GIIIIIIID

ELECTRIC BOAT GROTON, CONNECTICUT
A DIVISION OF GENERAL DYNAMICS

Photosynthetic Gas Exchange in the Closed Ecosystem for Space

Part III. Screening for Thermophilic Algae and Mutation Studies

NASA Contract No. NASW-95 1960-61

N. L. Richards

R. J. Benoit

GENERAL DYNAMICS CORPORATION
Electric Boat Division
Groton, Connecticut

Research and Development Department

Approved by: A. Bialecki

A. Bialecki, Head
Chemical Engineering Section

U411-61-107
August 1961

FOREWORD

This is the third part of the final report of a multiphase program directed toward the development of a photosynthetic gas exchanger suitable for use in a closed ecological system. The studies have been carried out under contract NASW-95 entitled, "Photosynthetic Gas Exchanger" issued to General Dynamic Corporation's Electric Boat Division by the National Aeronautics and Space Administration, Washington, D. C.

The authors gratefully acknowledge the advice and assistance of Dr. F. Trainor, Asst. Professor of Botany at the University of Connecticut for aid in the identification of algal isolates and of Dr. D. E. Nichols, Professor of Industrial Engineering at University of Rhode Island for statistical analyses of growth rate determinations. The technical assistance of Messrs. A. Stover and A. Savard, and the guidance and constructive advice of Dr. E. A. Zuraw and Mr. A. Bialecki (all of the Chemical Engineering Section) were also most helpful. Grateful appreciation is extended to colleagues throughout the world (Appendix A) who sent samples of algae for the screening program.

ABSTRACT

An algal screening and mutation study was undertaken to obtain algae superior to Chlorella 71105 for use in a photosynthetic gas exchanger. Of the forty-four thermophilic algae studied, eighteen appeared to have growth rates as great as Chlorella 71105. Optimization of the physical and chemical environments of these strains is recommended as a way to further improve growth rates and concommitant oxygen production. The mutation study revealed that Chlorella 71105 is relatively resistant to germicidal ultraviolet radiation. No high temperature mutants of Chlorella 71105 were found.

CONTENTS

Title	Page
FOREWORD	i
ABSTRACT	iii
PREFACE	ix
SECTION ONE: SCREENING FOR THERMOPHILIC ALGAE	1
INTRODUCTION	3
MATERIALS AND METHODS	5
Sample Procurement	5
Growth Units	5
Screening Procedure	6
Preliminary Isolations	7
Dilution of Samples	7
Growth Rate Determinations	8
Measurements of Growth Rates	8
Estimation of Growth Rates	9
Manometric Studies	11
RESULTS AND DISCUSSION	12
Morphology of the Isolates	12
Growth Measurements	15
Manometric Studies	18
SECTION TWO: MUTATION STUDIES	19
INTRODUCTION	21
EFFECTS OF ULTRAVIOLET RADIATION	23
PREPARATION OF CULTURES FOR IRRADIATION	24
MATERIALS AND METHODS	25
Cellular Morphology	25
Inactivation Curve Procedure	26
Procedure for Mutation	27
RESULTS AND DISCUSSION	29
BIBLIOGRAPHY	31

CONTENTS (Cont.)

Title		Page
APPENDICES		A
A	CONTRIBUTORS OF SAMPLES	A-1
B	SOURCES OF SAMPLES	A-2
C	STANDARD ELECTRIC BOAT MEDIUM FOR ALGAE CULTURE	A-7
D	OPTICAL DENSITY OF SUSPENSIONS OF *CHLORELLA* 71105	A-8
E	PROCEDURE FOR DRY WEIGHT DETERMINATIONS	A-9
F	SAMPLE CALCULATION OF MEAN CELL DIAMETERS, STANDARD DEVIATIONS, AND PERCENTAGES	A-10
G	CALCULATION OF KILLING CURVE	A-11

LIST OF TABLES

Table No.	Title	Page
1.	Growth Rate Determinations: Run 1	15
2.	Growth Rate Determinations: Run 2	16
3.	Growth Rate Determinations: Run 3	16
4.	Growth Rate Determinations: Run 4	17
5.	Growth Rate Determinations: Run 5	17
6.	Manometric Studies of O_2 Production of Selected Algae	18
7.	Cell Diameters in Culture Grown at Saturating Light Intensity	25
8.	Cell Diameters of Light-saturated Cells Placed in the Dark	26
9.	Distribution of Cell Sizes in a Population of Mixed Light and Dark Cells	26
10.	Inactivation of *Chlorella* 71105 by Ultraviolet Irradiation	89

LIST OF ILLUSTRATIONS

Figure No.	Title	Page
1.	Undesirable and Desirable Growth	9
2.	Actively Growing Chlamydomonas 13123 Culture, Showing Representative Morphological Types	13
3.	Chlamydomonas 13123 Mother Cell, Observed Under 970X	13
4.	Chlamydomonas 13123 Motile Cells Released from Mother Cell at Room Temperature.	13
5.	Mixed Culture Growing in Liquid on Primary Isolation, Observed at 440X	14
6.	Unidentified Green Algae Isolate (13118), Observed Under 440X	14
7.	13118 Observed Under 970X	14
8.	A Culture (12322), Rejected Because of Excessive Clumping, Observed Under 440X	14
9.	Effect of Incubating a Light-saturated Culture in the Dark	27
10.	The Inactivation of Chlorella 71105 by Ultraviolet Light	29

PREFACE

Several approaches to the development of a closed ecosystem are being explored by Electric Boat. First, and of primary importance, are engineering studies designed to provide data for an optimum design for a gas exchange apparatus with minimum power, weight, and volume. The second approach is the supporting biochemical studies on Chlorella 71105*, which consist of improving the chemical environment of the alga chosen for the photosynthetic gas exchanger. A third approach is the quest for a better alga than is presently being used in the research. In this quest, we have tried two attacks on the problem. First, soil and water samples were screened for fast-growing algal strains, and second, the existing strain of Chlorella was irradiated with ultraviolet light in an attempt to produce genetic mutants. Section I of this paper describes the screening program; Section II describes the mutation studies.

*A thermophilic strain of Chlorella was isolated from Texas soil by Sorokin and Myers (1953) and designated by them Chlorella pyrenoidosa 7-11-05. For convenience, we refer to the strain as Chlorella 71105.

SECTION I

SCREENING FOR THERMOPHILIC ALGAE

INTRODUCTION

The most reasonable approach to the isolation of an organism with specific growth characteristics is to screen samples from environments that are similar to the one in which the organism will be cultured. In the present study, for example, high-temperature, high light intensity algae were sought in areas exposed to high mean annual temperatures and intense sunlight.

There are a number of characteristics that an organism should possess if it is to be used in a photosynthetic gas exchanger.

1) Of primary importance is the generation time of the organism. Since oxygen production is related to growth, it follows that the organism selected should exhibit a high growth rate.

2) The organism should be photo-autotrophic in its nutrition and grow well in a chemically defined medium.

3) The strain should excrete minimal amounts of organic compounds into the medium. Otherwise, foaming, contamination, and growth inhibition may occur.

4) The alga should be tolerant of fluctuations in nutrient composition and temperature. Growth at high temperatures would be most desirable; cooling problems from high-intensity lamps or solar sources and the possibility of contamination by other microorganisms would be decreased.

5) The organism should be tolerant of fluctuations in hydrogen ion concentration. This characteristic could further discourage the growth of possible contaminants.

6) The organism should not have a tendency to clump or stick to surfaces.

7) The alga should be able to grow in high intensity light in order that it can be grown in a compact culture unit. Furthermore, the intense light will help control some bacterial contaminants.

8) The organism should metabolize urea and the major inorganic components present in human wastes.

9) The alga should be non-toxic and nutritious to man.

10) The organism should be able to grow free of association with other microorganisms.

An algal screening program was undertaken to obtain algae from nature with as many of these characteristics as possible. A spoonful of soil may contain billions of living microscopic organisms, including algae, molds, yeasts, bacteria, diatoms, viruses, insects, and nematodes, all of diverse type.

The algae present in the mixed flora of soil have different optimum growth rates and nutritional requirements. Samples were therefore exposed to selective laboratory environmental conditions which fostered the isolation of microorganisms with the following characteristics: 1) rapid growth rate, 2) photo-autotrophic metabolism, 3) growth at high temperatures, 4) growth with urea as a sole nitrogen source, 5) growth with CO_2 as a primary carbon source, and 6) growth in a medium with relatively high osmotic pressure.

As pointed out by Lewin (1959), the approach to the isolation of an algae should be determined by the ultimate objective. For example, thermophilic algae should be sought in relatively high temperature environments. By adaptation and natural selection, organisms with the desired characteristics will predominate in this environment.

In the Electric Boat screening program, soil and water samples were collected from many areas exposed to intense sunlight and high mean annual temperatures. Samples from tropical and sub-tropical countries, greenhouses, and hot springs were particularly sought.

Our standard laboratory medium was used in the screening studies for the following reasons:

1) The feasibility of growing Chlorella 71105 at its maximum growth rate using urea was well established. Urea, the primary nitrogeneous waste product of man, should serve as the sole nitrogen source to help maintain chemical balance in a closed ecosystem.

2) CO_2 is the sole carbon source. This allows the direct scrubbing of CO_2 from air. In addition, the possibility of bacterial contamination from other carbon sources is greatly reduced.

3) The medium is inorganic with the exception of urea, so bacterial contamination is further limited.

MATERIALS AND METHODS

The screening program was divided into five general areas:

1) Sample procurement
2) Fabrication of growth units
3) Screening procedure
4) Growth rate determinations
5) Manometric studies

SAMPLE PROCUREMENT

A circular letter was sent to scientists in various parts of the world. Those that expressed a willingness to cooperate were sent a package containing a mailing tube with international postal coupons to cover the cost of the return mailing. The tube contained a questionnaire requesting information about the sample and the site of collection. Also contained in the tube was a plastic envelope containing two sterile screw-cap shell vials. To the outside of each mailing tube was affixed USDA Plant Quarantine Form 21C, which authorizes the importation of soil samples without treatment. Soil samples have been kept in the covered vials since their receipt. No samples will be discarded without prior sterilization in the autoclave.

Microbiologists in many parts of the world cooperated by sending fresh soil and water samples. The samples were collected from environments such as hot springs, tropical soils, and greenhouses whose soils had been periodically steam-treated. Some samples from local and other temperature regions have been screened. All soil samples received and tested in this study are listed in Appendix B.

GROWTH UNITS

The test tube incubation unit consisted of an aquarium with a bank of four closely packed General Electric Powergroove fluorescent lamps. An automatic gas mixing system provided a $3.0 \pm 0.5\%$ CO_2-air mixture. A gas washing system consisting of two washing cylinders containing distilled water was immersed in the water bath. This system was used to warm and humidify the CO_2-air mixture.

During operation, excess water was disentrained from the humidified gas stream in a third cylinder. The gas mixture then passed through a bacteriological filter into gassing manifolds equipped with needle valves. This arrangement distributed approximately 500 cc per minute of warmed, humidified, sterile gas mixture to each test tube.

Clear lucite racks held aluminum-capped pyrex test tubes approximately four inches from the bank of lamps. The light reading at the surface of the test tubes was 2,000 foot candles.

The 50-gallon water bath was maintained at $38.5 \pm 0.5^{\circ}C$. A motor-driven stirrer was used to assure an even temperature. Two air-lift filters cleaned the water bath of debris. The gas distribution and filtering system was disassembled and autoclaved before each experiment.

Incubators for the petri dishes were fabricated from refrigerators by installing lights and thermoregulators which activated circulating fans to vent hot air to the room and draw in filtered cool room air.

SCREENING PROCEDURES

The following routine was used for the preliminary screening of soil and water samples.

1) Approximately 0.1 gram of soil was placed in three sterile test tubes and 50 milliliters of standard* medium was added to each.

2) Tubes were incubated at $38.5^{\circ}C$, $45^{\circ}C$, and $50^{\circ}C$ in the aquarium units until algal growth was apparent. If there was no growth after 48 hours, or longer in some cases, the tubes were discarded.

3) Step (1) was repeated with all samples that were negative in step (2).

4) If algal growth in a tube was apparent, a small portion (approximately 0.1 milliliter) was transferrred to fresh medium and the incubation was repeated.

5) After the sub-culture attained an optical density of 0.1-0.2 units (denser cultures were re-incubated as in step (4)), dilutions were made in standard medium, and 0.1 ml of each dilution were streaked on standard agar plates in triplicate and incubated in the lighted petri dish incubators. (Standard agar plates were prepared with Electric Boat Division standard medium and 2.5% agar.)

*The composition of Electric Boat standard medium is given in Appendix C.

6) Well isolated algal colonies were transferred to agar slants as soon as they could be seen with a dissecting microscope at 30X. Slants showing growth were then maintained as stock cultures on one-half strength standard agar slants. Only isolated colonies that appeared unialgal and bacteria-free were picked under a dissecting microscope using oblique transmitted lighting as described by Henry (1933).

7) To determine bacterial contaminants, slants were washed and plated out on nutrient agar. Some cultures proved to be free of bacteria after only one plating; others still had associated bacteria after several transfers.

Preliminary Isolations

According to Trainor (1960) and Hyatt and Levinson (1961), the growth of some microorganisms in resting stages may be stimulated by exposing them to heat for short periods of time. Therefore, ten soil samples that previously did not yield growth by the regular procedure were selected. Sub-samples of these were then exposed to 60°C for ten minutes and incubated in the regular way. This procedure did not result in the isolation of any algae from previously negative samples.

Dilution of Samples

The possible advantages of diluting soil samples before incubation were studied on the ten samples that gave no isolations in preliminary cultures. The following advantages were anticipated:

1) Soils that have been heavily fertilized with phosphates have been reported to inhibit algal growth. Dilution lowers the phosphate concentration.

2) High concentrations of soil permit many bacteria to grow because of increased organic nutrients. The secondary bacterial growth may inhibit algae growth as a result of the secretion of anti-metabolites, by the hydrolysis of urea, or by drastic pH changes.

Ten negative samples were diluted 1:10 and 1:100 and incubated in the regular way. This procedure did not result in the growth of algae in any of the ten cases.

Many soil samples yielded no isolates under the regular procedure, but others yielded several strains. Streak plates of test-tube cultures resulted in some plates covered with algae growth. Dilutions of these cultures yielded plates with isolated colonies. In some cases, bacterial and fungal colonies outnumbered the algae. Triplicate plates of each dilution increased the chances of obtaining well-isolated colonies.

In the regular procedure, slants showing growth were washed with standard medium and used to inoculate a test tube in the aquarium unit. After growth had proceeded in the tube, an aliquot was plated on standard agar supplemented with 0.1% polypeptone, encouraging the growth of any bacteria that might have been present. The stock cultures that resulted were apparently bacteria-free, unialgal, and able to grow at high temperatures in intense light and in the absence of soil nutrients. Stocks of isolated cultures were maintained on dilute standard agar slants in dim light to prevent rapid growth and eventual loss of viability.

The cellular morphology was observed from cells grown on slants, in test tubes, and in the liquid phase of slants flooded with medium and incubated to induce formation of motile phases. Cultures in test tubes kept in the dark at room temperature were also used for motility determinations. Nigrosine and iodine stains were used to detect capsules and internal starch formation.

GROWTH RATE DETERMINATIONS

The rate of production of algal cellular material is directly related to the rate of photosynthesis. However, the rate of production of cells can be much more easily and accurately measured than gas exchange in small scale runs. Rate of growth therefore was determined as a preliminary criterion for the selection of desirable strains.

Measurement of Growth Rates

Growth rates for the various isolates were determined in the aquarium units. The increase in optical density as a function of time was determined with a Fisher Electrophotometer at 550 millimicrons.

Growth rate determinations were standardized to insure measurements on actively growing cells. Stock cultures were first transferred onto fresh slants. The new growth on the fresh slants was washed off with standard medium and used to inoculate test tubes in the aquarium.

When the test tube cultures had grown to reasonable density, a second tube was inoculated and incubated. When this second sub-culture had grown to reasonable density (after 24-48 hours), a set of four or more test tubes was inoculated with varying small amounts of the culture. These cultures were grown overnight and their optical densities measured in the morning. This measurement was made by removing a tube from the rack, wiping it dry, and placing it directly in the Fisher Electrophotometer. (The 100-ml tubes are of the appropriate dimensions; tubes of adequate optical quality can be selected from stock lots with little difficulty.) The gassing tube was raised out of the light path during the photometric reading.

Some cultures, those that had received an inordinately heavy inoculum, were too dense after overnight growth; these were not diluted but discarded, since sterile technique was striven for in the growth measurements. Most tubes would have attained a density of about 0.1 units, but some few were less dense after overnight growth. Optical density readings were made hourly for periods from three to 14 hours, usually for eight hours. No further readings were made after cultures attained a density corresponding to full scale on the photometer. The relationship between packed cell volume (PCV) and optical density for Chlorella 71105 (given in Appendix D) is non-linear. The significance of this relationship to growth rate determinations is discussed in a later section.

The protocol used in the growth measurements was suitable from the standpoint of the effective use of available facilities and scheduling of steps in the procedure. The use of pure cultures, while obviously to be desired, gave rise to some problems. Autoclaving medium containing urea undoubtedly resulted in different degrees of hydrolysis. The amount of evaporation from tubes varied in spite of provisions for saturating the air-CO_2 mixture with water vapor at the growth temperature. The water loss was not made up before optical density measurements becuase of the difficulty of maintaining sterility while making periodic additions to cultures. No correction factor for volume was applied to optical density readings, but tubes whose evaporation loss was estimated in excess of 15% were not measured.

As growth rate determinations were being made, qualitative observations were made on cultural characteristics such as clumping, sticking, and settling tendencies of cells. An example of a desirable and undesirable cultural characteristic is given in Figure 1. The culture on the left exhibits undesirable clumping and settling; the other exhibits a desirable uniform distribution.

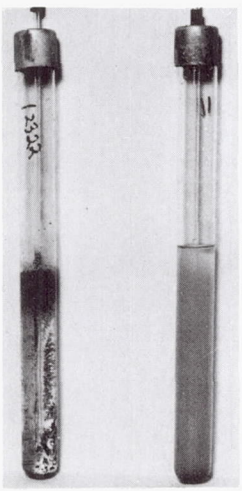


FIGURE 1
UNDESIRABLE AND DESIRABLE GROWTH

Estimation of Growth Rates

If a single algal cell is considered to divide to form two cells with a characteristic frequency (this is not strictly correct for many unicellular algae), the number of cells in a culture, N, at the end of an increment of time, t, is determined by the initial number, N_o, multiplied by 2 raised to a power, n, the number of doublings in time, t.

$$N = N_o \ (2^n)$$

If logarithms to the base 2 are taken;

$$n = \log_2 \frac{N}{N_o}, \text{ or } n = \log_2 N - \log_2 N_o$$

If periodic measurements of cell numbers are made, n can be calculated. Where the period of time is projected to 24 hours, n is the number of doublings per day, the growth rate constant of Myers (1957). This number is constant during the so-called logarithmic phase of population growth; if growth is observed during the log phase, the slope of the line relating log N to time is a direct measure of n or k (generation time). It was hoped that cell numbers could be measured in such a way that optical density would be a log function of the number of cells in the light path of the Fisher Electrophotometer. At 550 millimicrons the relationship between optical density and concentration proved to be curvilinear as shown in Appendix D. The relationship was unfortunately not logarithmic; a plot of log concentration against optical density was also curvilinear. In any case, optical density readings can be converted to concentrations for the calculation of k; but in our case, the hourly increment in optical density did not increase with time as it should during the logarithmic phase of growth but remained constant, as shown in the section on results. This fact coupled with the curvilinear but nonlogarithmic relationship between optical density at 550 mμ and concentration suggests that the range of culture densities used fell between the logarithmic phase and the linear phase of the growth curve for Chlorella and other strains of algae cultured in our set-up. This precluded any realistic estimate of k. Dr. Jack Myers in a personal communication has pointed out that relative growth in the linear phase may be a better index than the logarithmic growth constant for predicting the usefulness of an alga for the photosynthetic gas exchange application.

Since determination of conventional growth indices was precluded, growth rates were evaluated in three ways:

1) The equation and slope of the regression line was obtained from the optical density measurements on four replicate cultures.

2) The arithmetic mean of the hourly increments in optical density during the entire growth-rate determination period was calculated.

3) The maximum growth increment over any hourly reading was also noted as an indication of the maximum growth rate potential.

All three of these indices were considered in assessing the potential usefulness of the various cultures.

MANOMETRIC STUDIES

Oxygen production rates of three promising cultures were compared with *Chlorella* 71105 in a Bronwill illuminated Warburg apparatus. The following procedure was followed during the manometric studies:

1) Dilute cell suspensions were cultured in the aquarium unit to obtain an actively growing culture with an optical density of 0.17-0.27 units.

2) Cellular dry weight determinations were made from an aliquot of the suspension. This procedure is outlined in Appendix E.

3) Carbonic anhydrase with an enzyme activity of 1300 Philpot units per milligram was added to a stock mixture consisting of three parts 2.5 M $KHCO_3$ and one part 2.5 M K_2CO_3 as recommended by Dr. Dean Burk in a personal communication. The final concentration of carbonic anhydrase was approximately one milligram per ten milliliters.

4) Triplicate double sidearm flasks were inoculated with 1.6 milliliters of cell suspension in the main body of each flask; 0.2 milliliters of the enzyme-substrate mixture were added to each of the two flask sidearms and also to the center well.

5) The manometers and flasks were then attached to the shaker apparatus. Flasks were immersed in the water bath and agitated at 38.5°C for 30 minutes to allow the carbon dioxide concentration in the flasks to reach equilibrium.

6) The apparent photosynthetic rate was determined using the standard manometric techniques described in Umbreit, *et al* (1959).

7) The photosynthetic rate of the algae tested was expressed in terms of Q_{O_2} value which is defined as the microliters of oxygen evolved per hour per milligram of dry cellular material.

During the manometric studies, steps 3) and 4) above were done as rapidly as possible to conserve carbon dioxide in the flasks. Gas saturated cultures were pipetted directly into the Warburg flasks. The carbonic anhydrase solution was added to the flasks immediately after mixing, and the manometers were attached immediately after the mixture was added.

An analysis of the gas phase within the flasks at equilibrium was done with a Perkin-Elmer Model 154-C Vapor Fractometer. The equilibrium mixture contained approximately 1.5% carbon dioxide.

RESULTS AND DISCUSSION

Some 40 thermophilic algae cultures were subcultured and growth measurements were made on 33. Although most types were Chlorella-like in morphology, Oöcystis, filamentous blue-greens, Chlamydomonas, Chlorococcum, and Palmellococcus-like forms were also isolated. The morphology of the isolates is treated below. Some of the isolates are shown in Figures 2 through 8. Samples were screened at 38.5, 45, and 50°C, but all cultures except one were isolated at 38.5°C. The one 45°C strain, a filamentous blue-green, was deemed unsuitable because it grew poorly and adhered to the test tube as a mat.

MORPHOLOGY OF THE ISOLATES

Oöcystis-like 2156. This culture is grass-green in color and consists of nonmotile, solitary, ellipsoidal cells over 10 microns long. The cell wall is smooth and thin with cells occasionally having small polar nodules. Reproduction is by cell division with the parent cell containing many autospores. One chloroplast is present.

Oscillatoria-like 3292. The blue-green culture grows as an interwoven filamentous adhering mat. The straight, uniserate, unbranched trichomes have hundreds of cells and are without a sheath or heterocysts. The trichomes have a very uniform diameter throughout. Pigments are not localized in definite chromatophores and no flagellated cells were observed.

Chlamydomonas 13123. This microorganism consists of green algae having different stages: a motile zygote, palmella stage, and resting zygote. Motile cells are biflagellate, solitary, ellipsoidal and usually contain an eyespot at the base of the cell. Union of motile cells was occasionally observed, and asexual reproduction with formation of four or more daughter protoplasts within the parent cell wall was common. Palmella stages predominated at 38.5°C, but motile cells were released at room temperature. This indicates that 38.5°C is not the optimum temperature for this organism. Resting zygotes were not green, but appeared colorless. This isolate is shown in Figure 2 under 440 X.

Chlorococcum-like 12223. The solitary, spherical, green cells reproduced by forming autospores commonly and zoospores rarely. The cell wall is smooth. Cells have cup-shaped parietal chloroplasts.

Palmellococcus-like 1115. The solitary, ellipsoidal, smooth-walled cells are nonmotile and contain one or more chloroplasts. Reproduction is by formation of autospores.

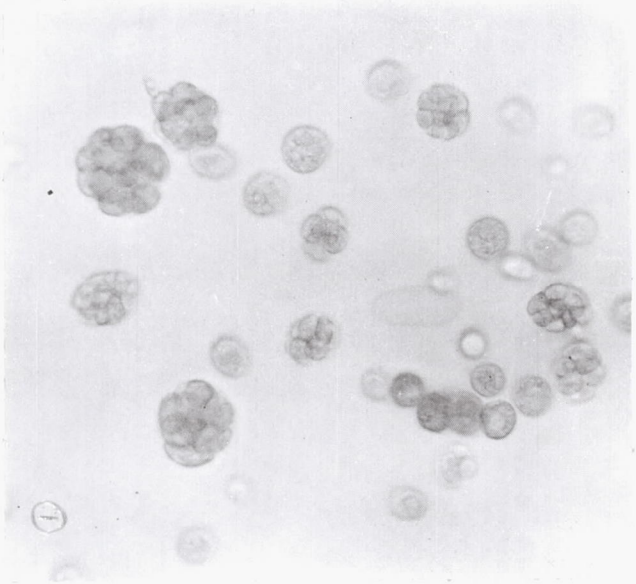


FIGURE 2

ACTIVELY GROWING CHLAMYDOMONAS 13123 CULTURE, SHOWING REPRESENTATIVE MORPHOLOGICAL TYPES. NOTICE PREDOMINANCE OF RESTING AND PALMELLA STAGES INDICATING THAT GROWTH CONDITIONS WERE NOT OPTIMUM.

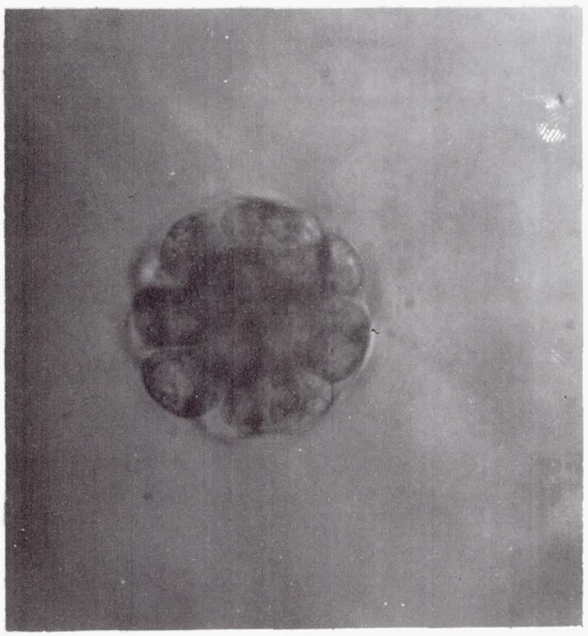


FIGURE 3

CHLAMYDOMONAS 13123 MOTHER CELL OBSERVED UNDER 970 x.

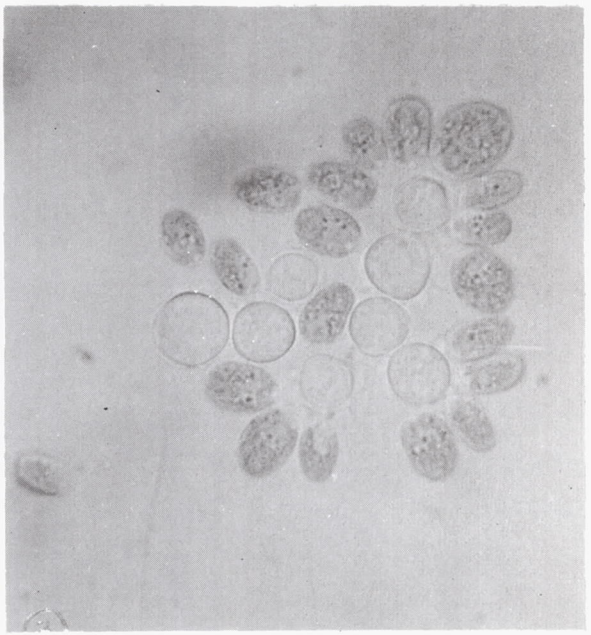


FIGURE 4

CHLAMYDOMONAS 13123 MOTILE CELLS RELEASED FROM MOTHER CELL WHEN THE CULTURE WAS PLACED AT ROOM TEMPERATURE.

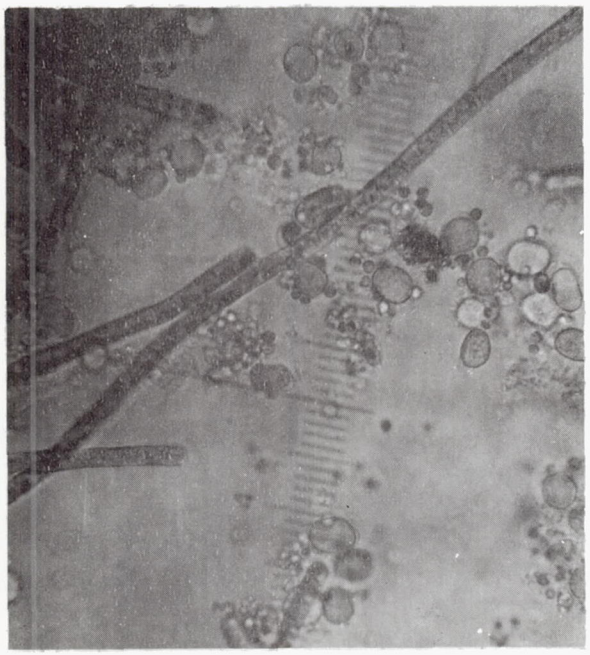


FIGURE 5

MIXED CULTURE GROWING IN LIQUID ON PRIMARY ISOLATION OBSERVED AT 440x. NOTE DIVERSE MORPHOLOGY OF CELLS.

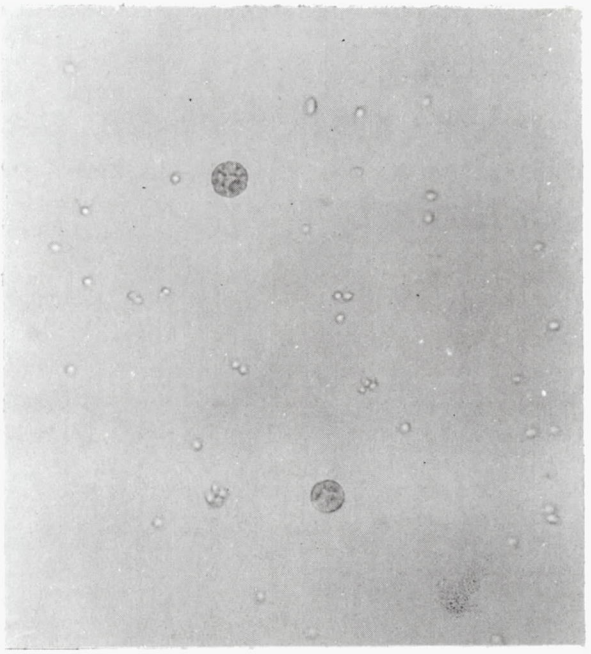


FIGURE 6

UNIDENTIFIED GREEN ALGAE ISOLATE 13118 OBSERVED UNDER 440x. LARGE MOTHER CELLS AND SMALL DAUGHTER CELLS ARE SHOWN.

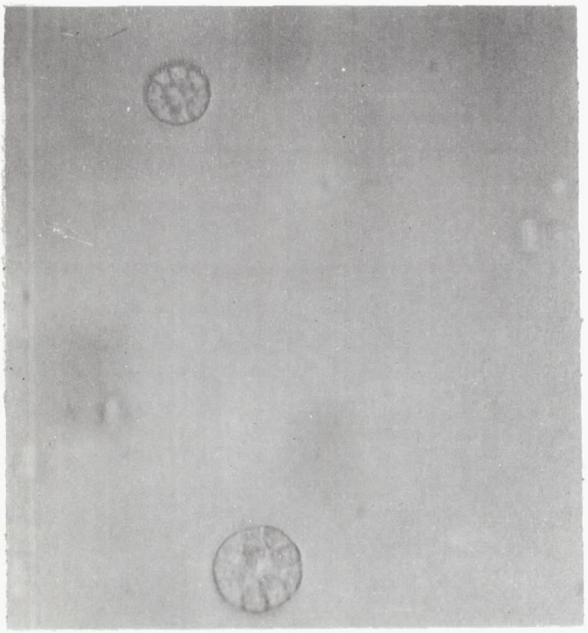


FIGURE 7

13118 OBSERVED UNDER 970x.

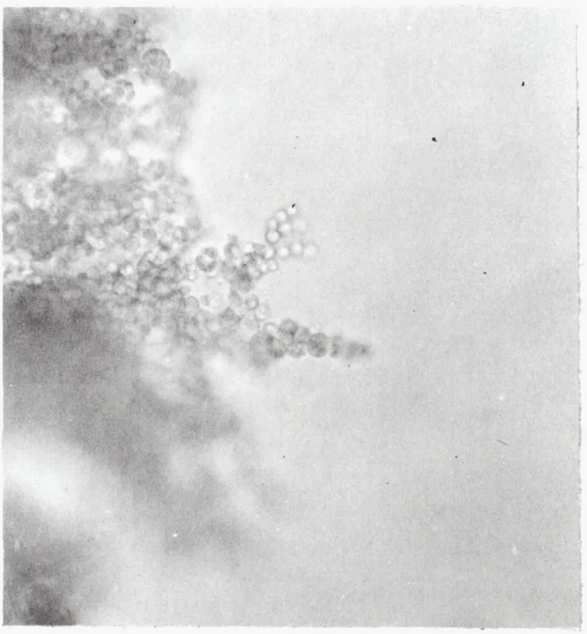


FIGURE 8

A CULTURE THAT WAS REJECTED BECAUSE OF EXCESSIVE CLUMPING, 12322, OBSERVED UNDER 440x. SINCE THE MASS OF CELLS EXTENDS IN THREE DIMENSIONS, ONLY A FEW REPRESENTATIVE ONES ARE CLEARLY SHOWN.

Chlorella-like. Most soil isolates were in this group. The spherical to ellipsoidal cells have a single parietal cup-shaped chloroplast. Reproduction is by means of autospores.

In addition, many thermophilic algae propagated on primary isolation, but could not be maintained on standard medium. Included in this group were algae that were similar to Hormidium, Tetraallantos, Scenedesmus, and other genera. No growth measurements could be made on these cultures.

GROWTH MEASUREMENTS

The results of growth measurements are given in Tables 1-5. Each run included a group of replicate cultures of Chlorella 71105. This set was included with each run to provide a control on each batch of medium; our misgivings about variable hydrolysis of urea have already been mentioned. The results in the tables represent average values over the set of replicates. The average initial density has also been included in the tables, since there was an apparent but weak and variable tendency for dense cultures to produce greater hourly increments in optical density. This is exactly what would be expected with cultures in the log phase. The calculated least squares regression between optical density and time was linear, however, in all but the few cases that are noted in the tables. For these cases, no regression line slope is given.

From Table 1, all strains are deemed worthy of further attention except 1311 and 12227. It is noteworthy that the maximum hourly increment serves to distinguish fast-growing from slow-growing strains as well as the average increment or the slope of the regression line does.

Table 1

Growth Rate Determinations; Run 1

Organism	Mean Initial Density	Regression Line Slope	Mean Hourly Increment	Maximum Hourly Increment
71105	0.15	0.087	0.11	0.19
1116	0.22	0.11	0.12	0.26
1311	0.08	0.031	0.033	0.09
11614A	0.12	0.085	0.085	0.18
12227* ***	0.17	--	0.055	0.12
13118**	0.13	0.089	0.088	0.20
13119*	0.17	--	0.077	0.19
13130**	0.12	0.11	0.12	0.20

*Regression of optical density on time departed significantly from linearity.
**See also Run 2
***See also Run 5

From Table 2, only strain 32411 seems worthy of note; again the maximum hourly increment as well as slope distinguishes the fast-growing strain.

Table 2

Growth Rate Determinations; Run 2

Organism	Mean Initial Density	Regression Line Slope	Mean Hourly Increment	Maximum Hourly Increment
71105	0.21	0.059	0.090	0.22
1319	0.09	0.036	0.039	0.10
2159*	0.25	--	0.027	0.11
13118**	0.25	0.019	0.023	0.09
13130**	0.11	0.038	0.053	0.13
32411	0.38	0.086	0.099	0.25

*Regression of optical density on time departed significantly from linearity.

**See also Run 1

In Table 3, regression line slope and mean hourly increment call attention to all but 2156 and 13123B. The maximum hourly increment of 0.19 for culture 13123B suggests that this strain is also worthy of further attention.

Table 3

Growth Rate Determinations; Run 3

Organism	Mean Initial Density	Regression Line Slope	Mean Hourly Increment	Maximum Hourly Increment
71105	0.48	0.14	0.15	0.18
1235C	0.27	0.084	0.094	0.20
2156	0.15	0.065	0.064	0.14
3241	0.52	0.10	0.11	0.19
3242A	0.10	0.082	0.085	0.14
3242B	0.33	0.089	0.086	0.20
13123B	0.14	0.062	0.073	0.19
21511B	0.72	0.12	0.12	0.18

Regression line slope values in Table 4 single out 1115, 1167, 3246, and 12329. Strain 1119A should obviously be added to the list because of its mean and maximum hourly increment. In Table 5, only strains 1115 and 11615 seem noteworthy.

Table 4

Growth Rate Determinations; Run 4

Organism	Mean Initial Density	Regression Line Slope	Mean Hourly Increment	Maximum Hourly Increment
71105	0.48	--	0.10	0.17
1115*	0.45	0.11	0.11	0.14
1119A	0.27	--	0.071	0.17
1167	0.30	0.084	0.083	0.13
1318	0.11	0.034	0.034	0.06
3246	0.32	0.093	0.092	0.16
12329	0.29	0.084	0.084	0.14
13122	0.20	0.043	0.052	0.08

*See also Run 5

Table 5

Growth Rate Determinations; Run 5

Organism	Mean Initial Density	Regression Line Slope	Mean Hourly Increment	Maximum Hourly Increment
71105	0.32	--	0.10	0.16
196A	0.17	0.049	0.053	0.12
196B	0.08	0.030	0.036	0.06
1115*	0.22	--	0.098	0.18
11615	0.14	0.058	0.061	0.11
12227**	0.22	0.052	0.028	0.06
21510	0.11	0.056	0.055	0.08
21512	0.11	0.031	0.046	0.07
32412	0.08	0.013	0.019	0.04

**See also Run 1

*See also Run 4

In all runs, 18 strains compared favorably with Chlorella 71105 in linear growth rate. These are 1115, 1116, 1119A, 1167, 1235C, 3241, 3242A, 3242B, 3246, 11614A, 11615, 12329, 13118, 13119, 13123B, 31130, 21511B, and 32411. With the exception of 1115, which is Palmellococcus-like and 13123B, which is Chlamydomonas-like, all of these strains are Chlorella-like. Strain 13123B grew rapidly but primarily in the form of mother-cells. That condition indicates a suboptimal chemical or physical environment. Future work with 13123B and other promising strains should begin with growing the algae in other media, especially calcium-rich media. The standard medium used in the screening contained only trace amounts of that element.

MANOMETRIC STUDIES

Oxygen evolution of a small fraction of a gram of cellular material was determined to prevent a light-limited condition during the measurement.

The results in Table 6 are expressed in terms of Q_{O_2}, the microliters of oxygen produced per milligram of dry cellular material per hour (uncorrected for respiration).

Table 6

Manometric Studies of O_2 Production of Selected Algae

Strain of Algae	Optical Density of Suspension	Q_{O_2}
71105	0.22	188
1115	0.17	200
11615	0.27	180
12227	0.23	176

The rates of oxygen production observed are equivalent to the production of 0.18, 0.19, 0.17 and 0.17 mg cells/mg/hour for strains 71105, 1115, 11615, and 12227, respectively. This equivalence has been based on the observation in Part I of this report that 1.03 liters of oxygen on the average are produced in the production of 1.0 gm of dry cellular material. The rate of production of oxygen observed for the four strains corresponds to a growth rate of about six doublings per day. The light intensity used for the manometric studies was limited by the design of the water bath to about 800 foot candles. A growth rate of six doublings per day is characteristic for Chlorella 71105 at 800 foot candles.

It is especially noteworthy that strain 12227 did not grow impressively in the aquarium unit at 2000 foot candles. Its growth was measured twice, in runs 1 and 5. In short-term measurements in the manometric apparatus it grew nearly as well as Chlorella 71105 and two other strains, 1115 and 11615, both of which were singled out on the basis of growth in test-tube experiments.

SECTION II

MUTATION STUDIES

INTRODUCTION

The purpose of the mutation study was twofold:

1) To attempt to induce mutants that would grow well at temperatures higher than Chlorella 71105's optimum temperature. Such mutants could reasonably be expected to have high growth rates and tolerate extremely bright light.

2) To determine the effect of ultraviolet irradiation on Chlorella, since this basic information has useful applications to closed ecosystems in space.

In nature, undirected, spontaneous mutations may be anticipated rarely in microorganisms. However, it is well known that the number of mutations may be increased several hundredfold by exposure to mutagenic agents, and that both spontaneous and induced mutations are almost invariably deleterious. Cases are known, however, in which beneficial mutations have been produced.

Induced mutations of microorganisms have been particularly useful for elucidating pathways in intermediary metabolism, for obtaining photosynthetic and chlorophyll mutants, and for obtaining increased yields in the fermentation industry. With high-temperature mutants of Chlorella 71105, the weight and bulk of a photosynthetic gas exchanger could be minimized because 1) less volume of culture would be required, and 2) the organisms could be placed in closer contact with the high intensity light source or its equivalent in solar radiation without solarization of the culture.

Friedman (1960) studied thermophilic bacteria and their mesophilic mutants; it was established that the protein of thermophiles was more heat-stable. The high-temperature protein had a different sequence and percentage composition in its constituent amino acids, resulting in more favorable conditions for hydrogen bonding. Hydrogen bonding reportedly stabilizes protein helices and protects the structure from denaturation.

Chlorella 71105 was used for the mutation studies because a unicellular, nonmotile, thermophilic, coccoid form would best meet the requirements of a photosynthetic gas exchanger for closed-cycle

ecological systems (Benoit et al, 1960). Some of the advantages of individual small spherical cells are:

1) They have greater surface area per cell volume, cellular wastes (including oxygen) and nutrients may be exchanged with the environment very rapidly.

2) Intracellular transport within small unspecialized cells is also facilitated.

In particular, Chlorella 71105 is especially suitable for use in photosynthetic gas exchangers because:

1) It has a simple asexual life cycle.

2) It maintains rapid uniform growth in liquid media.

3) It shows relatively little tendency to float, settle, or stick to surfaces in culture vessels.

4) It has the highest reported growth rate and concommitant oxygen production rate per unit quantity of cell material of any organism so far reported (Sorokin and Myers, 1953; Sorokin, 1959).

Ultraviolet was used to induce mutants in the present work for the following reasons:

1) It has been used successfully by many investigators for inducing mutants of Chlorella (Granick, 1948; Davis, 1952; Bogard and Granick, 1953; McLeod and McLochlen, 1959).

2) It is simpler and safer to use than X-rays and most mutagenic chemicals.

EFFECTS OF ULTRAVIOLET RADIATION

The short ultraviolet source used, a Thomas C-81 Ultraviolet Lamp equipped with a filter, was rated to deliver 163×10^{-6} joules per second per cm^2 to a target 18 inches away. This energy is primarily at 2537 Å. We placed the target suspension 6 inches from the source, consequently it received 9 times the amount of energy as at 18 inches. This is equal to $9(163) \times 10^{-6} = 1467 \times 10^{-6}$ joules per second per cm^2. The energy available at 2537 Å is 78×10^{-20} joules per photon. Particles at this energy level are non-ionizing; their mutagenic effects are the result of excitations in complex organic molecules in which electrons are raised to a state of higher energy. Chemical changes may occur in the affected molecules, but most excited molecules return to the original condition without permanent alteration. In such cases, excess energy is dissipated in the form of heat.

Absorption of ultraviolet radiation by cellular components depends on their chemical structure, since a quantum of a given energy will only be absorbed by electrons possessing the proper activating energy, which is determined by intra-atomic and inter-atomic forces. Nucleic acids, for example, absorb ultraviolet strongly through their purine and pyrimidine rings. The mutagenic rays produce changes in the deoxypentose nucleic acids localized in the cell nucleus.

Among other effects, these genetic changes could be reflected in abnormal protein metabolism. Since DNA determines the transmission of inherited characteristics, abnormalities such as altered protein metabolism would be passed on to the progeny.

PREPARATION OF CULTURES FOR IRRADIATION

During the life cycle of Chlorella, a number of stages occur. Individual cells pass through stages that differ:

1) in growth rate and metabolic activity (Tamiya, et al, 1953; Sorokin, 1957; Sorokin and Krauss, 1959),

2) in photosynthetic activity (Sorokin, 1957; Sorokin and Myers, 1957),

3) in sensitivity to environmental factors (Pirson, et al, 1959),

4) in accumulation of nucleic acids (Hase, et al, 1957), and

5) in chlorophyll content (Myers, 1946).

Since ultraviolet affects nucleic acids, cells in varying states of nucleic acid synthesis should have varying sensitivity to irradiation. However, the relative sensitivity of the various stages in the life cycle is unknown. A heterogeneous population of cell types was therefore exposed to insure the irradiation of some of each type.

MATERIALS AND METHODS

Cultures of Chlorella 71105 were aseptically grown at 38.5°C in the illuminated water bath incubator described in a previous section. All media used were prepared with water that had been passed through a mixed-bed ion exchange resin, and were sterilized by autoclaving after addition of 0.01 ml/L Dow-Corning Antifoam B.

CELLULAR MORPHOLOGY

Cultures were grown to an optical density of 0.1 units on a Fisher Electrophotometer at 550 mμ. These cultures were considered to be in the logarithmic phase of growth. An actively growing, light-saturated culture was divided in half: one portion was left in the light as a control, and a second portion was transferred to a capped, painted, opaque tube; both were gassed and incubated in the light.

Samples of both cultures were cooled in an ice bath and examined microscopically during the 5-1/2 hour experimental period. Cell diameters were determined at a 970X magnification using a calibrated eyepiece.

To obtain a mixed culture of "light", "dark", and intermediate cells, light cells were treated as follows. A culture of light cells was cooled in an ice bath, diluted to an optical density of 0.10, and mixed 1:1 with a cooled culture that had been in the dark for 5-1/2 hours. Cultures were cooled because Sorokin (1959) demonstrated this treatment prevents cell division.

Table 7 shows that the diameters of light cells fell almost entirely between 4.0 and 6.0 microns over the entire time the culture was observed. Under these conditions, the culture remained homogeneous and was deemed unsuitable for exposure to ultraviolet radiation, because it did not contain all representative cell types.

Table 7

Cell Diameters in Culture Grown at Saturating Light Intensity

Elapsed Time (hours)	Mean Cell Diameter (microns)	Standard Deviation	Per Cent of Sample at Indicated Cell Diameter									
			1.5μ	2.25μ	3.0μ	3.75μ	4.0μ	5.25μ	6.0μ	6.75μ	7.5μ	8.25μ
1	5.7	0.75	0	0	0	3	13	17	63	0	3	0
2	5.7	0.71	0	0	0	0	12	20	56	12	0	0
3.5	5.4	0.60	0	0	0	0	21	43	32	4	0	0
4.5	5.4	0.93	0	0	0	7	30	10	40	13	0	0
5.5	5.7	0.60	0	0	0	0	15	26	53	6	0	0

Table 8 summarizes the results obtained when a portion of the same culture was placed in the dark. The most striking change in cell diameter occurred during the first hour of darkness, but a new class of extremely small cells was observed after 5-1/2 hours in the dark. The results of Table 8 are shown graphically in Figure 9. The trend toward a population with small-diameter cells as a result of dark incubation can be seen.

Table 8

Cell Diameters of Light-saturated Cells Placed in the Dark

Elapsed Time (hours)	Mean Cell Diameter (microns)	Standard Deviation	Per Cent of Sample at Indicated Cell Diameter									
			1.5μ	2.25μ	3.0μ	3.75μ	4.0μ	5.25μ	6.0μ	6.75μ	7.5μ	8.25μ
1	4.0	0.78	0	0	27	18	50	0	5	0	0	0
2	4.1	0.78	0	0	21	26	46	8	0	0	0	0
3.5	4.5	0.77	0	0	4	30	52	0	15	0	0	0
4.5	4.1	0.60	0	0	7	48	38	7	0	0	0	0
5.5	3.4	0.59	0	6	37	31	25	0	0	0	0	0

A mixture of equal amounts of light and dark cells was made. Table 9 shows the distribution of cell size in the mixture. The mean size of the mixture was 4.8 microns, and the distribution bimodal. The mixture contained all cell types (sizes) and was deemed suitable for exposure.

Table 9

Distribution of Cell Sizes in a Population of Mixed Light and Dark Cells

Cell Diameter (microns)	Observed Frequency (percent)
2.25	3.8
3.5	15.1
3.75	17.0
4.5	18.9
5.25	11.3
6.0	26.4
6.75	1.9
7.5	5.7

An example of the method used to calculate the data in Tables 7 and 8 can be found in Appendix F.

INACTIVATION CURVE PROCEDURE

Cells were grown to obtain a mixture of life-cycle types as described in the previous section. The diluted mixture (approximately 116,000 cells/ml) in standard medium was then placed in open petri dishes six-inches from the ultraviolet lamp. Plate counts were taken during the experiment using the standard medium as a diluent and standard agar as a plating medium. Standard dilution and plating methods were used to determine the viable cell count during the exposure period. All plates were prepared in triplicate by spreading 0.1 ml of each dilution over the agar surface, allowing it to dry, and incubating in light at 38°C for five days before counting the colonies.

The results of the inactivation curve experiment are shown in Table 10 in the Results section. An equation describing the inhibition of Chlorella 71105 by ultraviolet irradiation is also derived.

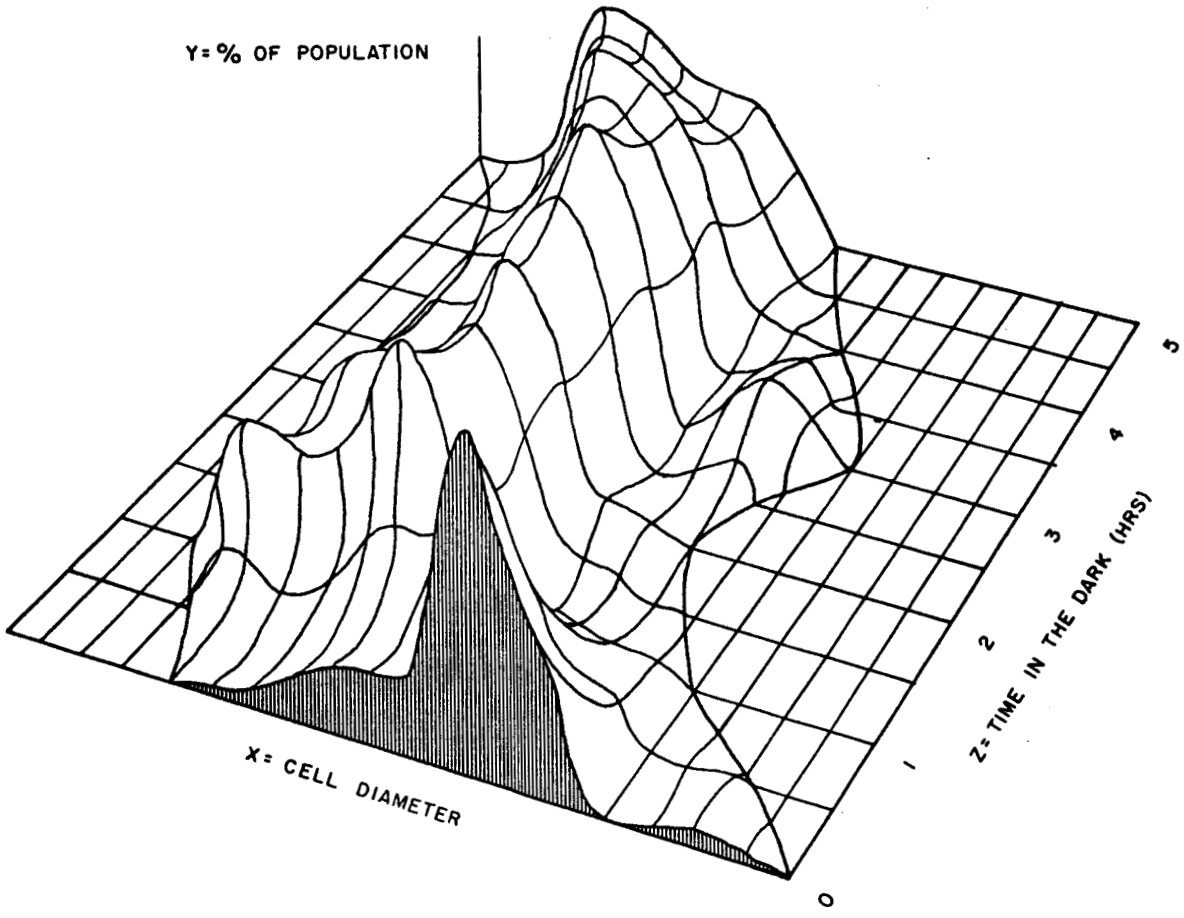


FIGURE 9

EFFECT OF INCUBATING A LIGHT-SATURATED CULTURE IN THE DARK

PROCEDURE FOR MUTATION

Twenty-five milliliters of mixed synchronized cells were constantly agitated during exposure periods adequate for 33%, 66% and 99% kill. Cultures were then diluted 1:1 with fresh medium and incubated in the aquarium unit at 45°C and 50°C for 48 hours. Following this incubation period, cultures were diluted with five parts of fresh medium and incubated an additional five days.

Following a similar exposure periods, other irradiated cells were first incubated for 24 hours at 38.5°C, 45°C, and 50°C as described above.

The pre-incubation at 38.5°C was deemed necessary because some mutants do not express phenotypic change in the first few cell divisions following exposure to ultraviolet (Demerec and Laterjet, 1946).

Since heat stability implies altered protein structure, it seemed likely that a high-temperature mutant would have an altered amino-acid metabolism and possibly a peptide or amino-acid dependence. Therefore, cultures were treated as described above (with and without pre-incubation) and cultured in media supplemented with either acid hydrolyzed casein (a source of amino acids), or an enzymatic digest of casein (a source of peptides and amino acids).

RESULTS AND DISCUSSION

The ultraviolet exposure figures shown in Table 10 indicate that Chlorella 71105 is killed by electromagnetic radiation at 2537Å. The equation describing the killing rate is developed in Appendix G and shown graphically in Figure 10. The results of the ultraviolet exposure indicate that direct sunlight in space, or growth under lamps which emit a high proportion of ultraviolet light, could inhibit Cholorella 71105 cultures. Of course, filtering by glass and/or water would prevent injury to the culture.

Table 10

Inactivation of Chlorella 71105 by Ultraviolet Irradiation

Time (min.)	Survivors (cells/ml)
0	116,000
1	95,000
2	13,000
3	560
4	200

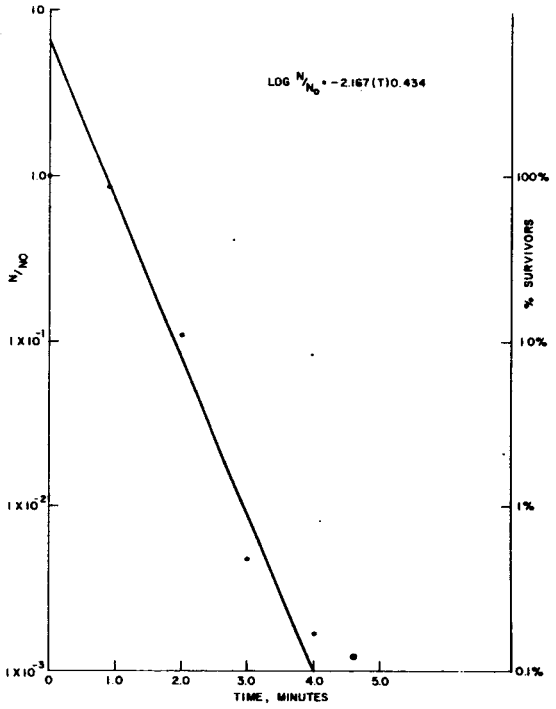


FIGURE 10
THE INACTIVATION OF CHLORELLA 71105 BY ULTRAVIOLET LIGHT

(General Electric quartzline lamps are currently being used in the NASA photosynthetic gas exchanger at Electric Boat. These lamps do not emit measurable germicidal ultraviolet radiation.)

Three petri dishes containing 25 milliliters of a dilute cell suspension were run for each of four ultraviolet exposures, and each experiment was repeated once. Therefore, a total of approximately 7.0×10^7 Chlorella cells were exposed to ultraviolet during the study. No high-temperature mutants were obtained under any of the imposed conditions, including incubation in amino acid and polypeptide media.

Time did not permit more ultraviolet exposures or the use of x-rays to obtain mutants. The advantage of using x-rays would have been that even greater numbers of cells could be exposed simultaneously, since x-rays penetrate dense cell suspensions better than ultraviolet. As a matter of fact, a thick paste of centrifuged cells could be exposed, increasing the probability of isolation of a mutant.

The use of mixtures of cell types had been recommended to us as prudent even though it seems reasonable that actively reproducing immature cells are most succeptible to radiation. In future work, the best type of life cycle intermediate for exposure should be established by determining the relative susceptibility of homogeneous populations of synchronized cells. The frequency of mutation could then be increased by exposing the most susceptible life-cycle type.

Another approach would be to concentrate synchronized cultures in a refrigerated centrifuge, expose the paste to x-rays in batches, and inoculate a large continuously diluted culture periodically with exposed cells. Operation of the mass culture unit at high light intensity and at high temperature would provide a selective environment favoring fast-growing mutants. After the unit were operated for a period, the dilution rate would be increased to favor the persistence of the most rapidly growing cell lineages in the culture.

The mean diameter of the cells exposed was 4.9μ: the total target area was thus approximately $19 \times 10^{-8} cm^2$. Since the ultraviolet lamp used emitted 19×10^{14} photons per second per cm^2, each cell was irradiated with 3.9×10^8 photons per second.

Therefore, if we define LD_{90} as the exposure time that will kill approximately 90% of the cells, the LD_{90} in our study was approximately 2 minutes. During this period of time each cell received 4×10^{10} photons on the average. Chlorella 71105 cells, then, are relatively resistant to ultraviolet radiation at 2537 Å under the conditions of this experiment.

The IES Lighting Handbook (Illuminating Engineering Society, 1959) lists the relative succeptibility of 44 common molds, yeasts, and bacteria to ultraviolet. All the microorganisms listed had LD_{90}'s lower than the Chlorella 71105 cultures; in other words, Chlorella is more resistant to ultraviolet radiation. The LD_{90} for Chlorella, 2 minutes exposure to 1450×10^{-6} joules/cm^2/sec, is equivalent to 174,000 microwatt-sec/cm^2. According to the Handbook, only two black mold spores have this order of resistance; vegetative stages, bacteria, and yeasts are only 1/10 to 1/100 as resistant.

BIBLIOGRAPHY

1. Benoit, R. J., Trainor, F., Bialecki, A., "Selection of an Alga for the Photosynthetic Gas Exchanger", WADC Technical Report No. 60-163, Wright Air Development Division, Wright-Patterson AFB, Ohio, 1960

2. Bogard, L. and Granick, S., "Protoporphyrin Precursors Produced by a Chlorella Mutant", J. Biol. Chem., Vol. 202, pp 793-800, 1953.

3. Davis, B., "Studies on Nutritionally Deficient Bacterial Mutants Isolated by Means of Penicillin", Experimentia, Vol. 6, pp 41-50, 1950.

4. Davis, E., "Photosynthetic Chlorella Mutants", Am. Jour. Bot., Vol. 39, pp 535-539, 1952.

5. Demerec, M. and Cahn, E., "Studies of Mutability in Nutritionally Deficient Strains of Escherischia coli", J. Bact., Vol. 65, p 27, 1953.

6. Demerec, M. and Laterjet, R., "Mutations of Bacteria Induced by Radiations", Cold Spring Harbor Symp. Quant. Biol., Vol. 11, pp 38-50, 1946.

7. Friedman, S. and Koffler, H., "Molecular Differences between Flagellins Isolated from a Themophile and its Mesophile Mutant", Bact. Proc., pp 149-50, 1960.

8. Granick, S., "Magnesium Protoporphorin as a Precursor of Chlorophyll in Chlorella", J. Biol. Chem., Vol. 175, pp 333-342, 1948.

9. Hase, E., Morimura, Y. and Tamiya, H., "Some Data on the Growth Physiology of Chlorella Studied by the Technique of Synchronous Culture", Arch. Biochem. Biophys., Vol. 69, pp 149-165, 1957.

10. Hase, E., Morimura, Y., Mihara, S., Tamiya, H., "The Role of Sulphur in the Cell Division of Chlorella", Archiv. Mikrobiol., Vol. 31, pp 87-95, 1958.

11. Henry, B. F., "Dissociation in the Genus Brucella", J. Inf. Dis., Vol. 52, pp 374-402, 1933.

12. Hyatt, M. and Levinson, H., "Interaction of Heat, Glucose, L-Alanine, and Potassium Nitrate in Spore Germination of Bacillus megatherium J. Bact., Vol. 81, pp 204-211, 1961.

13. Illuminating Engineering Society, IES Lighting Handbook, Third Edition, New York, pp 25-26, 1959.

14. Lewin, R., "The Isolation of Algae", Revue Algologique, Vol. 3, pp 181-198, 1959.

15. McLeod, O. and McLochlen, J., "The Sensitivity of Several Algae to Ultraviolet Radiation at 2537 Å", Physiologia Plantarum, Vol. 12, pp 306-309, 1959.

16. Myers, J., "Culture Conditions and the Development of the Photosynthetic Mechanism", Jour. Gen. Physiol., Vol. 29, pp 419-427, 1946.

17. Myers, J., "Study of a Photosynthetic Gas Exchanger as a Method of Providing for the Respiration Requirements of the Human in a Sealed Cabin, II", Report to the Air Research and Development Command, Air University School of Aviation Medicine, USAF, Randolph AFB, Texas, 1958.

18. Pirson, A., Lorenzen, H., and Koepper, A., "A Sensitive Stage in Synchronized Cultures of Chlorella", Plant Physiol., Vol. 34, pp 353-355, 1959.

19. Sorokin, C., "Changes in Photosynthetic Activity in the Course of Cell Development in Chlorella", Physiologia Plantarum, Vol. 10, p 659,1957.

20. Sorokin, C., "Tabular Comparative Data of High and Low Temperature Strains of Chlorella", Nature, Vol. 184, pp 613-614, 1959.

21. Sorokin, C., "Kinetic Studies of Temperature Effects on the Cellular Level", Biochemica et Biophysica Acta, Vol. 38, pp 197-204, 1960.

22. Sorokin, C. and Krauss, R. W., "Maximum Growth Rates of Chlorella in Steady State and in Synchronized Cultures", Proc. Nat. Acad. Sci., Vol. 45, pp 1740-1744, 1959.

23. Sorokin C. and Myers, J., "A High Temperature Strain of Chlorella", Science, Vol. 117, pp 330-331, 1953.

24. Sorokin, C. and Myers, J., "The Course of Respiration During the Life Cycle of Chlorella Cells", J. Gen. Physiol., Vol. 40, p 579, 1957.

25. Tamiya, H., Iwamura, T., Shibata, K., Hase, E. and Nihei, T., "Correlation Between Photosynthesis and Light-Independent Metabolism in the Growth of Chlorella", Biochim. et Biophys. Acta, Vol. 12, pp 23-40, 1953.

26. Umbreit, W., Burris, R. and Stauffer, J., Manometric Techniques, Third Edition, Burgess Publishing Company, Minneapolis, 1959.

APPENDICES

A

APPENDIX A

CONTRIBUTORS OF SAMPLES

The following persons generously supplied algal samples for the screening program:

J. Alcade, Spain

J. Cabanyes, Spain

J. V. Bhat, India

S. Minton, Pakistan

H. Guclu, Turkey

R. Dikman, Turkey

K. Birol, Turkey

M. Keskin, Turkey

J. J. Fernando, Ceylon

B. L. Elisberg, Malaya

L. J. Wells, Jr., S. Viet Nam

S. Taha, United Arab Republic

C. G. Smith, Venezuela

C. D. Vial, Chile

B. Fernandez, Costa Rica

R. Tanimoto, Hawaii, U.S.A.

G. B. Fernandes, Hawaii, U.S.A.

A. Phelps, Texas, U.S.A.

Special recognition is due to J. L. Davenport, Charles Pfizer Co., Maywood, N.J. who supplied many algal samples from various parts of the world.

APPENDIX B

SOURCES OF SAMPLES

	Code Number Assigned	Geographical Location	Type of Sample
1)	741	Thompson, Connecticut	Pond water-algal bloom
2)	11161	Texas	Moist, soil, green
3)	12201	Milford, Conn. USFWS Expt. Station	Stock culture *Duniella primolectrica*
4)	12202	Milford, Conn. USFWS Expt. Station	Stock *Chlorella stigmatophora*
5)	12203	Milford, Conn. USFWS Expt. Station	Stock *Duniella tertialectrica*
6)	12204	Milford, Conn. USFWS Expt. Station	Stock *Duniella* GL-2
7)	12221	New London, Conn.	Greenhouse #1 soil
8)	12222	New London, Conn.	Greenhouse #1 soil
9)	12223	New London, Conn.	Greenhouse #1 soil
10)	12225	New London, Conn.	Greenhouse #2 soil
11)	12226	New London, Conn.	Greenhouse #2 soil
12)	12227	New London, Conn.	Greenhouse #3 soil
13)	12228	New London, Conn.	Greenhouse #4 soil
14)	12229	New London, Conn.	Greenhouse #4 soil
15)	122210	New London, Conn.	Greenhouse #5 soil
16)	122211	New London, Conn.	Greenhouse #5 soil
17)	122212	New London, Conn.	Greenhouse #5 soil
18)	122213	New London, Conn.	Greenhouse #5 soil
19)	122214	New London, Conn.	Greenhouse #5 soil
20)	122215	New London, Conn.	Greenhouse #5 soil
21)	191	Denu, Nigeria	Soil
22)	192	Butte, S. Australia	Soil
23)	193	Butte, S. Australia	Soil
24)	194	Butte, S. Australia	Soil
25)	195	Roshanara, India	Soil
26)	2155	Spain	Soil
27)	2156	Spain	Soil

APPENDIX B (CONT)

	Code Number Assigned	Geographical Location	Type of Sample
28)	2157	Hawaii	Soil
29)	2158	Hawaii	Soil
30)	2241	Ceylon	Soil
31)	2242	Ceylon	Soil
32)	3243	UAR	Soil
33)	3244	UAR	Soil
34)	3245	Kepong, Malaya	Soil
35)	3246	Sentosota, Malaya	Water 40°C
36)	3247	Adana	Soil
37)	3248	Van, Turkey	Soil
38)	3249	Izmir-Narlikdere, Turkey	Soil
39)	32410	Afyon, Turkey	Soil
40)	32411	El Sombrero, Venezuela	Soil
41)	32412	San Juan, Venezuela	Soil
42)	C32	Japan	Stock Culture
43)	196	Kingsway, India	Soil
44)	197	Ashoka, India	Soil
45)	198	Rohtak, India	Soil
46)	199	India	Soil
47)	1910	Denn, Nigeria	Soil
48)	1911	Denn, Nigeria	Soil
49)	1912	Denn, Nigeria	Soil
50)	1913	Nigeria	Soil
51)	1914	Rani-Paawa, Turkey	Soil
52)	1915	Sallentar, Turkey	Soil
53)	1111	Gorkha, Turkey	Soil
54)	1112	Gorkha, Turkey	Soil
55)	1113	Rani-Paawa, Turkey	Soil
56)	1114	Sallentar, Turkey	Soil
57)	1115	Trisuli, Turkey	Soil
58)	1116	Gorkha, Turkey	Soil

APPENDIX B (CONT)

	Code Number Assigned	Geographical Location	Type of Sample
59)	1117	Karahji, Turkey	Soil
60)	1118	Trisuli, Turkey	Soil
61)	1119	Oakey-Queensland, Australia	Soil
62)	11110	Oakey-Queensland, Australia	Soil
63)	11111	Oakey-Queensland, Australia	Soil
64)	11112	Oakey-Queensland, Australia	Soil
65)	11113	Oakey-Queensland, Australia	Soil
66)	11114	Dalby-Queensland, Australia	Soil
67)	11115	Dalby-Queensland, Australia	Soil
68)	11116	Panajachel, Guatemala	Soil
69)	1161	Eschulita, Guatemala	Soil
70)	1162	Eschulita, Guatemala	Soil
71)	1163	Eschulita, Guatemala	Soil
72)	1164	Eschulita, Guatemala	Soil
73)	1165	Tontonicapan, Guatemala	Soil
74)	1166	Panajachel, Guatemala	Soil
75)	1167	Panajachel, Guatemala	Soil
76)	1168	Kandy, Ceylon	Soil
77)	1169	Matale, Ceylon	Soil
78)	11610	Chandigarh, India	Soil
79)	11611	Chandigarh, India	Soil
80)	11612	Agra, India	Soil
81)	11613	New Delhi, India	Soil
82)	11614	New Delhi, India	Soil
83)	11615	Bihar, India	Soil
84)	1231	Velugumatla, India	Soil
85)	1232	Velugumatla, India	Soil
86)	1233	Velugumatla, India	Soil
87)	1234	Chintakani, India	Soil
88)	1235	Masuliatna, India	Soil
89)	1236	Chintakani, India	Soil

APPENDIX B (CONT)

	Code Number Assigned	Geographical Location	Type of Sample
90)	1237	Tekulapelli, India	Soil
91)	1238	Munchagunda, India	Soil
92)	1239	Masulipatnam, India	Soil
93)	1314	Ansonia, Conn.	Soil
94)	1315	Norwich, Conn.	Soil
95)	1316	Ellington, Conn.	Soil
96)	1317	Putnam, Conn.	Soil
97)	1318	Norwich, Conn.	Soil
98)	1319	Wapping, Conn.	Soil
99)	13110	Wapping, Conn.	Soil
100)	13111	Brookfield, Conn.	Soil
101)	13112	Storrs, Conn.	Greenhouse
102)	13113	Storrs, Conn.	Greenhouse
103)	13114	Litchfield, Conn.	Soil
104)	13115	Litchfield, Conn.	Soil
105)	13116	Wapping, Conn.	Soil
106)	13119	Wapping, Conn.	Soil
107)	13120	Litchfield, Conn.	Soil
108)	13121	Norwich, Conn.	Soil
109)	13122	Storrs, Conn.	Greenhouse
110)	13123	Milldale, Conn.	Greenhouse
111)	13124	Ansonia, Conn.	Soil
112)	13125	Manchester, Conn.	Soil
113)	13126	Storrs, Conn.	Greenhouse
114)	13130	Storrs, Conn.	Greenhouse
115)	2151	Texas	Soil
116)	2152	Texas	Soil
117)	2153	Texas	Soil
118)	2154	Texas	Soil
119)	12310	Chintakani, India	Soil
120)	12311	Gosala, India	Soil

APPENDIX B (CONT)

	Code Number Assigned	Geographical Location	Type of Sample
121)	12312	Jocotan, Guatemala	Soil
122)	12313	Yucatan, Mexico	Soil
123)	12314	Yucatan, Mexico	Soil
124)	12315	Yucatan, Mexico	Soil
125)	12316	Guatemala	Soil
126)	12317	San Mateo, Orotina, Costa Rica	Water
127)	12318	Aqua Caliente, Cartego, Costa Rica	Hot spring 50°C
128)	12319	Hawaii	Soil
129)	12320	Hawaii	Soil
130)	12321	New Delhi, India	Soil
131)	12322	Aggra, India	Soil
132)	12323	Bogor, India	Soil
133)	12324	Orissa, India	Soil
134)	12325	Khatmandu, India	Soil
135)	12326	Vepricuitla, India	Soil
136)	12327	Vepricuitla, India	Soil
137)	12328	Chintakani, India	Soil
138)	12329	Velugumatta, India	Soil
139)	12330	Poranki, India	Soil
140)	12331	Litchfield, Conn.	Soil
141)	12332	Portland, Conn.	Soil
142)	1311	Storrs, Conn.	Soil
143)	1312	Wapping, Conn.	Soil
144)	1313	Goshen, Conn.	Soil

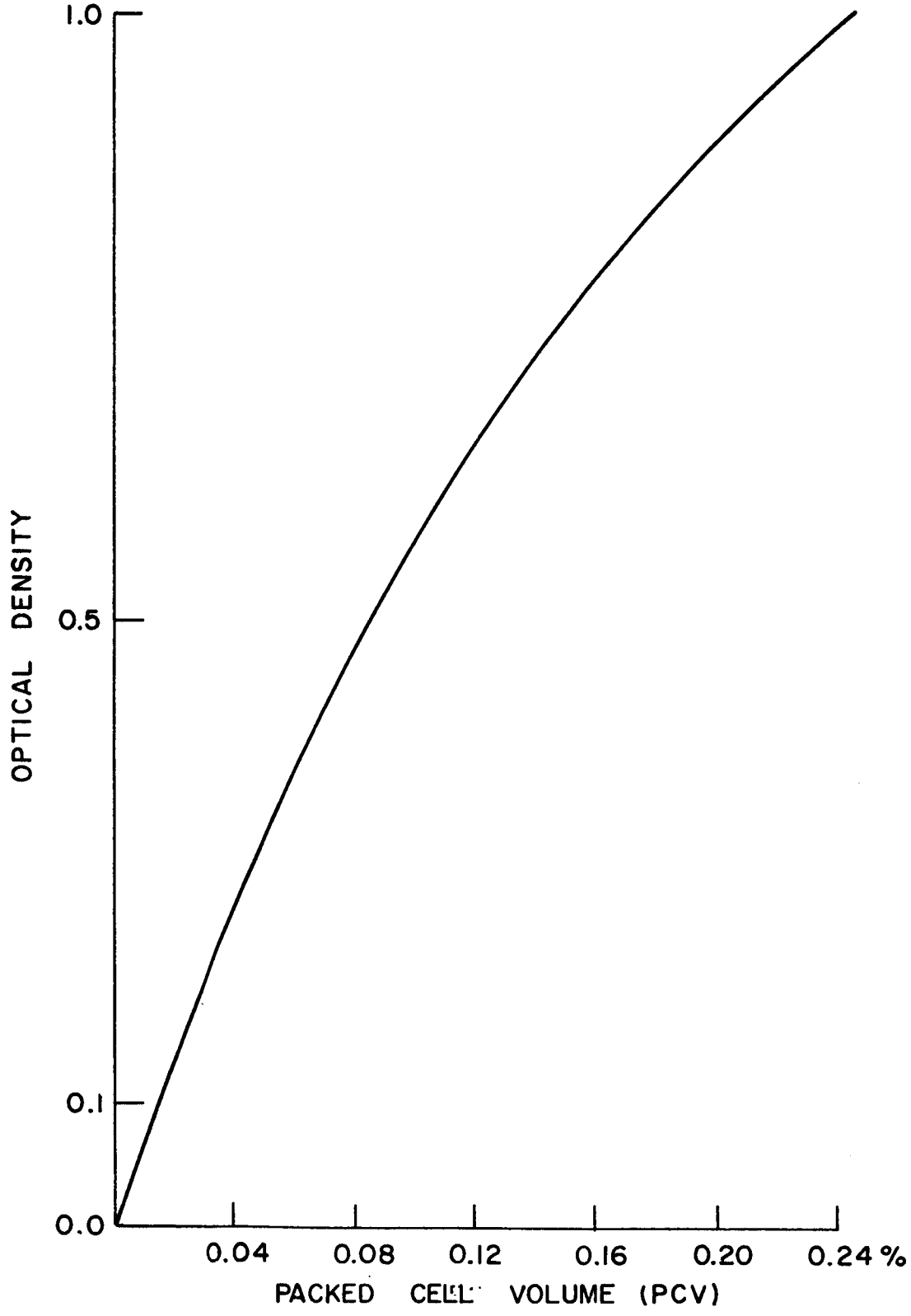
APPENDIX C

STANDARD ELECTRIC BOAT MEDIUM FOR ALGAE CULTURE

Constituent	Concentration
$MgSO_4 \cdot 7H_2O$	5.0 grams/L
KH_2PO_4	2.5 grams/L
NaCl	2.0 grams/L
Urea	186 mg N/L
Na_2Fe EDTA	0.56 mg Fe/L
$CaCl_2$	8.0 mg Ca/L
H_3BO_3	0.99 mg B/L
$Mn\ Cl_2 \cdot 4H_2O$	1.0 mg Mn/L
$ZnSO_4$	0.18 mg Zn/L
$CuSO_4 \cdot 5H_2O$	0.04 mg Cu/L
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.003 mg Mo/L
$Na_3VO_4 \cdot 16H_2O$	0.009 mg V/L

APPENDIX D

OPTICAL DENSITY OF SUSPENSIONS OF CHLORELLA 71105



APPENDIX E

PROCEDURE FOR DRY WEIGHT DETERMINATIONS

1. Measure 20 ml of the algal culture with a volumetric pipette.
2. Centrifuge the suspension; discard the supernatant.
3. Resuspend the packed cells in de-ionized water; wash and re-centrifuge three times.
4. Adjust the volume to 10.0 ml.
5. Distribute into a previously dried and weighed aluminum weighing dish.
6. Dry for 24 hours at 105°C, cool and weigh.

APPENDIX F

SAMPLE

CALCULATION OF MEAN CELL DIAMETERS, STANDARD DEVIATIONS, AND PERCENTAGES

1 Hour Light

Cell Diameter (micrometer Units) x	f	fx	fx^2	$\frac{f}{n} \times 100 = \%$
2.5	2	5	12.5	$\frac{2}{60} \times 100 = 3.3$
3.0	8	24	72.0	$\frac{8}{60} \times 100 = 13.3$
3.5	10	35	122.5	$\frac{10}{60} \times 100 = 16.7$
4.0	38	152	609.0	$\frac{38}{60} \times 100 = 63.3$
4.5	0	0	0	
5.0	$\frac{2}{60}$	$\frac{1.}{226}$	$\frac{50.0}{866}$	$\frac{2}{60} \times 100 = 3.3$

$$\bar{x} = \frac{\Sigma fx}{N} = \frac{226}{60} = 3.8 \times 1.5^* = 5.7\mu$$

$$s^2 = \frac{N(\Sigma fx^2) - (\Sigma fx)^2}{N(N-1)} = \frac{60(866) - (226)^2}{(60)(59)} = 0.25$$

$$S = \sqrt{\text{variance}} = \sqrt{0.25} = 0.5$$

*Microscopic factor (for conversion to microns) = 1.5

$S = 1.5 \times 0.5 = 0.75\mu$

APPENDIX G

CALCULATION OF KILLING CURVE

The first-order reaction describing the inactivation of algae by ultraviolet irradiation may be expressed by the differential equation:

$$-dN = kNdt \qquad (1)$$

By integrating the equation we obtain

$$-\int_{N_o}^{N} \frac{dN}{N} = k\int_{t_o}^{t} dt$$

$$\ln \frac{N_o}{N} = kt + \ell n\, i \qquad (2)$$

or

$$\frac{N_o}{N} = e^{(kt + \ell n\, i)} \qquad (3)$$

where:

N = number of survivors at time, t.

N_o = number of cells at time, $t = 0$

t = duration of exposure to ultraviolet

k = a constant

i = another constant.

The data from Table 3 can be applied to the above equation to determine the constants k and i:

Time (t)	Survivors (N)	N/N_o	$\ell n\ N/N_o$
0	116,000	1.0	0.00
1	95,000	0.82	-0.20
2	13,000	0.11	-2.21
3	560	0.0048	-5.34
4	200	0.0017	-638

Equation (2), when plotted on semilog paper, is a linear equation and may be represented:

$$y = bx + a \qquad (4)$$

where:

$$y = \ell n\ N/N_o$$

$$b = k$$

$$x = t$$

$$a = \ell n\ i$$

With this simplification the method of least squares may be applied to the data to obtain a and b, using the following equation:

$$a = \frac{\Sigma x^2\ \Sigma y - \Sigma x\ \Sigma xy}{n\ \Sigma x^2 - (\Sigma x)^2} \qquad (5)$$

$$b = \frac{n\ \Sigma xy - \Sigma x\ \Sigma y}{n\ \Sigma x^2 - (\Sigma x)^2}$$

The above equations are applied with the following assumptions:

(1) The uncertainty in the measurement of x is negligible with respect to the measurement of y.

(2) The scatter of the y values in a series of measurements at the same value of x would have a normal distribution.

For our data:

$\Sigma x = 1.0$

$\Sigma y = -14.13$

$\Sigma xy = -46.16$

$\Sigma x^2 = 30$

$\Sigma x^2 \Sigma y = -423.90$

$\Sigma x \Sigma xy = -461.6$

$(\Sigma x)^2 = 100$

$n = 4$

$n\Sigma x^2 - (\Sigma x)^2 = 20$

Therefore:

$$a = + \frac{37.7}{20} = 1.885$$

$$b = - \frac{43.34}{20} = -2.167$$

On semilog paper, the line will intercept the y axis at t = 0; a second point on the line can be determined as follows:

Let $y = \ln 1.0$ as the second point.

$$\frac{y_o - y_1}{x_o - x_1} = \text{slope} = -2.167$$

$$-2.167 = \frac{1.885 - 0}{0 - x}$$

Therefore:

$$x_1 = \frac{1.885}{2.167} = 0.87 = t_1$$

From the two points (6.6,0) and (1, 0.87) the line may be drawn on semilog paper. The equation for the line is therefore:

$$\ell n \frac{N}{N_o} = -2.167t + 1.885 = -2.167t + \ln 6.60$$

or

$$\log N/No - 1.885 = -2.167t \ (0.434)$$

The time required to kill a given percentage of the population can be calculated or read directly by examination of the killing curve. Since 99%, 66% and 33% kills were used for induced mutations, irradiated cultures were removed after 174 sec., 84 sec., and 66 sec.