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The Effect of Antibiotics on Thermophilic Blue-Green Algae

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AN ABSTRACT OF THE THESIS OF Erleen Blanche Christensen for the
Master of Science in Biology presented May 24, 1971.

Title: The Effect of Antibiotics on Thermophilic Blue-Green Algae

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The nature of the action of penicillin on the cell walls of bacteria is reviewed. The composition of bacterial cell walls is compared to cell walls of blue-green algae.

The test organisms used were thermophilic: Anaerostipes nidulans grew optimally at 35°C, where as Synochococcus lividus grew best at 45°C. Growth was recorded by reading optical densities.

Cells of these two thermophilic blue-green algae were treated with varying dosages of penicillin and streptomycin. Penicillin inhibited

growth of Anacystis nidulans and Synechococcus lividus in concentrations of 3.0 $\mu\text{g/ml}$ and 0.03 $\mu\text{g/ml}$ respectively. However, when 0.3 $\mu\text{g/ml}$ of penicillin was added to Anacystis nidulans, an initial lag phase of growth was observed. Possible reasons for this lag are suggested and results of pertinent experiments are discussed. No similar lag was noted in Synechococcus lividus.

Streptomycin inhibited growth of both species when present in 0.03 $\mu\text{g/ml}$.

THE EFFECT OF ANTIBIOTICS ON THERMOPHILIC BLUE-GREEN ALGAE

by

HELEN BLANCHE CHRISTENSEN

A thesis submitted in partial fulfillment of the
requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

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1971**

TO THE OFFICE OF GRADUATE STUDIES:

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INTRODUCTION

Blue-green algae differ from other algae, in that they lack well defined nuclei, mitochondria, and other membrane enclosed organelles normally found in the cells of eucaryotic algae. In these respects, blue-green algae are more similar to bacteria and it is from this point of view that this project has been done. Because antibiotics act upon bacteria in various ways, the question arises if they similarly affect blue-green algae, closely related procaryotes. In bacteria, penicillin interrupts the synthesis of the cell wall and primary attention here has been aimed at determining whether or not this is also the case in these algae. A comparison of the effects of streptomycin has also been made.

Mucopeptide In The Cell Wall

Bacteria are divided into gram-negative and gram-positive organisms on the basis of a differential stain (gram), which may be due to different permeability properties of the cell wall. All bacteria investigated have in their cell walls, mucopeptides and DAP (diaminopimelic acid), which are apparently only found in procaryotic organisms. Gram-positive organisms may have greater than 50% of the cell wall composed of mucopeptide while gram-negative organisms have 5-10%. This mucopeptide is a polymer of glucosamine and muramic acid with peptides attached to form a complex multimolecular network that provides considerable strength to the wall (Mandelstam and McQuillen, 1968; Novikoff and Heltsman, 1970).

The repeating units of the mucopeptide, glucosamine and muramic acid, are shown in Figure 1.

Figure 1. Structures of glucosamine and muramic acid.

The amino group in these compounds is usually not free but is combined

with an acetyl radical to give $\text{N}-\overset{\text{O}}{\parallel}\text{C}-\text{CH}_3$ instead of NH_2 . The peptides, which are attached to the muramic acid residues, are shown in Figure 2.

Figure 2. General structure for cell wall mucopeptide.

(From Flynn and Godsenki, 1967).

N-AcGlu = N-acetylglucosamine; N-AcMur = N-acetylmuramic acid;

glu = glutamic acid; lys = lysine.

Arrows indicate probable cross links between peptide side chains.

The peptides are composed of a limited range of amino acids. Alanine and glutamic acid are always found, together with either diaminopimelic acid (DAP), L-lysine, 2,4-diaminobutyric acid, D- or L-ornithine, glycine, or D-aspartic acid (Rogers, 1963; Mandelstam and McQuillen, 1968). Most of the glutamic acid and about half of the alanine is present as the so-called 'unnatural' D-isomer and this, together with the occurrence of muramic acid and DAP may be partially responsible for the properties possessed by the mucopeptide. The biosynthesis of cell wall mucopeptide is shown in Figure 3.

Evidence For Presence Of Mucopeptide In Blue-Green Algae

Salton (1960) reported the presence of mucopeptides in blue-green algae. It is known that in bacteria the synthesis of this compound is blocked by penicillin (Park and Strominger, 1957). Because of the presence of mucopeptide in the wall of blue-green algae, these structures may also be sensitive to the biosynthetic block brought about by penicillin treatment and would be potential substrates for lysozyme. Lysozyme hydrolyses many of the susceptible bonds between N-acetylmuramic acid and N-acetylglucosamine, and the cell wall is digested. It has been demonstrated that both direct treatment of isolated walls with lysozyme and growth in the presence of penicillin could be used effectively in studying the anatomy of cell walls of the blue-green alga, Phormidium uncinatum (Frank, Lefort, and Martin, 1962). Quantitative chemical analysis of isolated cell walls of this organism showed that the mucopolymer component resembles the composition of the cell walls of gram-negative bacteria. Studies on gram-negative bacteria have established

Figure 1. Biosynthesis of cell wall mucopeptide. (From Mandelstam and McQuillen, 1968).

that the mucopolymer layer is electron dense and lies next to the cytoplasmic membrane (Salton, 1964). Further work on bacteria indicates that lysozyme removes this layer in the formation of spheroplasts (Murray et al., 1965). The inner layer of the cell wall of Anacyctis nidulans was removed by the lysozyme in the formation of spheroplasts, indicating a possible similarity between this layer and the electron-dense mucopolymer layer in gram-negative bacterial cell walls (Lindsey et al., 1971). Similar ultrastructural investigation of Phormidium by Frank (1962) showed that the mucopolymer supporting membrane, which is apparently responsible for the rigidity of the cell wall (Weidel and Primosigh, 1957; Weidel et al., 1960), could also be digested by lysozyme.

Lysozyme is a one-chain protein with a sequence of 129 amino acid residues known (Mahler and Cordes, 1966). Lysozyme exerts its most pronounced effect on young bacterial cells (Chatterjee and Williams, 1955). The decrease with age in the percent of spheroplast formation of synchronous A. nidulans cells (Lindsey et al., 1971), could be due to metabolic differences in the cells or to variations in the amount of N-acetylglucosamine polymers. These polymers are the specific substrate for lysozyme action upon the cell wall. Lindsey and his group feel that the response to lysozyme action may be altered by the frequency and accessibility of the linkages in the substrate, by any factor affecting the stability of the cell surface structure, or by any substance blocking the interaction of lysozyme with its substrate (e.g. sheath or capsular substances). However, lysozyme easily attacks organisms surrounded by mucilaginous sheaths. Synechococcus was found to be resistant to lysozyme

(Crespi, 1962) and it is without an evident gelatinous sheath.

Synechococcus may have a more complicated cell wall or Crespi may be correct by inferring that distinctly sheathed algae probably contain relatively greater amounts of muramic acid and mucopolypeptide as important constituents of the cell wall.

Holm-Hansen (1965) reported DAP was present in A. nidulans and other blue-green algae. DAP was found in the bound form as a component of a mucopeptide of the cell wall. DAP, unique to procaryotes, has a structure similar to lysine (See Figure 4).

Figure 4. Similar structures of DAP and lysine.

Lysine is sometimes replaced by DAP in the mucopeptide.

Drews and Meyer (1964) found the mucopolymer components N-acetylglucosamine, N-acetylmuramic acid, glutamic acid, diaminopimelic acid, and alanine in the blue-green algae, Chlorogloea and Anacystis. Isolated cell walls were lysed by lysozyme, but whole cells were transformed into spheroplasts only by adding ethylene diamino tetra-acetic acid. These facts again suggest that blue-green algal walls are equipped

with a rigid layer of mucopolymer, like bacteria. The demonstration in members of the Cyanophyta of a mucopolymer cell wall component closely resembling that previously found in cell walls of bacteria provides further evidence for a taxonomic relationship between the two classes of organisms (See Table I).

Mechanism Of Action Of Penicillin

Penicillin affects mucopeptide synthesis in the cell wall of procaryotic organisms (Pitts-James, 1965). The percent of inhibition is apparently dependent on the ratio of analogue/natural substrate. Inhibition by penicillin begins at some stage after the formation of UDP-N-AcMur pentapeptide. There are several theories given for penicillin's ability to act. It has been shown (Collins and Richmond, 1965) that penicillin has a structural resemblance to the molecular topography of muramic acid (See Figure 5). Penicillin could be competing with muramic acid for the active center of polymerizing enzymes.

Secondly, since penicillin is believed to inhibit the transpeptidation reaction, it could be an analogue to the D-alanyl-D-alanine fragment of the linear glycopeptide, which is important in this transpeptidation. If there is no interpeptide linkage, a structurally weak mucopeptide results which cannot contain the protoplast and the organism will lyse.

Another analogue suggested is the L-alanine-D-glutamic acid region of glucopeptides. The free α -carboxyl group of the penicillin molecule would correspond to the free α -carboxyl group of D-glutamic acid in the mucopeptide (Wise and Park, 1965).

TABLE I			
PRINCIPAL COMPONENTS OF CELL WALLS			
COMPONENT	GRAM + BACTERIA	GRAM - BACTERIA	BLUE-GREEN ALGAE
Mucopeptide	+	+	+
Polysaccharide	+	+	+
Teichoic acid	+	+	
Teichuronic acid	+		
Protein	+	+	+
Lipid		+	+
Lipopolysaccharide		+	?
Lipoprotein		+	?

Information on bacterial cell walls from Mandelstam and McQuillen (1968).
 Information on algal cell walls from Drews and Meyer (1964) and Punnett
 and Derrenbacker (1966).

Figure 5. A comparison of the structure of N-acetyl muramic acid (a) and penicillin (b)

N-Acetyl muramic acid:

O_3 , ether oxygen of laetyl side chain

O_7 , carbonyl group of carboxyl attached to C_7

O_{10} , carbonyl oxygen attached to C_{10} in the N-acetyl residue.

Penicillin:

N_1 , triply-substituted nitrogen of β -lactam ring

O_1 , carbonyl oxygen attached to C_1 in carboxylic acid residue

O_9 , carbonyl oxygen attached to C_9 in the side chain

O_8 , carbonyl oxygen attached to C_8 of β -lactam bond.

(Taken from Richmond, 1966).

If the carboxyl group of the penicillin molecule is modified, there is decreased activity. Oxidation of the sulfur in the thiazolidine ring lessens the inhibitory effect greatly. All gradations of activity are possible by altering the acyl group on the amino position. The integrity of the β -lactam ring is critical to its activity (Wise and Park, 1965). The enzyme penicillinase breaks this lactam ring and thereby antagonizes the antibiotic's effect.

The Action Of Streptomycin

Streptomycin is known to attach to a specific ribosomal site on the 30-S subunit of the ribosome (Cox, 1964; Mahler and Cordes, 1966). This attachment does not interfere with the subsequent interaction of the particle with the mRNA nor with protein synthesis per se. What does happen as a result of this interaction, however, is that this altered ribosome-mRNA complex binds different amino acyl-tRNAs than does the corresponding complex in the absence of the antibiotic. This leads to a wrong translation of the advancing messenger tape. Clearly, such massive synthesis of faulty proteins should be lethal to the cell.

Previous Work Of Antibiotic Effect On Blue-Green Algae

When growing filaments of Phormidium were exposed to penicillin, localized defects appeared in the mucopolymer layer of longitudinal walls and in the corresponding layers of complete and incomplete cross wall (Lang, 1966). These defects may indicate regions of recent wall growth. Isolation and properties of streptomycin and penicillin resistant strains

of Phormidium mucicola has been reported by Srivastava (1969, 1970). He found that parent strains could not survive beyond 0.2 and 0.1 $\mu\text{g/ml}$ of streptomycin and penicillin respectively. Gupta and Kumar (1970) found similar results for Anacystis which would grow in 0.25 $\mu\text{g/ml}$ of streptomycin and 0.06 $\mu\text{g/ml}$ of penicillin. Srivastava reported that a penicillin resistant strain of Phormidium mucicola was not stable until three contracts with penicillin had been made. This was not the case with streptomycin. With Anacystis nidulans Pikalek (1967) got an increase of resistance of 100-fold with penicillin in five steps and 200-fold with streptomycin in seven steps. This differs substantially from the analogous processes known in bacteria (Szybalski, 1953), and could indicate a different kind of genetic control of resistance to these drugs in blue-green algae.

Cell-Wall Growth And Division In Prokaryotes

Growth of the cell wall in gram-positive and negative organisms may vary. Salton (1960) saw gram-positive cell wall growth occurring in a limited zone of the dividing cell, whereas gram-negative bacteria appear to have growth at various sites (Conti and Gettner, 1962). These two principal ways of distributing new wall material may be of considerable importance in terms of the action of penicillin. Multiplicity of wall forming sites in gram-negative bacteria would account for their greater resistance to penicillin, despite the smaller amount that cells need to synthesize.

Mandelstam and McQuillen (1968), however, state that the type of division in all bacteria may be remarkably similar. It may occur by an

inward growth of a septum before cell division, as is the case in blue-green algae (Desikachary, 1959).

Description Of The Problem

The purpose of the experiments done was to study the effects of varying concentrations of penicillin and streptomycin on growing axenic cultures of two species of blue-green algae, Synechococcus lividus and Anacystis nidulans. Growth curves were obtained on the organisms growing in antibiotic-containing media and electron micrographs were taken in an effort to illustrate any visible changes in the cell walls.

The blue-green algae used in this study were thermophilic: Synechococcus lividus grows optimally at 45°C and Anacystis nidulans grows best at 35°C. This type of organism may be more important in the future as the temperature of our waters rises with increasing pollution. In nature, Synechococcus outgrows other blue-green algae to give a dense suspension bloom (Castenholz, 1969). Many blue-green algae have higher temperature optima than eucaryotic organisms of the same waters (Batterton and Van Baalen, 1968), so as the temperature rises these blue-greens become more of a problem. It seems quite probable that habitats of thermophiles will increase in the future.

MATERIALS AND METHODS

Axenic cultures of Synechococcus lividus strain Y-52-J and Anacystis nidulans were provided by Dr. Richard Castenholz of the University of Oregon. Stock cultures were maintained on Cg-10 agar slants (Van Baalen, 1967) (See Table II). For experimental work involving growth curves, the algae were grown in defined D medium (Castenholz, 1969) (See Table III). (Media that is known to be specific for blue-green algal cultures, Kratz and Myers (Kratz and Myers, 1955) and Cyanophycean (Starr, 1964), did not support growth of these species). Optimal growth for S. lividus was 45°C whereas A. nidulans grew best at 35°C; therefore, experimental work was carried out at these temperatures. The 45°C temperature was achieved by use of a constant-temperature water bath with a light intensity of 480-510 ft-c. using cool-white fluorescent tubes. The 35°C temperature was attained in a Percival environmental growth chamber model E-57. Light intensity varied from 400-550 ft-c. at the surface of the liquid. Stock cultures of S. lividus were maintained at 45°C and the stocks of A. nidulans were kept at room temperature (ca. 25°C).

A 0.2 ml inoculum from a culture containing 8.0×10^6 cells/ml was placed in a sterile tube containing 9.7 ml D medium of pH 7.1 and 0.1 ml of an antibiotic. The drugs used were streptomycin and benzyl penicillins (the potassium and sodium salts of penicillin G). Final concentrations of penicillin ranged from .003 $\mu\text{g/ml}$ to 10 mg/ml . Streptomycin dosages varied from .0003 $\mu\text{g/ml}$ to 10 mg/ml . The solutions

TABLE II
Cg-10 MEDIUM

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	g/l
0.25	
K_2HPO_4	0.050
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	0.025
KNO_3	1.00
Na_2EDTA	0.010
$\text{Fe}_2(\text{SO}_4)_3 \cdot 6\text{H}_2\text{O}$	0.004
Glycylglycine	1.00
1.0 ml of A-5 microelements Stock Solution.	

A-5 Microelements Stock Solution

H_3BO_3	g/l
2.86	
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.81
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.222
$\text{MoO}_3(85\%)$	0.018
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.079
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.010

pH adjusted to 8.0.

TABLE III

MEDIUM D

Double distilled water	1,000 ml
Nitrilotriacetic acid	0.1 g
Micromineral solution	0.5 ml
FeCl ₃ solution	1.0 ml
CaSO ₄ ·2H ₂ O	0.06 g
MgSO ₄ ·7H ₂ O	0.10 g
NaCl	0.008 g
KNO ₃	0.103 g
NaNO ₃	0.689 g
Na ₂ HPO ₄	0.111 g

MICRONUTRIENT SOLUTION

Distilled water	1,000 ml
H ₂ SO ₄ (concd)	0.5 ml
MnSO ₄ ·H ₂ O	2.28 g
ZnSO ₄ ·7H ₂ O	0.50 g
H ₃ BO ₃	0.50 g
CuSO ₄ ·5H ₂ O	0.025 g
Na ₂ MoO ₄ ·2H ₂ O	0.025 g
CoCl ₂ ·6H ₂ O	0.045 g

FeCl₃ solution consists of 1,000 ml of distilled water and .1822 g FeCl₃·6H₂O.

Designed by R. P. Sheridan, medium D is prepared as a 10-fold concentrated stock and stored at 4 C unautoclaved. After dilution with double distilled water, the pH is 3.8. It is adjusted to 8.2 with 1 M NaOH. The final pH is 7.1 after the autoclaved medium has cooled and cleared completely.

of antibiotics were prepared in double distilled water and sterilized with either a millipore filter or chloroform. The penicillin solutions were always stored in the refrigerator at 4°C and solutions older than 2 weeks were discarded.

Growth was recorded by reading the optical density on a Klett-Summersen Photoelectric Colorimeter provided with a blue filter (#42). The cultures were mechanically dispersed to an even turbidity by using a Vortex Jr. mixer. Cultures were grown in test tubes that fit a special adapter in the colorimeter. The tubes were closed with cotton-gauze plugs or Morton stainless steel closures. The cotton-gauze plugs were preferred because growth was more rapid (Castenholz, 1969), although the problem of evaporation was greater, especially in the growth chamber.

Cell numbers were correlated with the optical densities by haemocytometer counts. Plate counts were attempted using Cg-10 agar and D medium solidified with agar (ca.1.5%).

A Corning model 7 pH meter equipped with a Sargent-Welch combination electrode was used for determining hydrogen ion concentration of the medium. This electrode made it possible to make readings directly in the culture tubes. Solutions of 0.1 M NaOH and 0.1 M HCl were used to alter the initial pH of the D medium when desired.

Experimental procedures used for testing the effect of penicillin and streptomycin varied. In some experiments, the antibiotics were added at various points in the growth curve. In others, cells from a culture growing in the presence of penicillin were again inoculated into D medium containing varying penicillin concentrations.

An extract was taken from an actively growing culture containing penicillin and was tested to determine if penicillinase was present. This was done by comparing the effect of the extract with the action of penicillinase (Bacto-Penase, Difco), obtained from Bacillus sp. These were tested on Anacystis nidulans and the bacteria Escherichia coli and Sarcina lutea. These organisms were fixed and stained by the gram stain.

Cells were measured using a calibrated ocular micrometer on a Zeiss light microscope, model WL. The film used for pictures on the light microscope was Kodak Panatomic X, FX 120.

For electron microscopy, cells were sedimented by centrifugation (10 min, 1500 rpm) and fixed with glutaraldehyde for two hours, rinsed with 0.2 M PO_4 buffer, and fixed with 1% osmium tetroxide overnight. The next day the samples were dehydrated in a graded series of acetone-propylene oxide, and embedded in Epon 812. Thin sections were cut on a Porter-Blum MT-2 microtome with glass knives and picked up on carbon-coated formvar supports. These were double stained with uranyl acetate and lead citrate, and photographed in a Zeiss electron microscope, Model 9S-2, on Agfa-Gevaert Scientia film at initial magnifications of 9500 X.

The graphs were generated on a Control Data Corporation 6400 computer by a program written in the FORTRAN language and plotted on a CALCOMP 670 plotter.

The Organisms

Synechococcus, a member of the Cyanophyta, Order Chroococcales, was first noted by Nägeli in 1849 (Smith, 1950). The cells of this

genus are usually solitary although they may be in groups of 2 to 4 aligned pole to pole (See Figure 6). The cells are ellipsoidal or cylindrical and are without an evident gelatinous sheath. Reproduction is by transverse cell division, normally followed by separation of daughter cells. If separation does not occur, 2-4 celled colonies will result. Individual rods measure 1.2 to 2.2μ wide and 5 to 7.5μ long.

Anagystis is also assigned to the Order Chroococcales. The cells are ellipsoidal or cylindrical with broadly rounded poles. A sheath is evident around each cell although portions of sheaths of cells may be confluent within a colony which may number hundreds. The individual cells are 1.2 to 2.2μ wide and $1\frac{1}{2}$ to 3 times longer than broad. Under certain culture conditions the cells tend to elongate and cells up to 20μ were not uncommon (See Figure 6).

(a)

(b)

(c)

Figure 6. Microphotographs of Synechococcus lividus and Anacystis nidulans. a) S. lividus. X 1350 b) S. lividus after 4 hour treatment with 4.5 g/ml Pen-K. X 1350 No morphological differences can be noted between a and b. c) A. nidulans. X 1400 Elongated cells are not due to penicillin treatment, but occurred under normal laboratory conditions.

RESULTS

Optimal Temperatures For Thermophilic Organisms

Synechococcus lividus is considered a true thermophile since its maximum growth occurs at 45°C (See Figure 7). However, Anacystis nidulans may be called a mesophile since it grows optimally at 35°C (See Figure 8). These temperatures are too high to allow survival of most eucaryotic organisms. Although many proteins (enzymes) normally are not active at these temperatures, it appears that the cells carry on photosynthesis and other metabolic activities at these comparatively high temperatures. Brock (1967) found the maximal rate of ^{14}C photo-incorporation in natural populations of Synechococcus taken from several different temperature regimes was very close to the temperature from which they had been collected. For this reason, assuming that the effect of antibiotics would be greater on healthy, growing cultures, experiments were carried out at the temperature giving optimal growth for each organism.

D medium contains the requirements known for growing blue-green algae (Castenholz, 1966; Gerloff, 1969). However, in D medium diluted 10 times more than specified, neither organism showed growth until 12 days after inoculation. This growth occurred only at their optimal temperature. Anacystis, which can normally be maintained at room temperature, would not grow in this instance; nor could Synechococcus grow at 35°C, a temperature that before had provided rapid growth. When nutrients were minimal, the other conditions had to be optimal.

FIGURE 7. SYNECHOCOCCUS LIVIDUS AT VARYING TEMPERATURES.
 ■=45 DEGREES X=35 DEGREES ○=25 DEGREES
 GROWTH VERSUS TIME.

FIGURE B. ANACYCTIS NIDULANS AT VARYING TEMPERATURES.
■=45 DEGREES X=35 DEGREES ○=25 DEGREES
GROWTH VERSUS TIME.

Cell Counts

Plate counts were attempted in an effort to correlate these with cell numbers, which were determined by microscopic counts at various optical densities. However, since neither organism grew on 1.5% agar of D medium, this was not possible. Anagystis nidulans was able to grow on Cg-10 plates, but the results could not be quantitative because single colonies were not obtainable upon dilution. This has been a problem before with Anagystis (Van Bealen, 1967; Allen, 1968). Indeed, in an attempt to illustrate sexual exchange in blue-green algae, Kumar (1962) could not assert categorically that recombination occurred within individual cells because he was unable to grow clones of Anagystis on solid media from single cells.

Synechococcus did not grow upon any of the agar plates. The main reason for this failure may have been that the Petri dishes could not be put in a water bath and therefore were maintained only at 35°C. This suboptimal temperature would only hamper an already difficult problem.

Effect Of pH On Growth

The pH of the defined D medium was usually found to be 7.1 after autoclaving. As this medium is essentially nonbuffered the pH rises as cells metabolize; so it was found that cells could grow in this medium if the initial pH was more basic. Even slightly more acidic conditions allowed growth to occur.

Hydrogen ion concentration usually dropped slightly after the log phase, so that all tubes that showed growth had essentially the same pH at the end of the growth curve, regardless of the starting point (See

Tables IV and V). All growing cultures of Synechococcus lividus attained a pH of 7.8 - 8.4 at the end of the growth cycle. The final pHs of cultures of Anacystis nidulans in stationary phase (tubes 3-11) range from 7.7 - 8.4. Tube 2 had a low pH at five days because the culture had not yet begun to grow. Similarly, it's final pH is high because growth is continuing. No growth was recorded in tubes 1,9, and 10--lowest and highest initial pHs.

The Gram Stain

The gram stain is used to differentiate procaryotic organisms, apparently due to the different permeability of their cell walls. Different permeabilities imply different compounds or amounts of them present in the cell wall. The gram stain was run on both test organisms, along with Escherichia coli, a known gram-negative rod, and Sarcina lutea, a gram-positive coccus. The results for the bacteria were as expected, Sarcina staining purple (gram-positive) and E. coli staining pink (gram-negative). Anacystis and Synechococcus both appeared to be gram-negative in this test. From this it was inferred that penicillin might not have as great an effect on the algae if their cell wall was so similar to gram-negative bacteria. In adding penicillin to E. coli, a chelating agent such as $MgSO_4$ may be used to make the organism more susceptible to the antibiotic. No chelating agents were used with the blue-green algae when it was determined that penicillin was inhibiting their growth.

TABLE IV
CHANGES IN pH ACCOMPANYING GROWTH OF
Synechococcus lividus

TUBE	INITIAL pH	DAY GROWTH BEGAN	pH @ 5 DAYS	TOTAL DAYS OF GROWTH	FINAL pH
1	5.1	No growth	5.1	25	5.4
2	6.0	6	8.2	30	8.1
3	6.5	5-6	9.8	30	7.8
4	6.8	4	-	25	8.2
5	7.0	3-4	-	25	8.4
6	8.0	4	10.3	30	8.1
7	9.0	4	10.4	30	8.2
8	10.0	3-4	10.4	30	8.5
9	11.0	3-4	10.6	30	8.4

TABLE V

CHANGES IN pH ACCOMPANYING GROWTH OF
Anaerostipes nidulans

TUBE	INITIAL pH	DAY GROWTH BEGAN	pH @ 5 DAYS	TOTAL DAYS OF GROWTH	FINAL pH
1	5.2	No growth	5.2	25	5.4
2	6.0	18	7.2	30	9.1
3	6.5	5	9.9	25	7.7
4	7.4	3	-	25	7.8
5	8.0	3	9.5	30	8.0
6	9.0	2-3	10.1	30	8.0
7	10.0	2	10.4	30	8.0
8	11.0	1-2	11.0	30	8.4
9	12.0	No growth	12.0	25	9.9
10	12.5	No growth	12.5	25	12.1

Effect Of Penicillin On *Anacystis nidulans*

The sodium and potassium salts of penicillin G were added to D medium at the same time as the inoculum. The lowest concentration that inhibited growth in most cases was $3.0 \mu\text{g/ml}$. Growth appeared to be normal at concentrations of $0.03 \mu\text{g/ml}$ and below. An initial lag phase of 6-17 days was observed with $0.3 \mu\text{g/ml}$ present, whereas controls had a lag of about 2 days (See Figures 9,10, and 11).

The sodium salt of penicillin G (Pen-Na) appeared to give a shorter lag, but lag phase was so variable that this cannot be definitely stated. Although differences were not as great, it should be noted that some variation occurs in normal growth curves.

Probable Explanations For Survival In Penicillin

Several experiments were conducted to test possible explanations for the survival of *Anacystis nidulans* in the presence of penicillin. Cells treated with penicillin may have become resistant to it by a mutation allowing them to survive. These mutated cells could grow and outnumber the sensitive ones present. If this were the case, these cells should more readily survive another contact with the antibiotic. However, an inoculum from such a culture gave the same length lag phase as stock cells; nor were the treated cells able to survive higher concentrations of the drug. Therefore, it does not seem likely that the origin of drug resistant mutant strains was a factor here.

Secondly, survival in the presence of penicillin could be due to the breakdown of the antibiotic by the enzyme penicillinase. If this enzyme were being produced by the algae, growth should occur with little

FIGURE 9. ANACYSTIS NIDULANS WITH PEN-NA PRESENT.
GROWTH VERSUS TIME. * = CONTROL. ○ = WITH 0.3 MICROGRAM/ML PEN-NA.

FIGURE 10. EFFECT OF PEN-K ON ANACYCTIS NIDULANS.
GROWTH VERSUS TIME. *=CONTROL. x, o=DUPLICATE TUBES CONTAINING
0.3 MICROGRAM/ML PEN-K.

FIGURE 11. EFFECT OF PEN-NA ON ANACYCTIS NIDULANS WITH INOCULUM TAKEN FROM A 5 DAY OLD CULTURE. ■=CONTROL. X=.03 MICROGRAM/ML PEN-NA. ⊙=0.3 MICROGRAM/ML PEN-NA.

or no lag phase. An extract from Anacystis nidulans that had been growing with penicillin present was prepared and compared with known penicillinase of Bacillus. The known penicillinase was extremely effective in counteracting the action of the penicillin when tested on cultures of Anacystis. In all but one experimental tube, the algal extract did not appear to contain the enzyme (See Figure 12). Cells in the test tube with 0.3 $\mu\text{g/ml}$ of penicillin plus the extract began growing at about 8 days after inoculation. Otherwise, the cultures either showed a greater initial lag phase in response to 0.3 $\mu\text{g/ml}$ or growth was entirely inhibited by the penicillin. Since subsequent experiments of this type did not reveal any further exception, it seems improbable that the algae are producing a penicillinase.

In addition, the penicillinase from Bacillus and the algal extract were tested on Sarcina lutea which is gram-positive and therefore very sensitive to penicillin. Addition of the antibiotic caused the cells to lyse, except when penicillinase was included. The extract from Anacystis did not counteract the effect of the antibiotic.

Effect Of Penicillin On Synechococcus lividus

The initial lag phase noted in the response of Anacystis to penicillin was not observed in similar experiments with Synechococcus. No growth was observed in cultures to which 0.03 $\mu\text{g/ml}$ were added at the time of inoculation. However, cells treated with 0.003 $\mu\text{g/ml}$ of Pen-Na showed no difference in growth patterns from the controls.

FIGURE 12. TEST FOR EFFECT OF PENICILLINASE ON ANACYCTIS NIDULANS
 ■=0.3 MICROGRAM/ML PEN-NA WITH PENICILLINASE. x=0.3 MICROGRAM/ML WITH
 EXTRACT FROM ANACYCTIS. ○=0.3 MICROGRAM/ML PEN-NA. GROWTH VERSUS TIME.

Effect Of Streptomycin On Anacystis And Synechococcus

When concentrations of streptomycin of $0.03 \mu\text{g/ml}$ or above were added at the time of inoculation, growth was inhibited in both organisms. No initial lag phase was observed in these experiments and results showed either normal growth or complete inhibition. Synechococcus survived with $0.003 \mu\text{g/ml}$, whereas Anacystis grew with $0.006 \mu\text{g/ml}$ added.

When 1.5 mg/ml streptomycin was added to a Synechococcus in log phase, growth leveled off for approximately one week and then began to decrease. The decrease in Anacystis however, was more rapid and quite obvious the first day after streptomycin was added (See Figures 13 and 14).

FIGURE 13. EFFECT OF STREPTOMYCIN ON ANACYCTIS NIDULANS.
GROWTH VERSUS TIME.

FIGURE 14. EFFECT OF STREPTOMYCIN ON SYNECHOCOCCUS LIVIDUS. GROWTH VERSUS TIME. * = CONTROL, O = WITH STREPTOMYCIN.

DISCUSSION

Anacystis nidulans and Synochococcus lividus were primarily chosen experimental organisms because it was deemed highly desirable to use pure cultures and these were the species that were fortuitously available in axenic condition. As work progressed it became readily apparent that these algae were advantageous for other reasons as well. They grew reasonably well in the media used and reached comparatively large cell numbers per unit volume. In addition, since growth was recorded by employing optical density techniques, it was necessary to have homogeneous solutions. Of the many blue-green algae tested, these two thermophilic species showed homogeneous growth or, in some cases, formed a pellet which could be readily and evenly dispersed throughout the medium. Also, because increasing temperatures of lakes and streams are current world problems, it seems that more information on thermophiles will be of value.

A wide range of initial pHs allowed growth of both test organisms. Although D medium is an essentially unbuffered solution, it was observed that final pHs were in the narrow range of 7.7 - 8.4. The fact that higher initial pHs tended to give slightly higher final pHs was not considered to be significant.

The literature seems to indicate an agreement among workers that the cell walls of blue-green algae are similar to gram-negative bacteria (Frank, Lefort, and Martin, 1962). Drews and Meyer (1964) say the mucopeptide layer in blue-green algae is similar to that found in gram-positive bacteria. Recently, M. J. Bazin (Personal communication) notes the

contradiction of Anacystis nidulans being sensitive to quite low concentrations of penicillin, and yet being classed as gram-negative. The gram stain showed both Anacystis and Synechococcus to be gram-negative. With only this evidence, however, it would perhaps be tenuous to state they are more similar to gram-negative bacteria, even though it is more generally agreed that this is the case.

Although penicillin does not usually affect gram-negative organisms as easily as gram-positive, there was the appearance of an initial lag phase in tests with Anacystis. The reason for the observed lag in Anacystis with 0.3 $\mu\text{g/ml}$ of penicillin present, cannot be definitely stated. Experiments gave no evidence that mutant strains may have arisen, since cells growing with penicillin present did not react differently than untreated cells when again exposed to penicillin.

There was virtually no experimental evidence to suggest that penicillinase was being produced by Anacystis and therefore the possibility that this could be the factor causing the initial lag phase is ruled out. Since there was only one instance in which the presence of the algal extract appeared to inhibit the action of penicillin it may be that this was due to experimental error. This ability to counteract the activity of the drug in this exceptional case could possibly be due to an earlier mutational event. However, this culture was not tested again before discarding and no conclusions can be made.

It is known that penicillin rapidly deteriorates when kept at temperatures above 4°C. It is probable, then, that some cells survive until the high temperatures destroy the penicillin; then after an apparent "lag", continue to grow.

It is also possible that the nature of the inoculum is critical in determining whether or not there is an initial lag phase, since different age cultures were used as sources of the original inoculum. However, experiments showed that cells taken from five days, fifteen days, and from older cultures in the stationary phase of growth, gave the previously noted lag phase.

In an experiment just recently completed, inocula from a nine day old culture were unable to survive in $0.03 \mu\text{g/ml}$. These cells, from the log phase of growth, were probably able to begin growth immediately and their inability to synthesize new cell wall did not allow survival. Cells from the stationary or lag phase may require an initial lag before they can begin growth in new media again. It would seem that the 5-day old cultures should have given the same results, since they appear to be in the log phase also. In view of the fact that these results seem contradictory, it may be that the physiological condition of the cells vary inconsistently as they go from one phase to the next.

Pen-Na appeared to cause a shorter lag phase (about 6 days) than did Pen-K (9-17 days). This difference may be due to slightly different binding properties, which in turn are the result of different ions present. Pen-Na may not be as close an analogue to the specific cell wall component as Pen-K, which would mean fewer cells would be affected. Or, it may be that Pen-Na is less stable, and if it becomes inactive more quickly the surviving cells could grow more rapidly in its presence.

However, some variability occurred in the controls. Variability here is probably caused by inoculum size or slightly different stages in the growth cycle. Inocula from cultures that have been growing the same

number of days may not be in the same physiological condition. Pippetting errors, variable environmental conditions (incubation temperatures and light intensity), and the possibility of contamination of the cultures all can introduce experimental variability.

It seems a bit unusual that Synechococcus is so easily attacked by penicillin, and yet is resistant to lysozyme action. The cell wall of Synechococcus may vary distinctly from Anaerostis, which is affected by both.

Streptomycin had an inhibitory effect on both organisms at a certain concentration. This is understandable since enough functional proteins must be made for the cell to survive.

Also, it seems that the proteins (enzymes) of either organism cannot function at 55°C, since no growth occurred at this temperature. They have adapted to become thermophiles but they are not able to withstand increasingly higher temperatures.

Suggestions For Further Research

It has been apparent throughout the course of this investigation that certain refinements might have been desirable. The limited space available for growing thermophiles was a constant problem and perhaps the use of hot water baths in place of additional growth chambers was not as efficient. Also, no attempt was made to get cultures in synchronous growth and this might prove to be a useful method in determining the factor(s) controlling such observable phenomena as the initial lag phase response to certain levels of penicillin.

It was hoped that it would be possible to obtain electron micrographs which would prove valuable in determining the morphological changes

associated with failure of cell wall synthesis. Although much time and effort was spent in the attempt, the micrographs obtained have not been of sufficient quality to include in this research.

SUMMARY

Anacystis nidulans and Synechococcus lividus are affected by streptomycin and penicillin. In most cases A. nidulans is more resistant to both drugs. It survives with .006 $\mu\text{g/ml}$ streptomycin present and 0.3 $\mu\text{g/ml}$ of penicillin whereas S. lividus was only able to grow in 0.003 $\mu\text{g/ml}$ of both antibiotics.

The growth pattern of Synechococcus was affected in the same manner by both drugs. There was either normal growth or complete inhibition. Although streptomycin had a similar effect on Anacystis, it reacted differently to penicillin.

With the lowest concentration of penicillin allowing growth (0.3 $\mu\text{g/ml}$), Anacystis was observed to have an initial lag phase that was longer than the controls. This initial phase in the growth curve was not observed in Synechococcus. Once the lag period was over however, these cultures usually attained the same maximal growth as untreated cultures. A variety of possible reasons for this lag period are discussed.

CONCLUSION

The work here has, corroborated the results of the previous workers who have shown that the cell wall of blue-green algae is similar to bacteria. No previous reports of the effects of penicillin on Synechococcus have been seen, so this work has produced additional evidence to support the supposition that blue-green algae respond to antibiotics in much the same way as their procaryotic relatives: the bacteria.

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