 52 ppm phosphorous. A definite decline was observed between 0. 52 and 2. 6 ppm, which was followed by a tremendous rise at 5. 2 ppm phosphorous. This trend was identical for all three analyses. The poor growth at concentrations between 0. 52 and 2. 6 ppm phosphorous may have been due to some

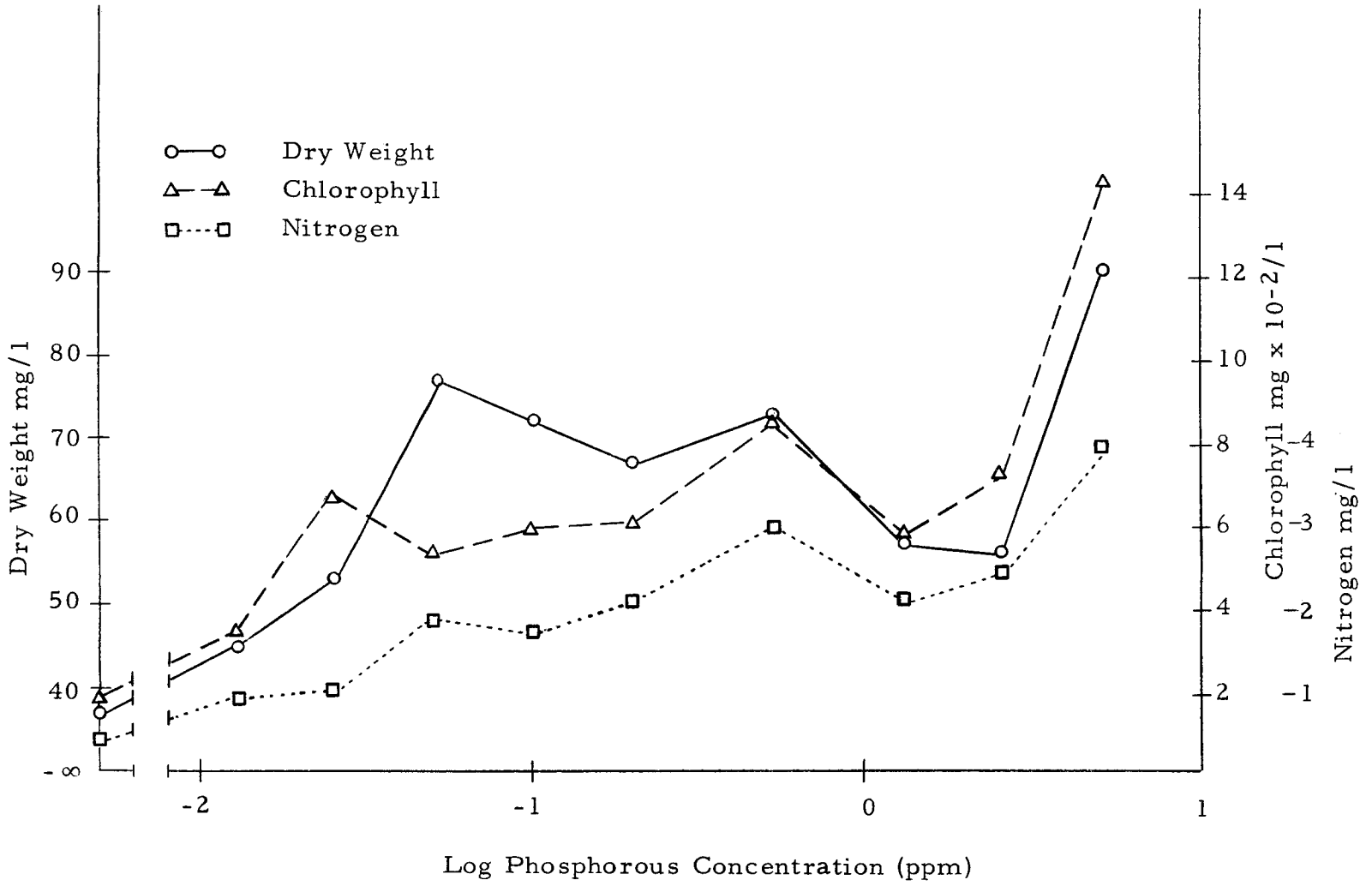

Figure 7. Effect of the concentration of phosphorous on growth of Anabaena.

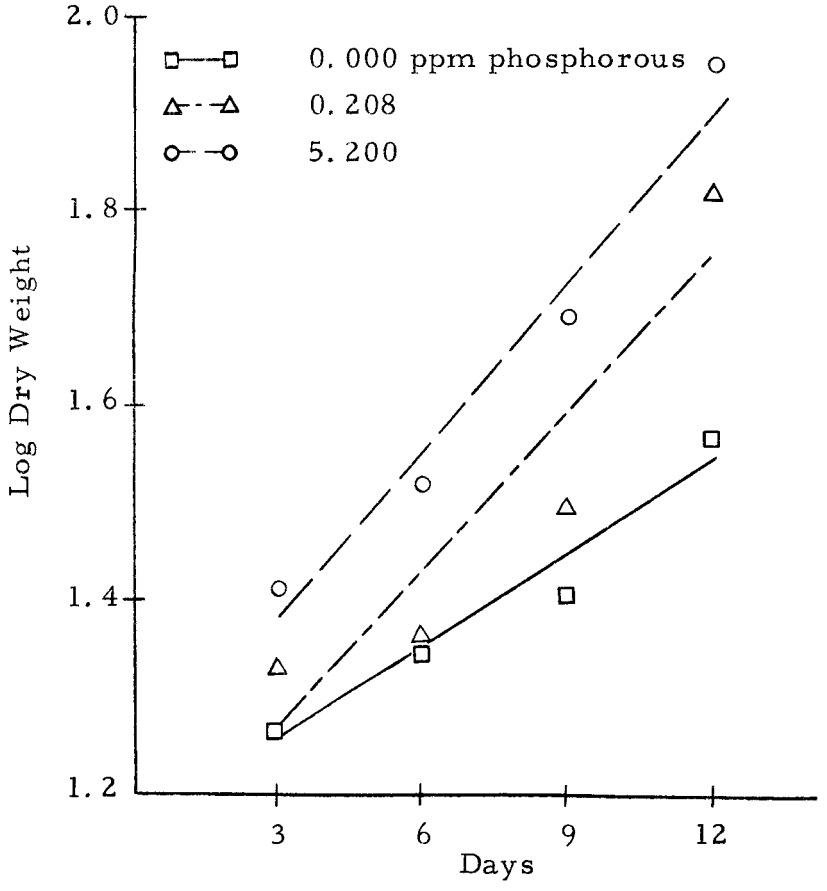

Figure 8. Effect of phosphorous concentration on growth of Anabaena as measured by dry weight.

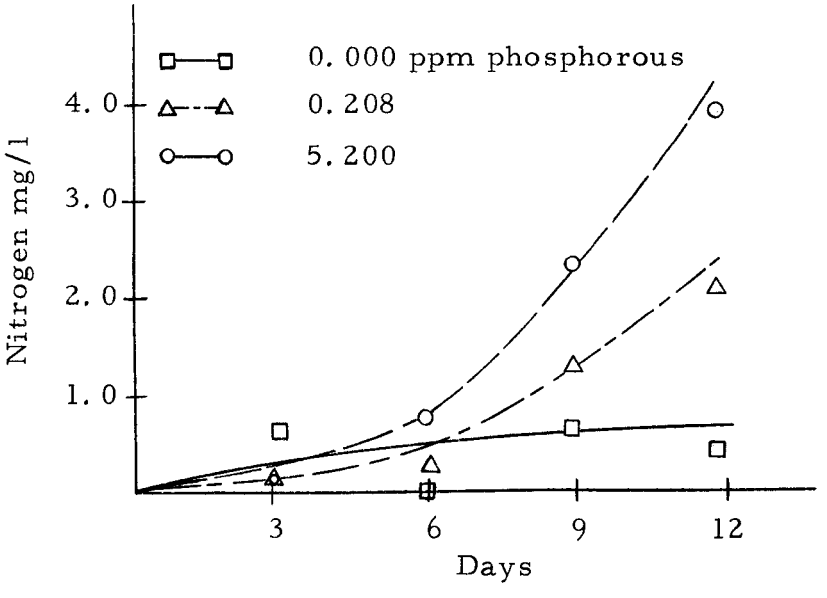

Figure 9. Effect of phosphorous concentration on growth of Anabaena as measured by nitrogen content.

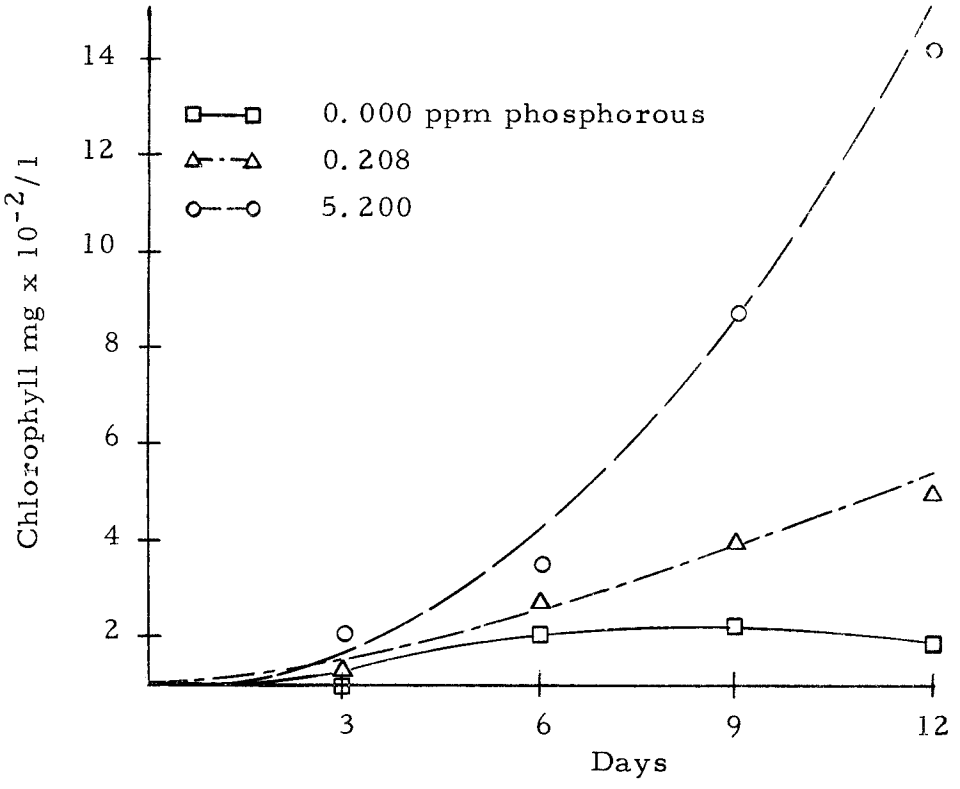

Figure 10. Effect of phosphorous concentration on growth of Anabaena as measured by chlorophyll content.

type of an ionic antagonism preventing phosphorous uptake. This antagonism might have been overcome by increasing the phosphorous content of the medium and this would account for the marked increase in growth occurring at 5. 2 ppm phosphorous.

Nitrogen

Two experiments were performed to test the effects of varying the concentration of nitrogen on the growth of Anabaena. The first consisted of varying the concentration of nitrate-nitrogen provided to the alga. The second experiment was designed to test the effect of various nitrogen sources.

Nitrate was provided as sodium nitrate and was added to the medium in concentrations sufficient to provide 0. 0, 0. 5, 1, 2, 4, 8, 16, and 32 ppm nitrogen. Analysis was based on the harvest of eight replicate flasks of each concentration after 12 days of growth. The initial inoculum was grown in a "no nitrate-nitrogen" medium for three months prior to the test. Some nitrogen (0. 84 mg/l) in the form of amino-nitrogen was present in the EDDHA addition but this was not thought to be sufficient to support good growth.

On the average, the growth of Anabaena was not influenced by the amount of nitrate-nitrogen present (Figure 11), except at 32 ppm where the increase in dry weight is probably due to stimulation of the production of sheath material. This unexpected result indicated

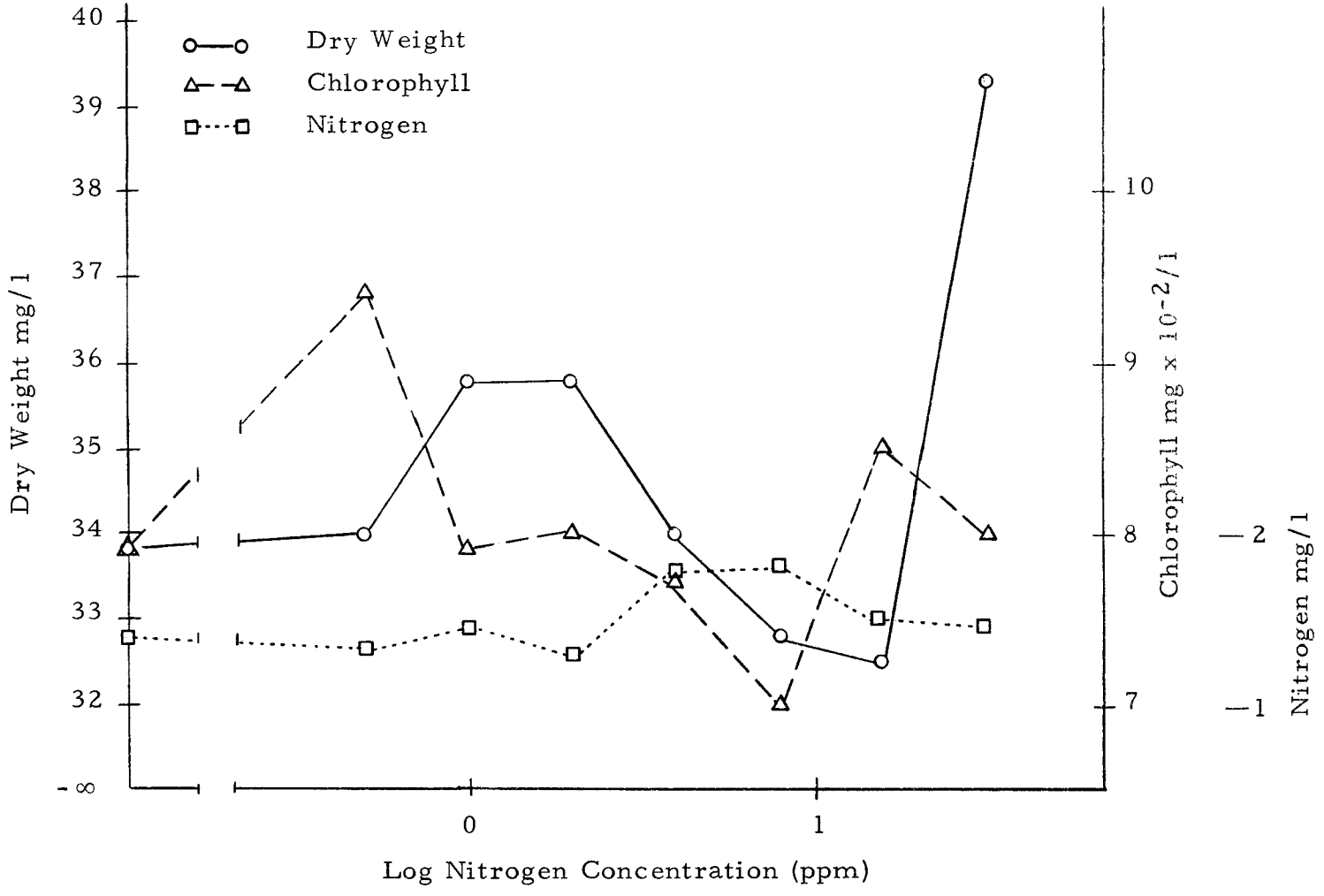

Figure 11. Effect of the concentration of nitrogen on growth of Anabaena.

that either Anabaena does not utilize nitrate or fixes atmospheric nitrogen with an efficiency equal to its use of nitrate-nitrogen.

To test nitrogen utilization further various sources of nitrogen were provided. Since it was possible that the growth observed in minus nitrate-nitrogen cultures could have resulted from nitrogen present in EDDHA, an organism which is incapable of nitrogen fixation but is capable of growth on nitrate or amino compounds was used as a control. The control organism was the Emerson strain of Chlorella obtained from Dr. Norman I. Bishop. Triplicate flasks were prepared in which various nitrogen sources were provided (Table 17).

Table 17. Growth of Anabaena and Chlorella on various nitrogen sources

Source	pH	Dry Weight mg/l	
		Anabaena[1]	Chlorella[2]
$NaNO_3$	7.7	42.12	22.28
$NH_2OH \cdot HCl$	6.1	-	-
Urea	8.8	33.33	34.09
Glycine	8.9	55.45	33.64
- Nitrogen	8.9	45.76	12.28

[1]Average of three replicas
[2]Average of two replicas

The nitrogenous compounds were added in concentrations providing 16 ppm nitrogen in the final medium. All compounds supported

growth except hydroxylamine-hydrochloride. This compound is extremely toxic to living systems and served to reduce the pH of the medium to such a low level Anabaena would have difficulty in becoming established. Chlorella grew very poorly in the no-nitrogen medium and the growth produced may be assumed to be due to the EDDHA source. The inhibition shown by Chlorella was not shared by Anabaena, which grew as well without an additional nitrogen source as with sodium nitrate. Growth of Anabaena was inhibited somewhat by addition of urea. This may have been due to release of ammonia within the cell raising the pH to an intolerable level. This toxic effect appeared less pronounced in Chlorella. Growth of the blue-green was greatly stimulated by addition of glycine. This is not surprising since the glycine supplied both nitrogen in the amino form and a carbon source to the alga.

It appeared that Anabaena spiroides fixed nitrogen with the same efficiency as it is capable of using nitrate. Stocks were, in fact, found to grow better and to reach denser concentrations before lysis in media lacking a nitrogen source. Most stock cultures are currently being maintained in a minus-nitrogen medium.

Magnesium

The effect of magnesium was studied using concentrations of

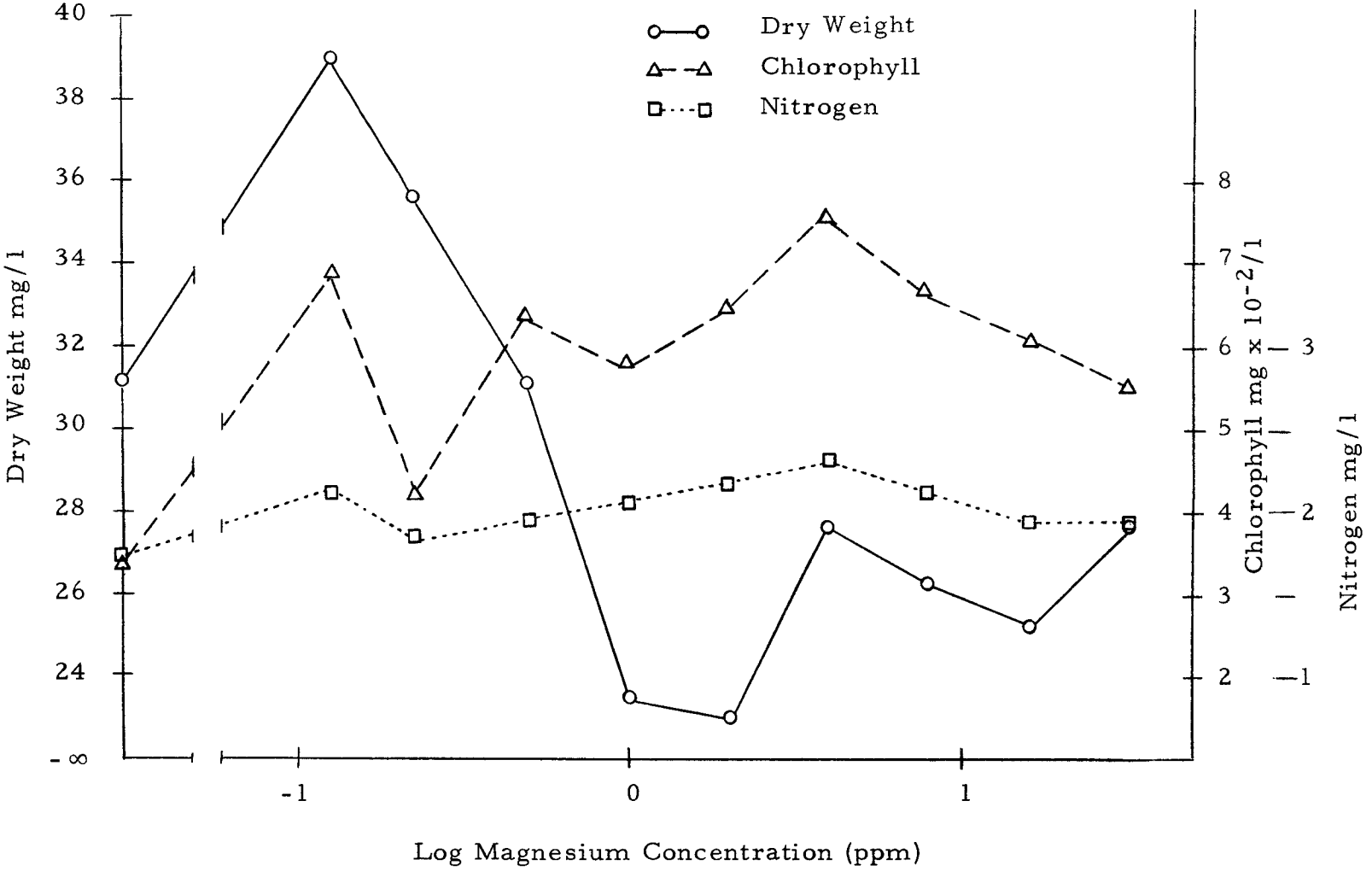

Figure 12. Effect of the concentration of magnesium on growth of Anabaena.

0.0, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 ppm magnesium added as magnesium chloride. Eight replicates of each concentration were harvested after 12 days of growth. The concentrations of chlorophyll and nitrogen remained uniform and were not influenced by increasing magnesium concentrations above 0.125 ppm (Figure 12). Maximum dry weight occurred with a concentration of 0.125 ppm followed by an increasing inhibition paralleling increasing concentration until a plateau was achieved at 1 ppm. This curve is almost the reverse of those obtained with the other elements where increase in dry weight due to sheath production occurred with higher concentrations. In this case it appears that increasing concentrations of magnesium tends to inhibit the formation of the sheath material. Concentrations ranging between 0.125 and 2 ppm were not detectable by the thiazole yellow method of magnesium determination, so it was impossible to determine at which concentration, if any, magnesium had become limiting. Analyses of the media containing 2-32 ppm magnesium showed a large proportion of the metal still present after 12 days of growth.

Calcium

The effect of variation in the concentration of calcium on the growth of Anabaena was studied, using $CaCl_2$ supplied in the range of 0.625 - 80 ppm calcium, each concentration being twice the

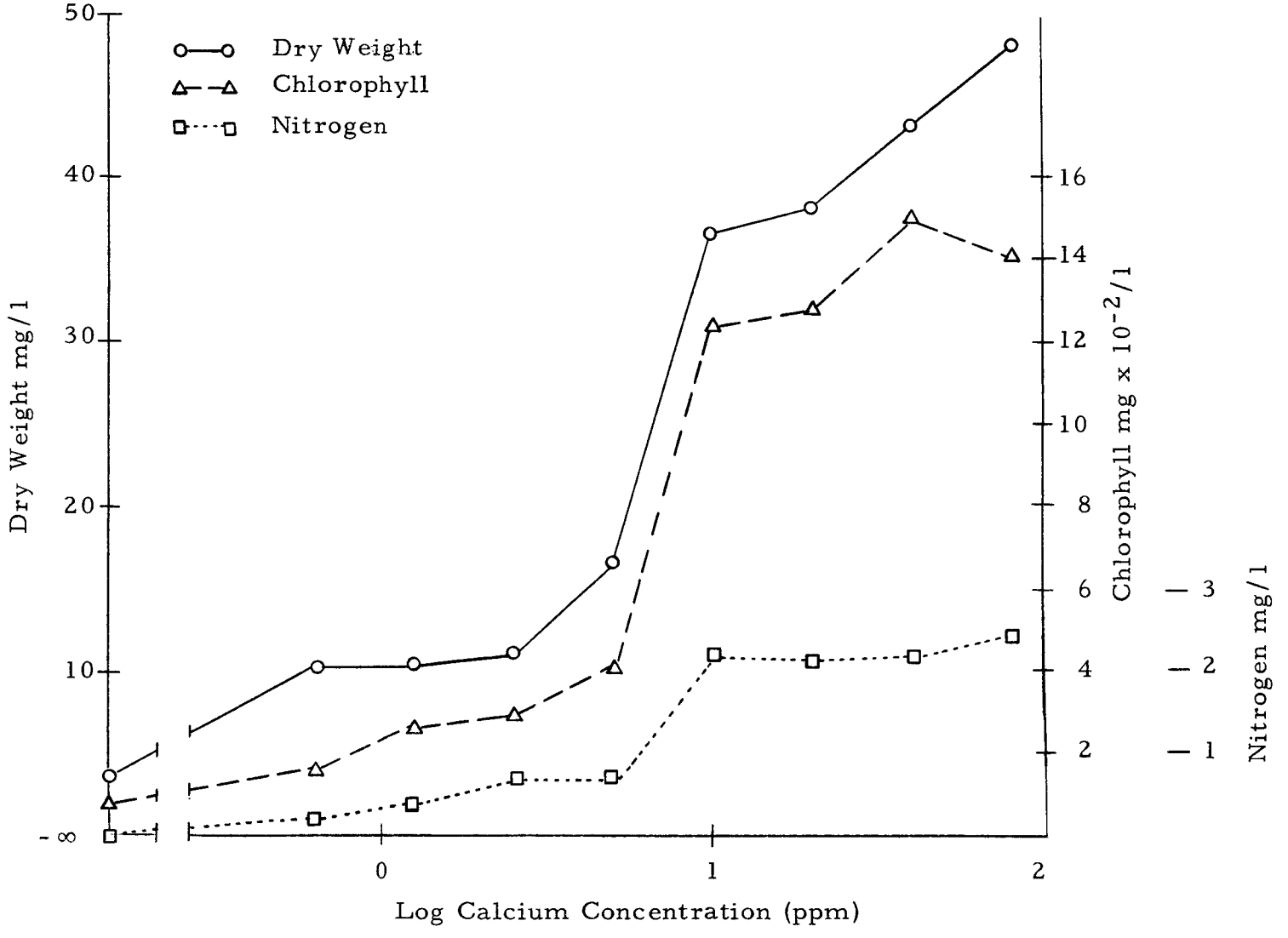

Figure 13. Effect of the concentration of calcium on growth of Anabaena.

preceding one. In concentrations less than 5 ppm, calcium had little effect on growth (Figure 13). As the concentration increased to 10 ppm a definite enhancement of growth occurred. This increase was little improved at concentrations higher than 10 ppm. The dry weight increased less than 10 mg/l with the addition of eight times as much calcium. This increase in dry weight may have resulted from increased production of carbohydrate sheath material which was not reflected by the other analyses, since the increase of chlorophyll and organic nitrogen at concentrations above 10 ppm is negligible.

Analysis of the calcium remaining after 12 days of growth showed an increase above that initially present in the media that contained 5 ppm and less. A decrease in calcium occurred, paralleling the increased rate of cell division in media that contained concentrations of 10 ppm or more. In no case did the rate of uptake warrant the high concentration of calcium required.

Sulfur

The effect of varying the concentration of sulfur on the growth of Anabaena was observed using 0.0, 0.36, 0.75, 1.5, 3, 6, 12, and 24 ppm sulfur added as sodium sulfate. The general trend (Figure 14) was an increase in dry weight and chlorophyll with increase in sulfur up to a concentration of 3 ppm. Above this concentration

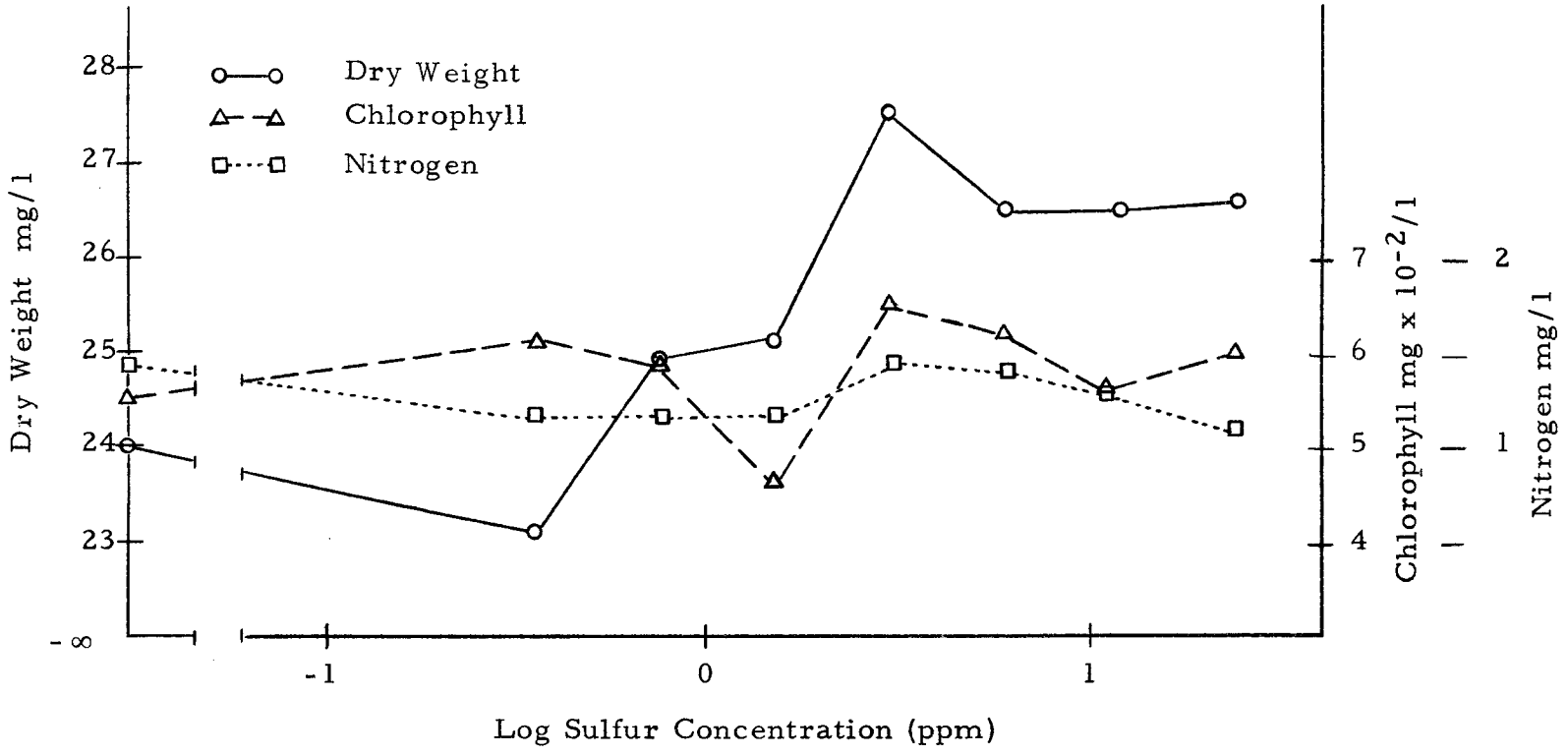

Figure 14. Effect of the concentration of sulfur on growth of Anabaena.

a plateau was reached where increased sulfur content did not affect growth. The organic nitrogen content also follows this trend but less dramatically than the chlorophyll content. The leveling off of the dry weight in coincidence with the chlorophyll and nitrogen indicates that production of sheath material did not occur rapidly at high sulfur concentrations.

Potassium

The effect of varying potassium on the growth of the alga was studied using concentrations increased from 0.625 to 40 ppm potassium. The organism was grown at 15°C and harvested after 12 days. Substantial growth occurred at 0 ppm potassium (Figure 15). This indicated that either the inoculum was not truly potassium deficient or that contamination of the chemicals used was sufficient to provide the basic requirement for this element. The chlorophyll and nitrogen analyses indicate that maximum growth occurred at 2.5 ppm potassium. This concentration produced a doubling in the amount of organic nitrogen above that present at 0 ppm while chlorophyll was increased by a factor of 1.26. This trend is paralled by an increase in dry weight which continued even after the content of nitrogen and chlorophyll leveled off. This latter increase was assumed to indicate production of extracellular sheath material rather than increase in cell numbers.

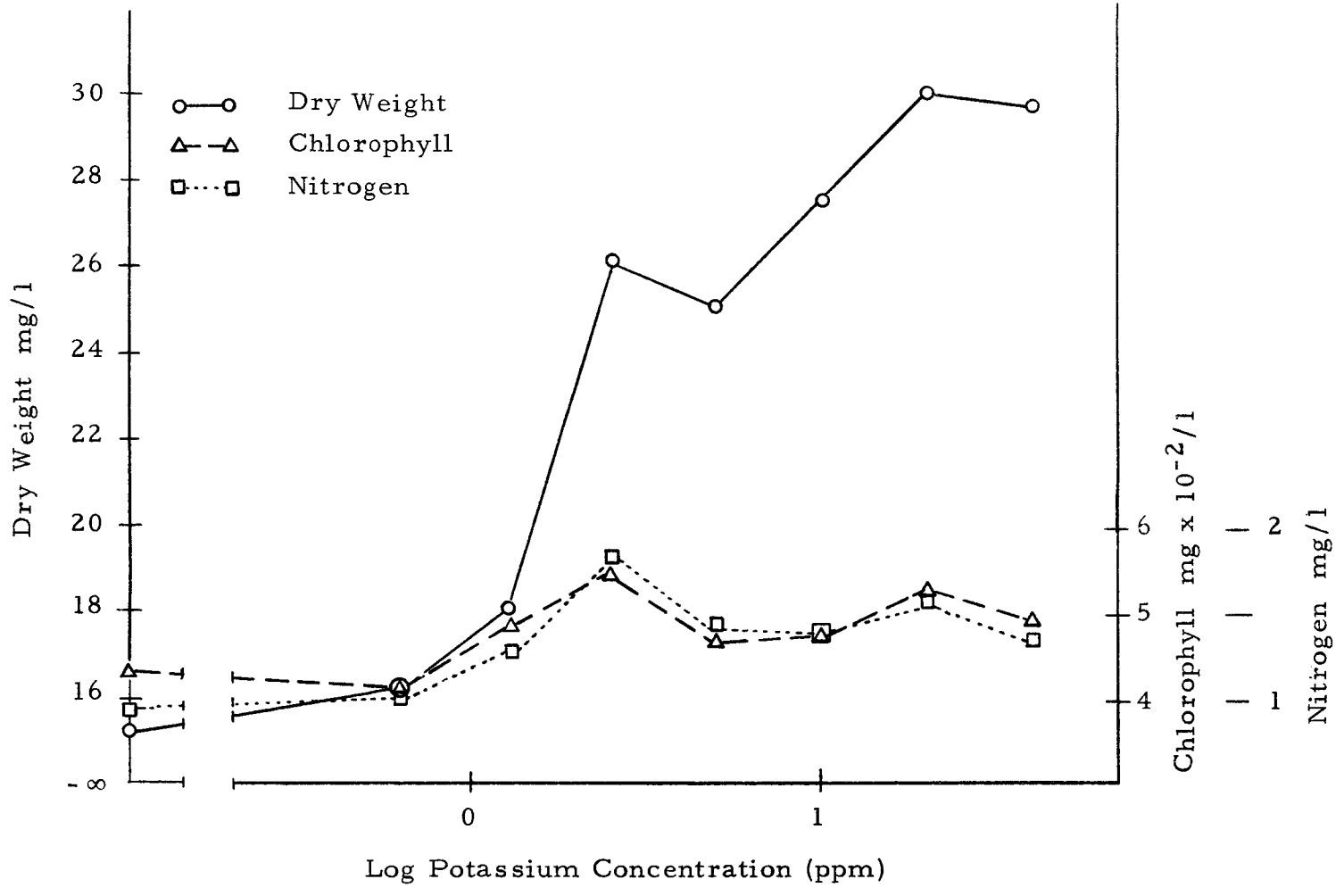

Figure 15. Effect of the concentration of potassium on growth of Anabaena.

Sodium Carbonate

To determine the effect of varying concentrations of carbonate on the growth of Anabaena, sodium carbonate was added to the medium at 0, 5, 25, 50 and 100 ppm. The initial pH of these media was 6.1, 6.7, 7.5, 7.9, 8.3, respectively. After 10 days of growth the pH had been altered to 7.4, 7.5, 7.9, 8.2 and 8.7. Dry weight and chlorophyll concentrations follow the same patterns (Figure 16A) except that dry weight increased enormously at 100 ppm sodium carbonate. This may have been the result of increased synthesis of sheath material. The incorporation of organic nitrogen was relatively uniform and showed no difference between cells grown at 0 ppm sodium carbonate and those grown at 100 ppm. Except for the decreased synthesis at 5 ppm, which cannot be explained, chlorophyll content also increased very little over the range of concentrations. It appeared, therefore, that addition of carbonate to the medium did not influence growth but may have stimulated sheath production. This stimulation may have resulted from the high pH obtained with high carbonate concentrations rather than a requirement for carbonate per se.

Minor Elements

Minor element nutrition was investigated in an exploratory experiment in which minor elements were supplied by addition of

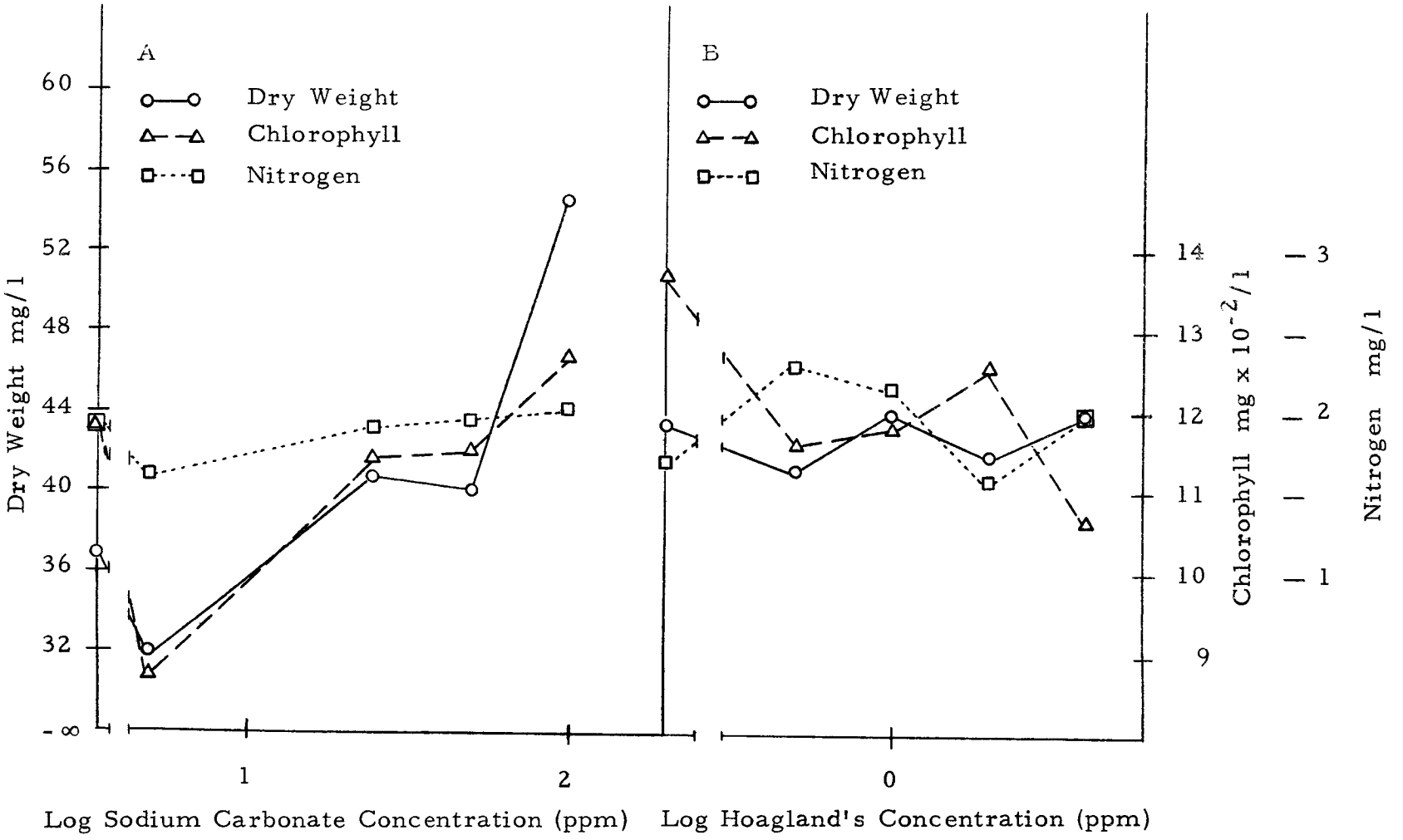

Figure 16. Effect of the concentration of (A) sodium carbonate and (B) Hoagland's minor element solution on growth of Anabaena.

Table 18. Concentration of the minerals in the media developed from nutrition studies (mg/l)

Element	Compound	Balanced Media							Unbalanced Medium
		+ Nitrogen				- Nitrogen			
		1	2	3	4	5	6	7	8
Mg	$MgSO_4$	16.63	16.63	16.63	0.52	16.63	16.63	16.63	33.6
	$Mg\ Cl_2$	--	--	--	--	--	--	--	1.2
S	$MgSO_4$	see above	see above	see above	see above	see above	see above	see above	--
	Na_2SO_4	--	--	--	--	--	--	--	26.6
K	K_2HPO_4	5.06	5.06	5.06	--	5.06	5.06	5.06	--
	K_2SO_4	--	--	--	5.56	--	--	--	--
	KCl	--	--	--	--	--	--	--	2.5
Ca	$Ca_3(PO_4)_2$	21.03	--	--	2.6	21.03	21.03	--	--
	$Ca(NO_3)_2$	235.69	235.69	235.69	--	--	--	--	--
	$CaCl_2$	--	--	--	33.02	146.74	146.74	146.74	146.74
P	$Ca_3(PO_4)_2$	see above	--	--	see above	see above	see above	--	--
	NaH_2PO_4	--	--	--	--	--	--	--	23.2
N	$Ca(NO_3)_2$	see above	see above	see above	--	--	--	--	--
Fe	EDDHA*	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
CO_3	$NaCO_3$	100.0	50.0	100.0	80.0	100.0	100.0	100.0	100.0
Hoagland's Solution**		1.0	1.0	1.0	--	1.0	1.0	1.0	1.0
TRIS Buffer		--	.24mM	--	--	--	.24mM	--	--

* Addition calculated in ppm iron.

** 1.0 ml of a 1/25 dilution per liter added.

Hoagland's A-Z minor element solution at a dilution of 1/25 that required by higher plants. Quantities of 0.5, 1, 2 and 4 ml were added per liter of culture solution. A great variation in dry weight and chlorophyll content of the cultures resulted from the different concentrations of minor elements (Figure 16B). The increase in organic nitrogen was more or less unchanged and the tendency would be to say that, overall, variation in concentration did not effect the growth of Anabaena to any significant extent.

Since Hoagland's solution contains many elements which added separately in sufficient quantity would prove toxic to the organism, it is natural to expect that certain concentrations of the solution of these combined elements would reach toxic proportions for a particular system. The next concentration, however, might provide a particular element in such optimal amounts that deleterious effects of others would be overshadowed. This may account for the irregularity of the dry weight and chlorophyll curves.

Development of an Optimal Growth Medium

The preceding nutritional study provided an indication of the concentrations of the various major elements necessary for the optimal growth of Anabaena under the culture conditions employed in these experiments.

The culture solutions developed, based on the previous ana-

lysis, consisted of seven physiologically balanced media and one unbalanced medium (Table 18). The dry weight obtained by growth in media numbers 1, 5 and 8 is greater than that in the control (Table 19). Each of these media contains the same concentration of essential elements except that in No. 8 all cations are provided as chloride and all anions as sodium salts. Number 5 is identical to No. 1 except that calcium nitrate was omitted and the calcium was reintroduced as calcium chloride.

Medium No. 1 was the only one which provided enhanced growth as measured by all three analyses. The dry weight increase was over twice that of the control but this may have been due in part to enhanced sheath production. The increase in chlorophyll and nitrogen is also substantial, however. Medium No. 1 (Table 20) is, therefore, recommended for the future culture of Anabaena spiroides.

Table 19. Growth of Anabaena after ten days in test media

Medium	pH	Dry Weight mg/l	Chlorophyll mg x 10^{-2}/l	Nitrogen mg/l
RGT (Control)	8.8	63.3	19.7	4.11
#1	8.6	139.3	21.9	5.36
#2	8.8	71.3	11.5	3.16
#3	9.2	88.0	11.1	4.22
#4	9.5	17.3	3.0	1.78
#5	8.5	101.3	16.5	3.95
#6	8.8	57.3	4.8	3.38
#7	8.9	66.0	15.8	3.38
#8	9.0	94.0	12.1	5.15

Each determination is a mean of three replicas.
Each medium was subcultured twice to allow for adaptation to the new conditions.

Table 20. Recommended medium for growth of Anabaena spiroides

Compound	Concentration mg/l	ppm Essential Element
$MgSO_4$	16.63	Mg . . . . . 4.0 S 5.3
K_2HPO_4	5.06	K . . . . . 2.5 P 5.2
$Ca_3(PO_4)_2$	21.03	Ca . . . . 48.9 N 28.0
$Ca(NO_3)_2 \cdot 4H_2O$	235.69	Fe 1.2
$NaCO_3$	100.00	
EDDHA	1.20 ppm Fe	
Hoagland's A-Z	1.00 ml	

DISCUSSION

McLachlan (1957) reviewed extensively the early literature concerning the culture techniques employed in growing green and blue-green algae under artificial conditions and the studies of the mineral requirements of these organisms. Since 1957 Lewin (1962), Gerloff (1963) and Hutner and Provasoli (1964) have provided additional excellent reviews on the subject.

The successful growth of blue-green algae in culture often is not as rapidly achieved as the growth of either green algae or higher plants. Perhaps much of the difficulty in culturing blue-green algae stems from the fact that their environmental requirements have not been studied extensively. The first medium used for growth of blue-greens was simply that of a soil extract (Pringsheim, 1912). More recently a number of synthetic media have been developed based on analysis of the requirements of specific organisms (Gerloff, Fitzgerald and Skoog, 1950, 1952; Provasoli, McLachlan and Droop, 1957; McLachlan and Gorham, 1961; Zehnder, 1963; Smith and Wiedeman, 1964).

ASMT medium developed by McLachlan and Gorham (1961) for Microcystis was used in the first attempts to introduce Anabaena spiroides to culture. Growth in this medium was extremely slow and at least one month intervened before the cell concentration became dense enough to warrant subculture. Although the concentration of

the medium was repeatedly varied, no dramatic enhancement of growth occurred. This initial work was based strictly on qualitative observations since the amount of material available and its extremely slow growth rate precluded quantitative determinations. The addition of a trace element solution developed by Arnon (1935) improved growth only slightly. The growth rate obtained in ASMT was so slow that any study of the growth or nutritional requirements of the organism was impracticable.

It has been noted by McLachlan (1957), Phinney (1958), Phinney and Peek (1960) and O'Flaherty (1966) that blue-greens have no trouble achieving tremendous populations in the waters of the Klamath Drainage Basin. It was thought that perhaps a medium possessing ionic concentrations similar to those in the lake from which Anabaena was isolated might stimulate growth. A medium was formulated to simulate the mineral ion composition of water from the Link River, an effluent river draining Upper Klamath Lake (Oregon State Sanitary Authority, 1964). This culture solution did not support growth of the alga at all. The LR medium possessed very low salt concentrations (see Table 2) and, although capable of supporting growth in nature, were insufficient for growth in culture. Natural water contains numerous organic compounds which act as chelating agents combining with metal ions present in the water. A chelate complex keeps metallic ions in solution and makes them more available for use by aquatic

organisms. In culture these natural chelating agents are absent and increased concentrations of the mineral ions must be substituted to provide the material in the necessary concentration, or a synthetic chelate supplied.

The medium developed by Chu (1942) and modified by Gerloff (1950) is a physiologically unbalanced medium in which the essential cations are supplied as chlorides and the essential anions as sodium salts. This medium provided conditions in which enhanced growth of Anabaena was obtained. By varying the concentration of the entire medium ($\frac{1}{4}$, $\frac{1}{2}$, 1, 2, 4, 8 times the original concentration) it was found that 2XG-10 promoted the best growth.

In order to stabilize the pH of the medium, a buffer for the pH range 7.0 to 8.5 extensively employed in media for growth of marine alga was utilized. Tris (hydroxymethyl) aminomethane (Tris) has been found toxic to certain blue-greens when used in high concentrations at high pH (McLachlan and Gorham, 1961, 1962; McLachlan 1962; Zehnder, 1962). This toxicity has been postulated to be due indirectly to potassium deficiency caused by Tris antagonizing the uptake of potassium (MacLeod and Onofrey, 1954; Pinter and Provasoli, 1958; Provasoli et al. 1957). McLachlan and Gorham (1962) determined that this antagonism did not occur with Tris concentrations of 4-10 mM at pH's ranging from 7.9 to 8.4. A concentration of 8 mM was employed in the 2XG-10 medium with pH ranges 7.7 to 8.4.

Tris was used as a buffer in preference to carbonate or phosphate buffers which provided additional nutrient ions. Tris is a large, complex molecule which cannot be used as a carbon or a nitrogen source in the growth of green algae (Wiedeman, 1964). This was assumed to be true also for Anabaena.

When nutritional studies were initiated it was found that high concentrations of salt would cause masses of yellow, flocculent precipitate. Colorimetric analysis of the medium showed that very little iron remained in solution. For this reason a chelated iron source was substituted for the ferric citrate-citric acid complex. Although Versene-Fe^3 was initially tested this chelate is unstable under alkaline conditions (Jacobson, 1951) and rapidly releases its iron. Another chelate, EDDHA, which is stable under alkaline conditions has been used in work with higher plants (Evans, 1963) and was tested as a substitute iron source in the 2XG-10 medium. One disadvantage to the use of this compound is the red color possessed by the metal-ligand complex. At 2 ppm iron, the concentration used in stock medium, the color imparted is distinct and it was feared that the quality of light available to the cells might be altered. The absorption spectrum for 2XG-10 + 2 ppm Fe as EDDHA (RGT medium) was run against a blank of 2XG-10 + ferric citrate-citric acid. The EDDHA medium absorbs approximately 15% of the light in the blue region of the spectrum. This reduction is not thought to be sufficient to inhibit

the photosynthetic processes of the organism. Light sufficient to saturate photosynthesis may be absorbed entirely from the red region. Since the absorption peak of phycocyanin (615 nm) is not in the area affected by the iron source it is doubtful whether photosynthesis would be at all influenced. The organism is planktonic and, as such, floats at or near the surface of the medium so the amount of liquid traversed by light prior to absorption by the cell organelles would probably be insufficient to greatly alter the quality or intensity.

That an iron source is important has been emphasized by Rodhe (1948) and the lack of an appropriate source may be a major obstacle in introduction of new organisms to culture (O'Flaherty, 1966). The amounts of iron required will, of course, depend upon the organism concerned, the pH of the medium and the nature of the iron source provided. Cooper (1937) has shown that as the pH of the medium increases the amount of iron in true solution declines rapidly and the greater portion of this iron consists of ferrous and $FeOH^{++}$ ions. Zehnder (1962) found that 1 - 2 mg of Versene-diol chelated iron per liter of culture solution was sufficient for maximum growth of Gloeotrichia echinulata while 0. 75 mg iron as ferric citrate-citric acid was found suitable for Microcystis aeruginosa (Zehnder and Gorham, 1960). McLachlan (1957) found 1. 12 ppm ferric citrate optimal for growth of Aphanizomenon while O'Flaherty (1966) found 5 ppm iron added as ferric chloride - sodium EDTA produced optimum growth.

Walker (1954) found that 30 µg iron were required for the growth of 1 gram of dried Chlorella in the absence of EDTA, while in the presence of EDTA higher concentrations were required. The same result was also found in our experiments where 5 ppm iron was required when added as Versene and 1.2 ppm and 0.3 ppm when additions were ferric citrate-citric acid and EDDHA respectively. Increasing chelating power in alkaline media appears to result in a decrease in amount of iron required.

In natural situations the availability of iron has often been thought to be a controlling factor in the establishment and cessation of algal blooms. Uspenski (1927) found that in a stream environment optimum concentrations of inorganic iron for individual algae ranged from 0.2 - 2.0 mg of ferric oxide per liter of water. In addition, he noted that concentrations exceeding this optimum by 2 - 3 times were toxic. The speed with which iron was removed from the waters was in direct proportion to the calcium content of the water. This relationship was also noted by Pringsheim (1951) who determined that high concentrations of calcium tended to precipitate the phosphates causing an increase in pH. It is through this rise in pH that iron is also removed from solution.

Gerloff and Skoog (1957a) studied the growth of Microcystis aeruginosa in the lakes of southern Wisconsin. They found that the critical levels for maximum growth of the organism was 100 ppm

iron and 4 ppm manganese. By analysis of the ionic concentration of these elements within the algal cell they concluded that the cell contents were in all cases so far in excess of the critical levels that it was unlikely that the availability of either element was a factor in the development of blooms in lakes.

Klamath Lake water, measured at station K4 on the Link River (Oregon Sanitary Authority, 1964), had an average iron concentration of 0.240 ppm between August, 1959 and May, 1961. This concentration was below that required by Anabaena in culture but the use of EDDHA decreased the in vitro requirement to 0.3 ppm. Since the average pH at station 4 is 8.1, the iron present must be bound as a stable chelate. As such it is readily available to the cells allowing more growth than would be expected at such a low concentration. That natural iron chelates exist is substantiated by the fact that when iron is added to culture media as a stable complex, concentrations providing optimum growth approach those in the lake. Studies by Peek (1963) showed that humic marsh water is capable of stabilizing iron and phosphorous at high pH.

The preference of Anabaena for alkaline media is not unique but appears to be a general requirement for blue-greens. Maertens (1914) performed the first exact measurements of the effect of pH and found that in no case would cultures of Oscillatoria brevis, O. tenuis, Nostoc sp., Cylindrosperum licheniforme or Calothrix stellaris grow

below pH 7. Gerloff (1950) found that Coccochloris peniocystis did not develop well at pH below 8 and Allen (1952) in her study of 23 species of blue-greens found that all cultures developed well at a pH of 8 to 8.5 but not below pH 7. Mc Lachlan and Gorham (1962) found Microcystis aeruginosa grew equally well throughout the pH range 6.5 to 10 when provided with suitable media and Zehnder (1963) showed Gloeotrichia echinulata to have a maximum growth rate at pH 9. Aphanizomenon flos-aquae , exhibited no measurable growth below pH 9 (McLachlan 1957) although O'Flaherty (1966) maintained his cultures in a range of pH 7.3 to 7.7. Kratz and Meyers (1955) demonstrated the pH optima of Anabaena variablis and Nostoc muscorum to range between 6.9 and 9.0 and that a 2 hour generation time could be induced in Anacystis nidulans if grown between pH 7.4 and 9.0. These requirements for highly alkaline media present special problems in developing precipitate-free culture solutions.

Phosphorous has been proposed as exerting limiting effects on the growth of phytoplankton in nature (Rodhe, 1948). In our experiments the time during which Anabaena was cultured in medium lacking phosphorous (40 days) did not appear sufficient to impede growth greatly. The growth of the alga was, of course, enhanced by addition of phosphate, but in its absence, increase in cell numbers was substantial. The increase indicates an accumulation of phosphorous in the cells which may be drawn upon in times of phosphorous deficiency.

Cultures have been maintained in the laboratory for more than 3 months in a phosphorous deficient medium without noticeable adverse effects.

Gerloff and Skoog (1954) studied the cell content of nitrogen and phosphorous of Microcystis aeruginosa as a measure of their availability for growth. They found that there could be almost a four-fold increase in the phosphorous content of the medium (0.12 - 0.46%) without a significant change in yield. Considerable growth was obtained after transfer from media containing high concentrations of phosphorous or nitrogen to solutions lacking either one of these elements. They proposed that algae in a lake could begin growth in a nutrient rich region such as the layer next to the bottom muds where they might absorb sufficient quantities of various elements to permit considerable growth after being carried into the nutrient deficient surface waters.

Anabaena spiroides overwinters in the mud at the bottom of Klamath Lake and returns to the surface as the light intensity and temperature increase in the spring months. During spring algal blooms the phosphorous content in the surface waters is negligible (less than 0.003 ppm) and although the lake is shallow and a constant replenishment of minerals to the upper layers may occur, available phosphorous may be deficient. A process of accumulation of phosphorous when it is present in excess would explain the luxuriant

growth obtained both in the lake and in culture under conditions where the cells are deprived of this element for relatively long periods of time.

Prior to the work of Chu the amount of phosphorous added to culture media varied from 9 - 100 ppm. Chu, (1943) who was the first to study the effect of phosphorous concentration on the growth of phytoplankton, found that the phosphorous concentration present in natural waters (0. 003 - 0. 02 ppm) was not inhibitory to growth and that toxic concentrations were not reached before a concentration of 17. 9 ppm phosphorous. The optimum concentrations for growth of phytoplankton species that have been studied appear to be above those occurring in natural waters (Rodhe, 1948; Gerloff, Fitzgerald and Skoog, 1950 and 1952; McLachlan, 1957; O'Flaherty, 1966).

Gerloff, Fitzgerald and Skoog (1952) demonstrated optimum growth of Anacystis at a range of phosphate concentrations of 0. 18 - 1. 8 ppm. McLachlan (1957) was unable to show a difference in growth of Aphanizomenon flos-aquae in concentrations of phosphorous ranging from 0. 1 to 2. 5 ppm while O'Flaherty (1966) obtained optimal growth at 1. 8 ppm.

Growth of Anabaena spiroides was little affected by a range from 0. 052 to 0. 52 ppm of phosphorous. The noticeable decline observed between 0. 52 and 2. 6 ppm is inexplicable except by the possibility that these concentrations may be such that either phosphorous

is antagonizing the uptake of some other ion in the medium or phosphorous uptake is being prevented.

The nitrogen metabolism of an organism is extremely important and, although some blue-greens are capable of accumulating nitrogen (Gerloff and Skoog, 1954), the quantity of nitrogen required is sufficient to greatly limit growth and development when nitrogen sources become unavailable. Nitrogen, for example, was the primary limiting factor for the growth of Microcystis, 5 mg of nitrogen being required for each 100 mg of algae produced (Gerloff and Skoog, 1957b). The N/P ratio for optimum growth was found to be 60:1, nitrogen appearing required in much larger quantities for this organism than phosphorous.

In 1889, Frank discovered that increases in combined nitrogen in soil cultures incubated in the light could be associated with the development of blue-green organisms. This was the first observation that blue-greens might be capable of fixing atmospheric nitrogen. Beijerinck (1901) grew copious amounts of Anabaena catenula and other Anabaena spp. in media, initially free from combined nitrogen, that had been inoculated with small amounts of soil and incubated in the light. Neither Frank's nor Beijerinck's observations constituted proof of nitrogen fixation by these organisms, however, since the cultures studied were contaminated with other microorganisms which might have been the nitrogen fixers. The first pure cultures of blue-

green algae were obtained by Pringsheim (1914) but neither he nor Maertens (1914) could find evidence for nitrogen fixation in these species , although several had been previously reported to have this capacity. It was therefore concluded that blue-green algae do not fix nitrogen by themselves and that they are capable of developing in media deficient in this element because they are in symbiotic association with bacteria possessing this capacity (Jones, 1930). Bacteria-free cultures of Anabaena variabilis and Nostoc punctiforme were isolated and shown to be able to fix nitrogen by Drews (1928).

Later work has amply confirmed that many members of the Myxophyceae are capable of nitrogen fixation. A number of good review articles have been written on the nitrogen metabolism of blue-green algae (Fogg, 1947; Fogg and Wolfe, 1954; Fogg, 1956; Fogg, 1962) and most of the cultural and nutritional work involving the Myxophyceae has been related to the study of nitrogen fixation in these organisms.

Not all of the blue-greens are capable of fixation and until recently the capability was thought to be limited to those organisms belonging to the Nostocaceae. In 1951 Fogg demonstrated that bacteria-free cultures of Mastigocladus laminosus, a member of the Stigonemataceae, fixes atmospheric nitrogen and studies by Williams and Burris (1952) on Calothrix parietina verified the existence of nitrogen fixation in the Rivulariaceae. Watanabe (1951) established

fixation in Tolypothrix tenuis, a member of the Scytonemataceae. Stewart (1965) stated that all blue-greens possessing heterocysts show nitrogen fixation but this may not be a valid rule (Williams and Burris, 1952; Allen, 1952; McLachlan, 1957; O'Flaherty, 1966).

The mineral requirements for nitrogen fixation have been studied by a number of workers. Molybdenum has been found an essential element for nitrogen fixation (Bortels, 1930). In studies with Nostoc sp. (Bortels, 1940), Nostoc muscorum (Eyster, 1959) and Anabaena cylindrica (Wolfe, 1954a,b; Allen, 1956) elimination of molybdenum from the culture medium prevented nitrogen fixation completely. The molybdenum requirement is specific (Allen, 1956) no other element being capable of adequate substitution. Although molybdenum is also required when a nitrate-nitrogen source is provided only one-half the amount necessary for optimal fixation is needed (Wolfe, 1954a). Growth is possible in the absence of molybdenum only when ammonium is supplied as the nitrogen source (Wolfe, 1954a; Eyster, 1959).

Calcium has also been found essential whether the organisms are fixing nitrogen or growing on nitrate. Twenty ppm calcium is necessary for optimum fixation in Anabaena cylindrica (Allen, 1956) and 30 ppm for Nostoc muscorum (Eyster, 1959). Strontium is not able to replace calcium in either of these requirements.

A requirement of 5-10 mg sodium per liter, which cannot be

replaced by another cation, has been found necessary for optimal growth of Anabaena cylindrica (Allen, 1956). Eyster (1959) also demonstrated an enhancement of growth with addition of sodium but stated that this requirement had no direct relationship to nitrogen fixation.

In addition to molybdenum and calcium, Eyster (1952) found boron essential for nitrogen fixation in Nostoc. The role that these elements play in the blue-greens is not definitely understood but Allison et al.(1937) suggested that calcium, at least, may play a catalytic role in nitrogen fixation as in Azotobacter (Burk and Lineweaver, 1931). It would not be at all surprising to find that nitrogen fixation in the blue-green algae very closely approximates that in the bacteria.

In studies of Anabaena cylindrica Cobb and Myers (1964) found a very close relationship between nitrogen fixation and photosynthesis. The ratio of carbon to nitrogen fixed is inversely related to the carbon-nitrogen ratio in the cell. Nitrogen fixation and carbon dioxide reduction are competitors for hydrogen donors and at light intensities higher than those required to saturate photosynthesis the rate of nitrogen fixation is reduced. This might well explain the observation that Anabaena spiroides grows best at low light intensities and is markedly inhibited by moderately intense light.

Obviously the only positive proof that a blue-green alga fixes

nitrogen is to demonstrate fixation in a bacteria-free culture, a difficult task in view of the problem of removing bacteria embedded within the gelatinous investment surrounding the algal cells. Anabaena spiroides was not obtained in pure culture and, although the evidence suggests that the alga is capable of fixing atmospheric nitrogen, it is realized that the growth obtained in nitrogen deficient media may result from nitrogen produced by associated bacteria. Although the contaminant bacteria were isolated in pure culture their ability to fix nitrogen was not tested. The slight difference between growth of Anabaena on a nitrogen deficient medium and on a nitrate-nitrogen medium was at first surprising but the same result was reported by Allen and Arnon in 1955 in their work with Anabaena cylindrica. Allen (1956) also found that the presence of combined nitrogen in the medium does not prevent nitrogen fixation of A. cylindrica whereas, in Azotobacter combined nitrogen inhibits formation of the enzymes involved in fixation. In the study of Anabaena spiroides a decrease in growth rate was expected when the organism became adapted to fixing atmospheric nitrogen after utilizing combined nitrogen. No such decrease was observed, and it is possible that the organism was fixing nitrogen even though combined sources were present. Such fixation could only be substantiated by the use of N^{15}, however.

Nostoc muscorum has been reported by Magee and Burris

(1954) to require a period of adaptation before it could utilize nitrate after fixing atmospheric nitrogen. It is assumed that the reverse might also hold true. The data do not seem to show such a lag period in the case of Anabaena. If any generalization can be made, it would be that the alga is not influenced by the amount of nitrate present in the culture medium.

The growth rate in the presence of glycine was greatly stimulated indicating greater efficiency of utilization of amino-nitrogen than atmospheric nitrogen. Since the end product is the same and reduction of nitrogen in fixation would require expenditure of more energy than would removal of the amino group from glycine the observation is not surprising. The increased growth rate may also result, in part, from the provision of a carbon source which may be incorporated and metabolized directly, freeing the organism somewhat from dependency on carbon dioxide fixation.

The capacity for nitrogen fixation would be of extreme advantage to the organism in nature since it would be independent of replenishment of nitrogen in the upper surface waters to maintain itself. Hutchinson (1944) found that Anabaena circinalis would appear in Connecticut lakes in bloom proportions when the inorganic nutrients were almost exhausted. The alga was most favored when nitrate was scarce indicating that when nutrient levels are high, other organisms become established with which Anabaena cannot adequately compete. When these organisms have depleted the nutrient

reserves Anabaena, because of its capacity for nitrogen fixation and its apparent tolerance for low nutrient concentrations, can dominate the environment. Nitrogen fixation by Anabaena may then produce a more favorable environment for succeeding organisms (Hutchinson, 1944) and is extremely important in the metabolic cycle of a lake containing appreciable quantities of the alga (Hutchinson, 1941). The same concept may apply to Anabaena spiroides which appears in Klamath Lake following blooms of Aphanizomenon flos-aquae, a non-nitrogen fixer (Williams and Burris, 1952; McLachlan, 1957; O'Flaherty, 1966). Aphanizomenon may exhaust the combined nitrogen present in the upper layers of the lake and thereby limit its own growth. The low nitrogen is then suitable for establishement of Anabaena which fixes sufficient nitrogen to meet its growth requirements. It is well known that blue-greens secrete extracellular nitrogenous substances (Fogg, 1952; Taha and Refai, 1962; Stewart, 1963; Whitton, 1965) and it may be that by excretion of the excess nitrogen fixed a non-nitrogen fixing codominant, Microcystis, may become established.

Magnesium is required as an integral part of the chlorophyll molecule and is required as a cofactor in many enzymatic reactions. It is not required in high concentrations, however. Most algal species require concentrations ranging between 0.1 and 1.0 ppm under cultural conditions (Gerloff, et al., 1952; Ketchum, 1954). Anabaena

did not require a high concentration of magnesium and although chlorophyll synthesis was slightly increased over the concentration range 0. 125 - 32 ppm, dry weight was diminished and the nitrogen content remained constant. The higher concentrations actually appear inhibitory, if only dry weight is considered. The decrease in dry weight is quite possibly due to decreased production of extracellular sheath material. Why magnesium should inhibit sheath formation is not clear. The sheath has been proposed to be a polysaccharide matrix (Virieux, 1910; Payen, 1938; Kylin, 1943; Jones, Hough, and Wodman; 1952; Bishop, Adams and Huges, 1954; Biswas, 1957) which is presumably transformed into a solid gel by linkages with calcium and/or magnesium ions (Foerster, 1964). If the dry weight determinations of the calcium and magnesium tests are compared it will be noted that magnesium exerts its effect at low concentrations while calcium is effective in increasing dry weight at high concentrations. In the study of magnesium the calcium content is constant and at low concentrations of magnesium the calcium utilization in the sheath is normal. As the concentration of magnesium is increased, however, the uptake of calcium is impaired and the production of a stable, insoluble sheath is impeded. It is unlikely that production of the component carbohydrates would be markedly influenced as long as enough magnesium was present to satisfy the requirements of the enzyme and chlorophyll systems. It is more

probable the unavailability of calcium, whether caused by antagonism of uptake or absence from the medium, prevents the bonding required to transform highly soluble carbohydrate components into an insoluble gel. The non-bonded material would pass undetected through the filter used in dry weight determinations. In tests varying the concentration of calcium the amount of magnesium was maintained at 2.4 ppm. At low concentrations of calcium this might be sufficient to antagonize the uptake of calcium and result in the low dry weights observed at concentrations below 10 ppm. Increase in calcium would overcome this effect and sheath material would again form in a solid state. This is a speculative explanation but the metal bonds formed by pectin-like compounds are rather specific and inhibition of calcium uptake by magnesium could well account for the apparent reduction in dry weight by inhibition of production of a solid sheath.

The requirement for calcium other than as a component in the sheath is seen by the increase in nitrogen and chlorophyll content between 5 and 10 ppm calcium. The requirement for calcium in nitrogen fixation by the blue-greens has already been discussed. Allison, Hoover and Morris (1937) found that neither calcium nor strontium was required for growth of Nostoc muscorum in the presence of combined nitrogen while Allen and Arnon (1955a) found that calcium is essential for growth of Anabaena cylindrica whether grown on atmospheric nitrogen or nitrate. They also found the requirement to be

specific and calcium could not be replaced by strontium. In those green and blue-green organisms for which no calcium requirement could be demonstrated (Rodhe, 1948; Gerloff et al., 1950; Ketchum, 1954) it is possible that the salts utilized contained sufficient contaminant calcium to meet the requirements. Although calcium is supposed by many to be required in trace amounts (Gerloff et al., 1952; Eyster, 1959) Allen found that 20 ppm was required for optimum growth in Anabaena cylindrica (1956) and I found 10 ppm optimal for Anabaena spiroides.

The sulfur requirement of the blue-greens has received very little attention. Gerloff (1950, 1952) found that sulfur was essential and required in larger quantities than phosphorous for growth of Coccochloris and Microcystis. In fact, next to nitrate, sulfate was the ion required in highest concentration. McLachlan (1957) reported enhanced growth of Aphanizomenon with 1.0 ppm sulfur and neither enhancement nor inhibition occurring at higher concentrations (maximum sulfur concentration tested was 3 ppm). Increased growth was observed in Anabaena spiroides at 3 ppm with no further effect either stimulatory or inhibitory at concentrations as high as 32 ppm. At higher sulfur concentrations there was no increase in dry weight due to sheath production.

Potassium is also considered an essential element in the development of phytoplankton although it very seldom becomes

limiting in the natural environment (Rodhe, 1948). Gerloff found that potassium was the cation required in highest concentration by Coccochloris (1950) and Microcystis (1952) in culture. Benicke (1898) found an Oscillatoria capable of growth in a medium in which all the potassium salts had been replaced by sodium compounds. Since that time several workers have found sodium to be a physiologically suitable substitute for potassium. Allen (1952) found 23 different Myxophyceae that could grow in media containing sodium and no potassium. Although sodium is capable of replacing potassium Ketchum (1954) remarked that there was no evidence that a sodium requirement existed in algal nutrition. Emerson and Lewis (1942) had found, however, that sodium was necessary for the maintenance of steady photosynthesis in Chroococcus and that it was possible to reduce the potassium concentration in the culture medium to as low as 1 ppm without depressing growth of the alga. Growth was inhibited, however, if sodium was eliminated completely. Kratz and Myers (1955) also found sodium required for maintaining high photosynthetic rates in Anabaena variabilis, Anacystis nidulans and Nostoc muscorum but potassium was also essential.

Allen (1956) and Allen and Arnon (1955a, b) demonstrated abnormal development of Anabaena cylindrica in the absence of sodium. They also determined the sodium requirement to be specific, irreplaceable by potassium, lithium, rubidium or cobalt. In Chlorella

and Ankistrodesmis Meyers (1951) indicated that potassium could be partially replaced by rubidium but not by sodium or cesium.

In the work with Anabaena spiroides increased growth was obtained with potassium concentrations increasing to 2.5 ppm. At higher concentrations no further enhancement of growth occurred. Similar results were obtained by McLachlan (1957) with Aphanizomenon flos-aquae. The increase in dry weight at high potassium concentrations may result from greater sheath production since the increase is not accompanied by increases in chlorophyll or nitrogen. The substantial growth obtained at 0 ppm potassium may indicate that the organism is capable of accumulating this element. The potassium contamination present in the other chemicals used may also have been sufficient to provide minimal requirements. Since an unbalanced medium was used in which the anions were supplied as sodium it was not possible to study the sodium requirements of this organism.

The addition of carbonate to the medium did not appear to greatly effect the growth of Anabaena as measured by chlorophyll and nitrogen contents. However, sheath production was tremendously enhanced at the highest carbonate concentrations. The absence of carbonate did not inhibit growth, as was also shown by McLachlan (1957) with Aphanizomenon. As Gerloff (1952) has suggested, it is also possible the favorable effects of sodium carbonate may concern

the pH of the medium rather than an essential requirement for the constituent elements.

The effect of trace elements on the growth of phytoplankton has received very little study. Trace elements are usually supplied to the medium in an arbitrary fashion as Hoagland's A-Z solution (Hoagland and Synder, 1933) or as Arnon's modification of this solution (Arnon, 1938). Boron, cobalt, copper, molybdenum and zinc have been determined to be essential for the growth of some species (Eyster, 1952; Fogg and Wolfe, 1954; Ketchum, 1954; Holm-Hansen, Gerloff and Skoog, 1954). Need for cobalt, molybdenum and boron in the nitrogen fixation processes was discussed earlier.

A more thorough investigation of micro-nutrient requirements is definitely indicated. The inclusion of a chance mixture of minor elements into a medium may do much to inhibit or enhance growth. That all of the elements provided in Hoagland's A-Z solution are necessary is extremely doubtful. At any particular concentration of the solution one or more of the components may reach toxic proportions for a particular system. That a requirement for minor elements cannot be demonstrated by using such a solution is shown by the results obtained using Anabaena spiroides. The inhibitory and stimulatory effects operate to produce an irregular curve of growth which is essentially meaningless. The blue-greens of Klamath Lake probably have a fairly high tolerance for minor elements since they

exist in a humic habitat where these elements are held in solution as chelate complexes. The only way to determine the appropriateness of adding the constituents of Hoagland's solution, of course, is to determine the essentiality of each component element, admittedly, a tremendous task.

The combination of the optimum concentrations of each element to produce a physiologically balanced medium was met with difficulty since most of the compounds containing the requisite anion-cation combinations are insoluble. The problem is also complicated by the association of the anion and cation, since addition of one member at a particular concentration necessitates a certain concentration of the other. For this reason it was not possible to produce a medium which contained the exact optimal concentrations indicated in each study. In the final medium, sulfur is in much higher concentration than would have been ideal. Since no drastic inhibition of growth was noted with high concentrations of sulfur an increase in sulfur was considered more desirable than a decrease in magnesium. The concentration of nitrogen did not appear to greatly affect the growth of the alga and the amount of calcium required determined the amount of nitrogen provided.

The increase in dry weight obtained in medium No. 1 probably resulted to a large extent, from an increase in carbohydrate sheath material. Since the organic nitrogen content, although increasing,

does not even approach doubling, it does not appear that the increase in dry weight resulted solely from increase in cell mass. The chlorophyll data also tend to substantiate this conclusion.

Medium No. 5, although it produced an enhanced dry weight did not produce concommitant increases in chlorophyll or nitrogen. The data, in fact, very closely approximated that obtained in RGT medium. The increase in weight in this case is probably entirely due to production of sheath material.

The growth obtained in medium No. 8 is reflected by increases in both dry weight and nitrogen. The unbalanced nature of this medium and its high sodium chloride content made this enhanced growth surprising and indicates that the organism can tolerate quite high salinity.

The addition of Tris (Nos. 2 and 6) did not enhance growth and, in fact, resulted in a marked decrease in chlorophyll and nitrogen content which may have resulted from an antagonism of potassium uptake as discussed previously.

The development of a medium providing rapid growth of Anabaena will be of definite advantage in providing cells in optimum condition for future investigations. The results obtained in the nutrition study indicates that the organism possesses a wide tolerance for variations in ionic concentrations and is also capable of considerable growth when a major nutrient element is eliminated from the medium

The implication is that either very minute amounts of most of the major elements are required while at the same time there is tolerance of very high concentrations, or surpluses are accumulated within the cell that may be drawn upon during times of depletion in the environment. The study presented here provides a starting point from which to determine the true explanation. Determination of the minimal mineral requirements of Anabaena would benefit our understanding of the physiology of these ubiquitous and often troublesome creatures.

The difficulty faced in obtaining pure cultures of the blue-greens is intriguing. Since the major obstacle lies in the presence of the gelatinous investment around the algal cells, studies on the sheath might lead to refined methods of purification. A number of unanswered questions exist concerning the sheath: What conditions favor sheath formation? What are the components and how are they joined together to produce the structure observed? Is sheath production regulated or unregulated? Is its formation linked in any way with the photosynthetic processes? Is the presence of a sheath beneficial? These and many other questions remain unstudied and need attention.

Aside from the problems of purification, the bacteral-algal association presents a relationship which might prove to be based on mutual benefit or even mutual necessity. Since the bacteria have

been isolated and maintained separately while all attempts to grow Anabaena spiroides in a pure culture have failed, it may be that the alga survives because of metabolic products released by the bacteria embedded in the sheath. Of course, this is speculative but presents a fascinating problem.

The nitrogen-fixing capability of Anabaena spiroides requires additional attention. Although preliminary data indicate that the organism is an active fixer this can only be proven by use of pure algal cultures, N^{15} tracer work, or by establishment of cell free nitrogen fixation utilizing the enzyme systems extracted from the alga.

A number of problems have been suggested in the course of this study that will be the subject of future research.

SUMMARY

This thesis describes some of the effects of variation of major mineral nutrients on the growth in culture of the blue-green planktont Anabaena spiroides Klebahn. In addition, some of the effects of variations in hydrogen ion concentration and light intensity were considered.

The alga was isolated from plankton collections from Klamath Lake, Oregon, and maintained in unialgal culture in Erlenmeyer flasks. Attempts were made to provide the organism with constant conditions of temperature and light intensity. Growth was determined by dry weight, chlorophyll a concentration, and nitrogen content, determined by the microKjeldahl procedure.

The organism was initially introduced to culture using McLachlan's ASMT medium but more rapid growth was later obtained using Gerloff's modification of Chu number 10 medium. The latter medium was modified by the addition of Tris buffer and substitution of EDDHA for ferric citrate-citric acid. This physiologically unbalanced medium (RGT) provided the basis for the nutritional studies.

The culture used during this investigation, although unialgal, was not bacteria-free. A number of unsuccessful attempts were made to rid this organism of bacteria by use of antibiotics, removal of the sheath, pasteurization, and ultra-violet irradiation. All major elements found necessary for growth of higher plants, except nitrogen,

were found to be necessary for growth of the alga. The independence shown with regard to nitrogen strongly suggests this organism is a nitrogen-fixer. Phosphorous, although required in high concentrations for optimum growth, was apparently accumulated within the cell allowing considerable growth when the element was eliminated from the medium. Iron, when supplied as the chelate EDDHA, was required in much lower quantity than supplied as either ferric citrate or Versene. When provided as the latter compound, a considerable quantity of the iron was precipitated from solution, because of the alkaline nature of the medium. A definite requirement for calcium was shown, and it appears that the interaction of calcium and magnesium ions influences solidification of the sheath.

A physiologically balanced medium based on the individual optimal nutrient concentrations was developed that allowed enhanced growth of the alga. When the nitrogen source was deleted from this culture solution, however, growth was the same as obtained in the physiologically unbalanced RGT medium.

Experiments varying the pH indicated that an alkaline medium favored growth of the alga. Very little growth occurred below pH 7 while pH 10 appeared optimal. Although the range of light intensities studied was not extensive it appears that the alga prefers low light intensities, growth being impeded as the intensity increases above 120 foot candles.

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