LIMITING FACTORS AFFECTING THE MASS CULTURE OF *SCENEDESMUS OBLIQUUS* (TURP.) KUTZ. IN AN OPEN SYSTEM.

by
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of the University of Maryland in partial
fulfillment of the requirements for the
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INTRODUCTION

Investigations of algal growth in nutrient solutions have been pursued since 1871 when Famintzin (25) added inorganic salts to cultures of algae being used for studies in development. The research requiring the use of artificial cultures has been varied. Smith (84), Chodat (14, 15), Pringsheim (75), and Bold (5) have extensively utilized unialgal cultures in developmental morphology and taxonomy. Emerson (21), Warburg and Negelein (95), and Burk et al.(12) investigating quantum yields based their conclusions on the data from algal cultures. Hutner (40), Gunderson (33), and van Overbeek (63) have used such cultures to study growth factors and vitamins both produced and required by the algae. Brannon and Bartsch (7), Yin (98), and Kylin (47) have determined the influence of auxins. Chu (17, 18), Hopkins (39), and Gerloff, et al.(30) have studied mineral nutrition by varying the nutrient solution at arbitrary levels or by employing the familiar triangle technique (Pratt, 71) and measuring differences in growth in an effort to determine a best solution or a best balance of principal ions. Emerson (23) has measured the effect of cations in increasing or decreasing the quantum yield in photosynthesis. Scott (81) in following growth also analyzed the algal material produced. Ketchum (41), and Ketchum et al.(42) in studies on nitrogen uptake from different levels were the first to analyze both cells and medium for this element. Organic nutrition has been studied extensively by Myers (54).

During recent years the possibility of more efficient utilization of solar energy for the production of carbohydrates has been discussed by Daniels (19), Riley (77), and Transeau (90). Basing his calculation

on the work of Arnold (27) with Chlorella Daniels (19) admits the possibility of a maximum possible photosynthetic efficiency of 20%. Transeau (90) had previously calculated the efficiency of the corn plant to be, at best, 1.6%. Therefore the utilization of algae as a source of food has caught the imagination of many. Yin (99) has strongly urged the utilization of algae as a food source. His optimistic calculations based on yields with Chlorella show a possible production of 4 tons of dry substance per acre in eight days, half of which would be carbohydrate.

Spoehr and Milner (86) have studied Chlorella grown in culture solutions of varying amounts of nitrogen. They have introduced a new term for organic carbon compounds called the R value which is a statement of the degree of reduction of carbon obtained in that compound. It is given in an arbitrary scale of 100 between the points of 0 for carbon dioxide and 100 for methane and can be computed by the following formula based on analyses:

$$R = \frac{[(\%\,C \times 2.664) + (\%\,H \times 7.936) - \%\,O] \times 100}{398.9}$$

This value is unique. It can be applied to mixtures as well as to pure compounds. The higher the R value the greater the reduction of the carbon compounds analyzed and the greater the amount of stored energy. Protein, carbohydrate, and lipid content can be calculated from the R value if the nitrogen analysis is also known. From such calculations the effect of various environmental factors on photosynthesis can be determined. It was observed that high R values were only produced when the nitrogen supply was at a minimum. Cells containing from 8.7% to 58% protein were produced by variations in the

nitrogen level. The direct production of such high protein materials by plants might obviate the use of animal intermediates in the future.

Prior to the initiation of the work presented in this paper a project was established by the Carnegie Institute of Washington to secure basic data concerning growth in mass in a closed system. A brief description of the experiments was published in 1950 by Cook (9).

The general literature concerning algal growth has been thoroughly reviewed by Kufferath (46), Bold (6), Brunel et al. (9), and Pringsheim (75). Additional reference to pertinent literature will be found where it applies to problems confronted in the text.

The purpose of this study was to gain information concerning the limiting factors which would affect the growth of algae if they were to be produced in large amounts. In designing the experiment consideration was given to the general type of equipment which might be used on a commercial scale. Though no attempt was made to erect a pilot plant the equipment used was to secure data in the dimensions necessary for the erection of such a plant. A primary decision was whether to grow the algae in an open system or in a closed bacterial-free system. The former was chosen for several reasons. First, since the early work of Beijerinck (4) most studies of algal growth have been in small, so-called pure, unialgal cultures. Though initiation is simple, great difficulty is inherent in maintaining such cultures and applying necessary sampling routines. This difficulty multiplies as the size of the culture increases. Such a complication, a continuing problem in the commercial growth of Penicillium, is discussed by Foster (26). Several methods other than a closed asceptic system were developed for its production prior to the engineering accomplishment

which made sterile culture possible. Second, if solar energy is finally to be used the efficiency of illumination would be reduced by confinement to a closed system. There is no maximum limit to the amount of light which can be utilized. The greater the intensity the greater the yield. Third, no data are as yet available concerning the feasibility of growth in such an open system nor has work been accomplished showing the possible effect of contaminants in such equipment. Cook (9) points out the need for information concerning the growth of Chlorella or other algae in an open system.

The size of the culture was limited to 100 liters at a minimum and 400 liters at a maximum because the large volume of solution needed for analyses made it advisable to have the culture large enough to prevent serious error caused by sampling. The continuous centrifuge system envisioned to prevent reduction of available light as the cell concentration increases would also require considerable volume for efficient operation.

The employment of a new technique made imperative the inductive approach. A total of five experiments was completed, ranging from two to three weeks each depending upon the information desired. An effort was made to secure a growth curve with the highest possible rate by varying certain factors in the environment. The changes in growth were reflected in the status of the culture solutions as determined by pertinent analyses.

MATERIALS AND METHODS

Variation and non-reproducibility of results from early studies have resulted from the growth of algae under environmental conditions which vary with the investigation. Variations in any one of the following will give information dependent on the unique set of conditions prevailing at the time of the experiment:

(1) light -- intensity, duration, spectral distribution.

(2) temperature.

(3) carbon dioxide supply -- amount, concentration, and duration.

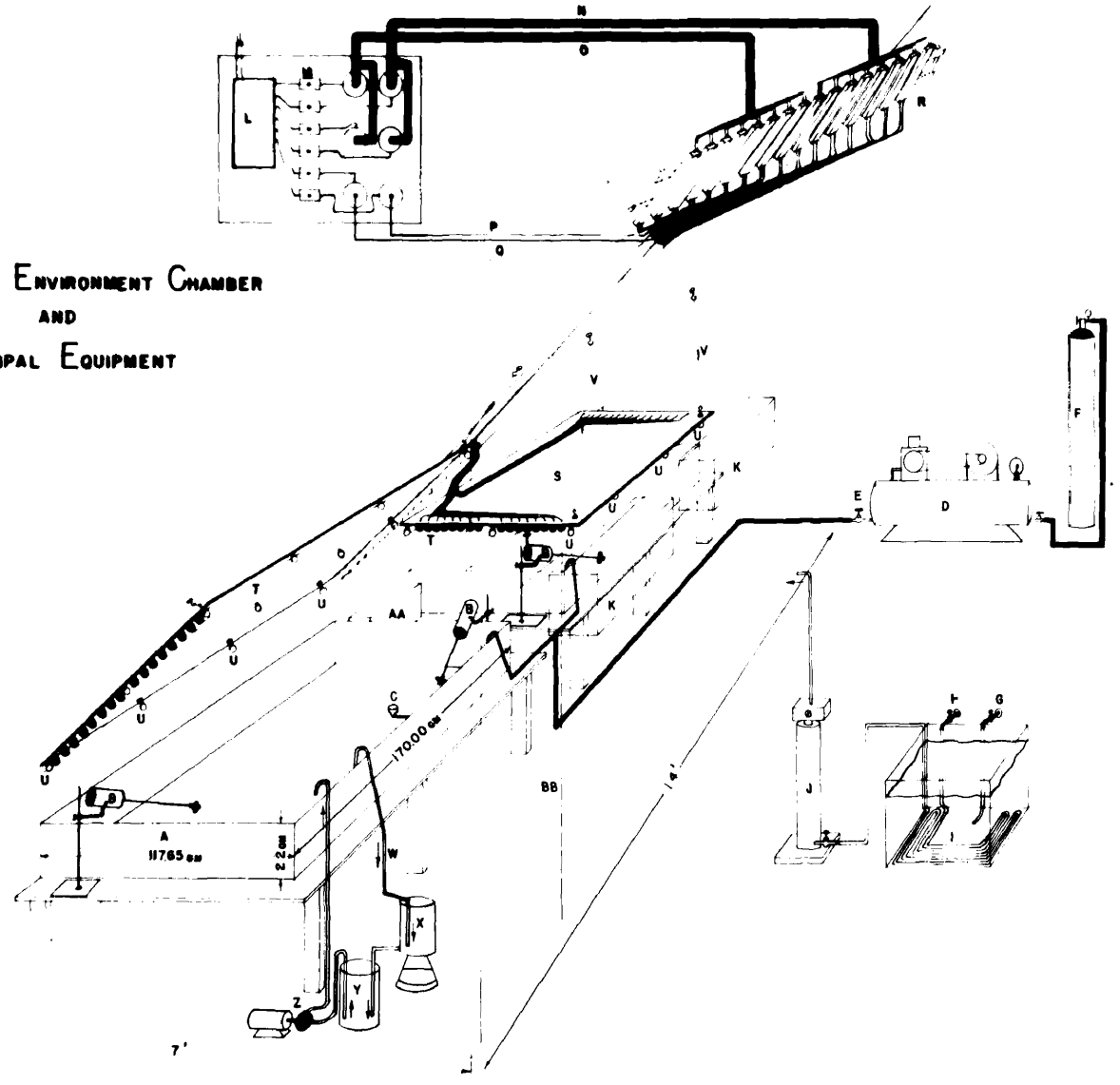
(4) water supply -- inorganic and organic nutrients, and bacterial contamination.

Two essentially different systems are available for regulation of the two physical factors usually causing the greatest variation, viz., light and temperature. First, light can be supplied by a constant artificial source close to the culture solution and the solution kept at a constant temperature by direct refrigeration or by surrounding the light source with a water bath, or a combination of these as used by Myers (57), Scott (81), Ketchum (43). Second, the light source can be held in an air-conditioned chamber where the ambient temperature is controlled by a thermostat and where radiation passes directly to the culture. The latter system offers the advantage of obviating the loss of light due to glass or water screens and avoiding fogging due to evaporation in a closed system. A controlled environment chamber was constructed as shown in fig. 1 to take advantage of the latter system.

Fig. 1. A diagrammatic, perspective view of the controlled environment chamber and principal equipment. To identify individual pieces of equipment refer to the following key.

A -- White, polyethylene-lined vats containing 400 liters at maximum
B -- Rheostat controlled stirrers with glass propellers
C -- Sintered glass aspirator at end of carbon dioxide line
D -- Pneumatic air compressor
E -- Constant-delivery diaphragm valve
F -- Liquid carbon dioxide cylinder
G -- Cold water line
H -- Hot water line
I -- Glass cooling coil in cold water bath
J -- Cation-anion exchange column
K -- Air conditioner and thermostat
L -- Main switch box
M -- Individual bank switches
N -- Line for right fluorescent bank
O -- Line for left fluorescent bank
P -- Line for right incandescent bank
Q -- Line for left incandescent bank
R -- 430 milliamp ballasts
S -- Top view, right reflector board, normal position
T -- Fluorescent tubes
U -- Incandescent bulbs
V -- Chain and turn-buckle support for the reflector board
W -- Siphon line to centrifuge
X -- Basket chemical centrifuge
Y -- Reservoir
Z -- Centrifugal pump and return line to tank
AA -- Recording hygro-thermograph
BB -- Door to chamber

Controlled Environment Chamber
and
Principal Equipment



The chamber which housed the equipment had a floor area of 98 sq. ft. The sloping roof was a feature of the available space and not of the design. The entire interior of the chamber was painted white with titanium dioxide paint for maximum reflection. The concrete floor was sealed by a special grey cement floor paint and covered with linoleum. The concrete tile walls and ceiling provided sufficient insulation for the study. However, it is recognized that cork insulation over the entire wall surface would have given even more steady environmental control.

Illumination close to the maximum for continuous artificial light was secured from two similar panels of sixteen, 6-foot fluorescent slim-line tubes (72-T-12), 4500 White, mounted one-quarter inch apart giving a combined wattage (per panel) of 9600 watts. In addition, twelve 60-watt incandescent bulbs, giving a wattage of 7200, were mounted evenly in three rows each 60 cm. apart. The value of balanced combinations of incandescent and fluorescent lamps has been discussed by Parker and Borthwick (64). The 4500 White fluorescent tube provides a radiation peak for blue and the incandescent provides a similar peak in the red so that maximum radiation approaches the absorption peaks of chlorophyll and the action spectrum for photosynthesis. In order to prevent overheating of the room the sixteen General Electric Tulamp ballasts (59-G-949), rated at 430 milleamps each, were mounted outside the room seven inches apart and far enough from the wall to allow adequate air circulation. The reflector boards were 1/4 inch masonite mounted on steel frames and suspended by chains anchored in the ceiling girders. Changes in elevation of the frame above the surface of the tanks could be attained by shortening or

lengthening of these chains. Final adjustment and leveling was achieved by the use of turn-buckles. The reflector boards were painted with titanium dioxide white-gloss for maximum reflection. The intensity and distribution of illumination are shown in tables I and II and by figure 2. The readings are in new candles (60) measured with a carefully standardized Weston Illumination Meter (Model 756). The readings were taken at the beginning of the third culture. The decrease in illumination after Cultures Four and Five was negligible. This slow decline, after the initial drop, is in agreement with the observations of Parker and Borthwick (65) on General Electric slim-line T-8 lamps. The illumination during the first 100 hours for fluorescent tubes is several hundred foot candles above that of the prolonged intensity, the decrease thereafter is slight until a sudden drop-off which can be easily detected by the light meter. The initial high intensity was removed by burning the tubes for seven days prior to the first culture.

The temperature was controlled by two Remington Professional Air-Conditioners (Model 8) with a cooling capacity of 9000 b.t.u. per hour. Both air-conditioners were mounted three feet from the floor in the front wall of the chamber each opposite a bank of lights. Cooling coils and fans were directed into the chamber. The compressor, however, was entirely outside the chamber so that operating heat could be dissipated. The fluctuation in ambient temperature was held within 2.5° F. by two White-Rodgers Temperature Controls, Type 201, Number 8, Style G1.* A constant record of ambient temperature was kept on a

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*The temperature in the culture tanks fluctuated even less, owing to the high thermal capacity of water.

TABLE I

LIGHT INTENSITY IN FOOT CANDLES PLOTTED ON A GRID OF THIRTY CENTIMETER SQUARES FOR THE LEFT VAT.

A. At the Bottom of the Vat

700	900	1000	1000	1000	900	700	500
800	1000	1200	1300	1300	1100	800	500
800	1100	1400	1500	1400	1300	900	600
800	1100	1300	1400	1400	1200	900	500
600	800	1000	1100	1100	900	700	400
500	600	700	700	700	700	500	300

B. At Surface Level of the Culture When Filled to 300 Liters

700	800	900	1000	1000	900	800	600
800	1000	1300	1300	1300	1200	900	600
800	1300	1500	1600	1600	1400	1000	600
700	1100	1400	1500	1500	1300	1000	600
600	900	1100	1200	1200	1100	800	500
400	500	600	600	600	600	500	400

TABLE II

LIGHT INTENSITY IN FOOT CANDLES PLOTTED ON A GRID OF THIRTY CENTIMETER SQUARES FOR THE RIGHT VAT.

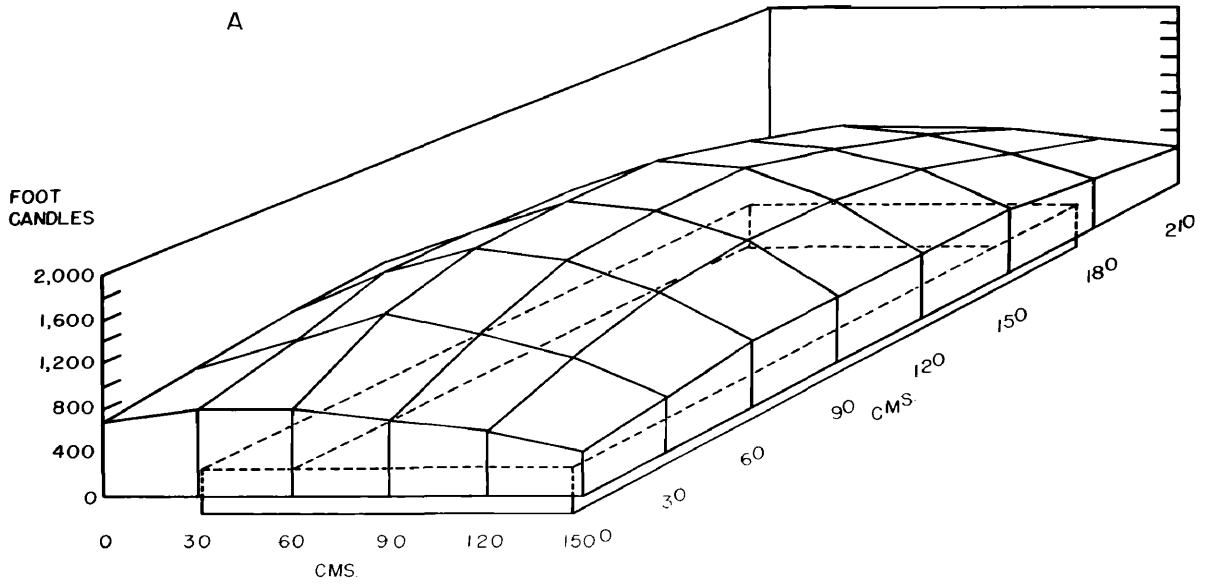
A. At the Bottom of the Vat

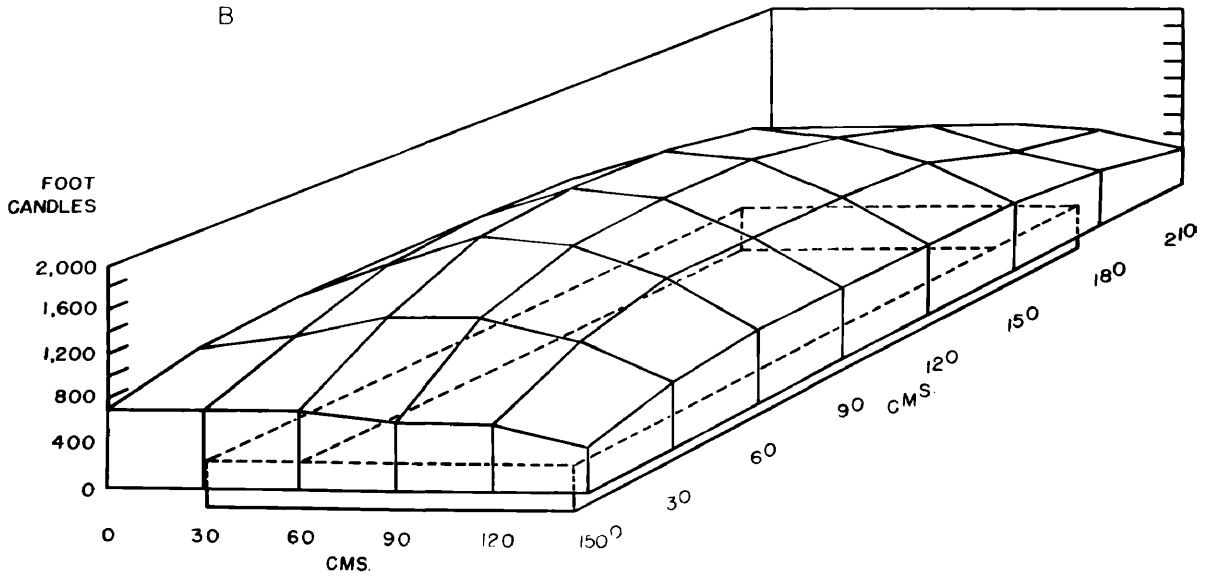
600	700	900	1000	1100	1000	800	600
500	900	1100	1300	1200	1100	900	700
600	1000	1300	1500	1500	1400	1000	800
600	1000	1300	1400	1400	1400	1000	700
500	800	1000	1100	1100	1000	800	600
300	500	700	800	800	700	600	500

B. At Surface Level of the Culture When Filled to 300 Liters

700	900	1000	1000	1100	1000	800	600
700	1000	1300	1400	1400	1200	1000	700
700	1200	1600	1700	1700	1500	1100	800
600	1200	1500	1600	1600	1400	1100	700
600	1000	1200	1200	1200	1000	800	600
400	600	700	700	700	700	600	400

Fig. 2. Distribution of light at the surface of the vat filled to 300 liters. (A) is the left vat; (B) is the right vat. Ordinates are in foot candles; the abscissae are in centimeters. The use of the third dimension prevents constant coordinates which must be estimated in perspective.


A
FOOT CANDLES
2,000
1,600
1,200
800
400
0
0
30
60
90
120
150
CMS.
30
60
90
120
150
180
210
CMS.


B
FOOT CANDLES
2,000
1,600
1,200
800
400
0
0
30
60
90
120
150
CMS.
30
60
90
120
150
180
210
CMS.

Frieze hygro-thermograph (Model 594). Daily readings of actual water temperatures were also recorded.

The construction of suitable vats presented several problems. First, it was essential that the vats be large enough to meet the maximum capacity of 400 liters, but still be light weight and easily handled. Second, they must be white in order to effect maximum light reflection. Early experiments in glass containers painted white or black on the outside showed that the cell count of Chlorella after seven days was twenty times greater in the white containers than in the black. Third, that the lining of the tanks would be chemically inert. Fourth, complete removal of all cells and organic material from each culture had to be readily attained. Several materials including glass, stainless steel, and galvanized steel with water proof paints were considered. In order to meet the requirements the tanks were constructed of 3/4 inch water-resistant plywood painted white with titanium dioxide gloss enamel and lined with a single sheet of transparent, four mill, polyethylene plastic sheeting. This material proved to be eminently satisfactory for it was water-proof, completely inert, easily folded into the plywood containers, and inexpensive enough to be discarded after each culture.

The inside dimensions of the tanks, 117.6 cm. wide by 170 cm. long by 22 cm. high, made each five centimeters in depth equal to 100 liters of solution. The vats were made smaller than the panel of lights above them so that the highest light intensity and most even distribution could be attained.

A supply of 5% carbon dioxide-in-air mixtures is usually obtained for studies of this sort by mixtures prepared at the factory. Such a

mixture was used for Culture Two only. The rapid exhaustion of the cylinders and the expense thereof prompted development of another method. Pure carbon dioxide was allowed to pass from a standard gas cylinder at 2000 lb. pressure into the reservoir of a Curtis Air Compressor (Model V-101). When the desired percentage of carbon dioxide had been delivered to the tank, as determined by the pressure gauge, the flow was cut off and the remaining pressure was obtained by running the compressor to fill the reservoir to capacity at 150 lb. pressure. A tank thus filled would deliver an even flow of carbon dioxide-in-air through sintered glass aspirators for approximately eight hours, after which time it was again filled by a measured amount of carbon dioxide and air. Delivery into the tanks was regulated by a constant flow diaphragm valve shown in figure 1.

Water for the cultures was passed through a column of cation-anion exchange resins in a Bantam Demineralizer, removable cartridge type (Model VD-1-DR). Except for Cultures One and Two no water was introduced into the vats if it showed a resistance of less than 200,000 ohms/cc. Such water is freer from contaminants than distilled water usually available in the laboratory and it can be produced rapidly. It is especially valuable in that copper and other heavy metals have been efficiently removed by the exchange resin. The use of an ion exchange column, however, does not remove bacterial contaminants. Despite the fact that an open system was used it was desirable to reduce initial biological contamination to a minimum. For Culture Three and later cultures water from the hot water line was used. This water was delivered from the tap at 70° C. four floors above the boilers where it must have been heated close to 100° C. This water

was passed through 300 cm. of pyrex glass coil immersed in a cold water bath shown in figure 1 prior to passage through the exchange column. After initial filling of the tanks water for replacement was stored in 18-liter carboys so that additions necessary to replace evaporation could be measured.

In order that the information secured from the initial cultures could be compared to earlier work the nutrient solution was a modified Knop's solution* using the following salts:

$Ca(NO_3)_2 \cdot 4 H_2O$	100 grams
KCl	25 grams
$Mg SO_4 \cdot 7 H_2O$	25 grams
KH_2PO_4	25 grams
$FeCl_3$	0.2 gram

Water to make 300 liters.

The above solution has been used successfully in more routine cultures than any other. A calculation of the individual ions in p.p.m. and m.e./L. is given in the tables of mineral analyses. This solution was used in Cultures One, Two, and Three, but was modified in Cultures Four and Five.

A major problem in culturing algae in containers of any dimensions is their tendency to settle. In order to prevent this sedimentation two glass propellers run by Fisher Versatile Stirrers (Type NSE-11-R) at the maximum rate of 5000 r.p.m. were employed in each tank. The rapid cyclosis prevented sedimentation except in the corners where an

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*Bold (6) has emphasized the inaccuracy and confusion found in citations of culture media by reference to the author. It is necessary that the composition as well as the "original" user be cited. By way of illustration the above composition is commonly called Detmer's solution.

additional stirring was periodically applied with a third glass stirrer run at 5000 r.p.m.

The organism selected for these experiments was Scenedesmus obliquus Turp. (Kützing). It was secured from the stock of pure cultures at the Woods Hole Marine Biological Laboratory. The strain was maintained in a stock nutrient solution and on agar. Inoculation for each succeeding mass culture was obtained from stock cultures derived from the preceding mass culture and maintained until needed in Detmer's solution. Each inoculation contained enough stock culture to provide an initial concentration of 1000 cells/cc. in the tank. The inoculations were from stock cultures of similar age, though Uhran (92) observed little difference in rate of nitrogen uptake and growth using inocula from cultures as much as one year old. The amount of stock culture added per 300 liters was between 50-100 cc.* Growth was followed once or twice daily by means of cell counts, using a Levy-Hauser Hemocytometer counting 100 squares on the improved Nebauer ruling. This is considerably more than necessary for statistical significance. Growth was concomitantly followed by per cent transmission in a Fisher Electrophotometer using a #425 filter. A formula was derived for converting from cell count to transmission, but for all calculations direct cell count was used. In Cultures Four and Five the cell count was supplemented by dry weight determinations of 100 ml. samples of cultures. These samples were centrifuged, washed with distilled water, and centrifuged again. They were dried

*Pratt and Fong (73) maintain that Chlorella produces a growth inhibiting substance which is manifest in lesser growth when large inocula are used. The minute size of the inoculum should minimize such an effect if present in Scenedesmus.

in an oven at 70° C. for 16 hours and transferred to a $CaCl_2$ desiccator and allowed to cool for four hours prior to weighing.

Analyses

Routine determinations of pH were made at least daily with a Beckman glass electrode pH meter. The conductivity was determined by means of a conventional conductivity bridge. Bacterial counts of the water for Cultures Four and Five were made using the method in Scott (82). For Cultures Three, Four, and Five, 400 ml. samples of the solution were taken every other day. The samples were centrifuged to remove the cells and decanted into 500 ml. bottles for storage. In order to prevent biological modification of the sample one cc. of a 1500 p.p.m. solution of mercuric chloride was added to each sample giving somewhat more than the lethal concentration of 3 p.p.m. as given by Salle (78).

A series of methods for inorganic analysis was tested. After experimentation the following were selected as being most applicable to this study:

Potassium.

The Perkin Elmer Flame Photometer (52-C) was used for potassium determinations. The readings were taken balanced against a lithium internal standard as described in the manual for the flame photometer (69). The method was highly sensitive and an error of less than $\pm$ 5% is estimated.

Calcium.

The soap titration method given by Burgess and Breazeale (11) was used for Culture Three. Results were entirely unsatisfactory in samples containing all ions though reasonable

accuracy was secured for knowns containing only calcium and magnesium. For Cultures Four and Five the Perkin Elmer Flame Photometer was employed with good results, though the error was probably greater than for potassium.

Magnesium.

For Culture Three magnesium was determined by means of the soap titration method of Burgess and Breazeale (11). This method was not satisfactory, so the Titan Yellow Method of Peech, Michael, and English (68) was employed for Cultures Four and Five. This method also proved unreliable. Something in the samples interfered with the proper formation of the lakes and the results are questionable. Magnesium was considered to be the only element for which a better method of analysis would be desirable.

Nitrate.

The classical Devarda Method modified by the use of a long water condenser rather than the usual air-cooled condenser as given by Lepper et al. (48) proved completely satisfactory.

Phosphate.

The method of Lindner (49) was modified by the omission of the 2.5 N sodium hydroxide solution usually required to prevent excess acidity.

Sulfate.

The determination by the Benzidine Method as given by Reitemeier (76) was employed without modification.

Chloride.

The silver nitrate titration as given by Lepper et al. (48) was employed for chloride.

Bicarbonate and Carbonate.

This determination was made in Culture Three along with the routine analyses. However, in order to secure greater accuracy in Cultures Four and Five the carbonate-bicarbonate titration was performed immediately after taking the sample and centrifuging. The method is given by Lepper et al. (48).

RESULTS

Except for environmental factors, modified as described specifically for each, all cultures were maintained under the same constant conditions. Maximum light intensity as plotted in figure 2 was maintained for all cultures. The radiation measured on a General Electric Radiation Meter was 0.2 gram calories per cm. per second in the highest central area of illumination and graded off to 0.1 gram calories per cm. per second in the areas of lowest intensity at the edges of the tank. The temperature was held at 21° C. and did not vary more than 1.5° F. above or below this point. The depth of solutions was maintained at 15 cm. giving a total volume of 300 liters. The Knop's solution was modified only in Cultures Four and Five. The water supply for Cultures One and Two was introduced directly to the cation-anion exchange column and delivery was not allowed to fall below 100,000 ohms. In Cultures Three and Four, however, hot water was utilized and was allowed to fall to only 200,000 ohms. For convenience in review the presentation of results attained from each experimental culture is followed by a brief discussion which presents the information considered necessary for the steps taken in the subsequent cultures.

Culture One. The purpose of the first culture was to establish the general growth curve with pure carbon dioxide supplied at brief intervals of time each day. It was not expected that maximum yield would be secured in this manner, but information was secured which demonstrated the effect of rapid growth on a culture solution.

The growth curve and data for changes in pH are given in table III and figure 3.

TABLE III

CELL COUNTS, TRANSMISSION, pH AND ADDITION OF PURE CARBON DIOXIDE FOR CULTURE ONE.*

Days after inoculation	Cells/cc X 10^6	Per cent Transmission	Hours CO_2 Added	pH
0	0.0	100.0	.5	5.1
1	0.0	100.0	1.0	5.3
2	0.0	100.0	0.0	5.3
3	0.0	100.0	0.0	5.4
4	0.0	99.0	.5	5.7
	.1	98.5		5.9
5	.2	96.5	2.0	6.2
	.4	92.5		6.5
6	1.2	90.0		6.9
	1.3	89.0	1.0	7.0
7	1.5	82.0		7.6
	1.6	79.5	2.0	5.3
8	2.0	61.5		8.1
	2.5	59.0	1.0	8.7
9	4.4	49.5		9.0
	5.0	62.0	1.0	8.4
10	6.3	43.0		8.5
	6.9	35.0	1.0	10.0
11	7.5	34.0		9.5
	8.9	32.0	1.0	10.5
12	9.1	29.0		9.3
	10.6	27.0	1.0	10.5
13	10.3	26.5		8.9
	10.3	25.5	1.0	9.9
14	13.0	22.0		9.1
	13.2	20.0	1.0	9.9
15	14.5	18.0		8.8
	14.8	17.5	1.0	9.7

TABLE III

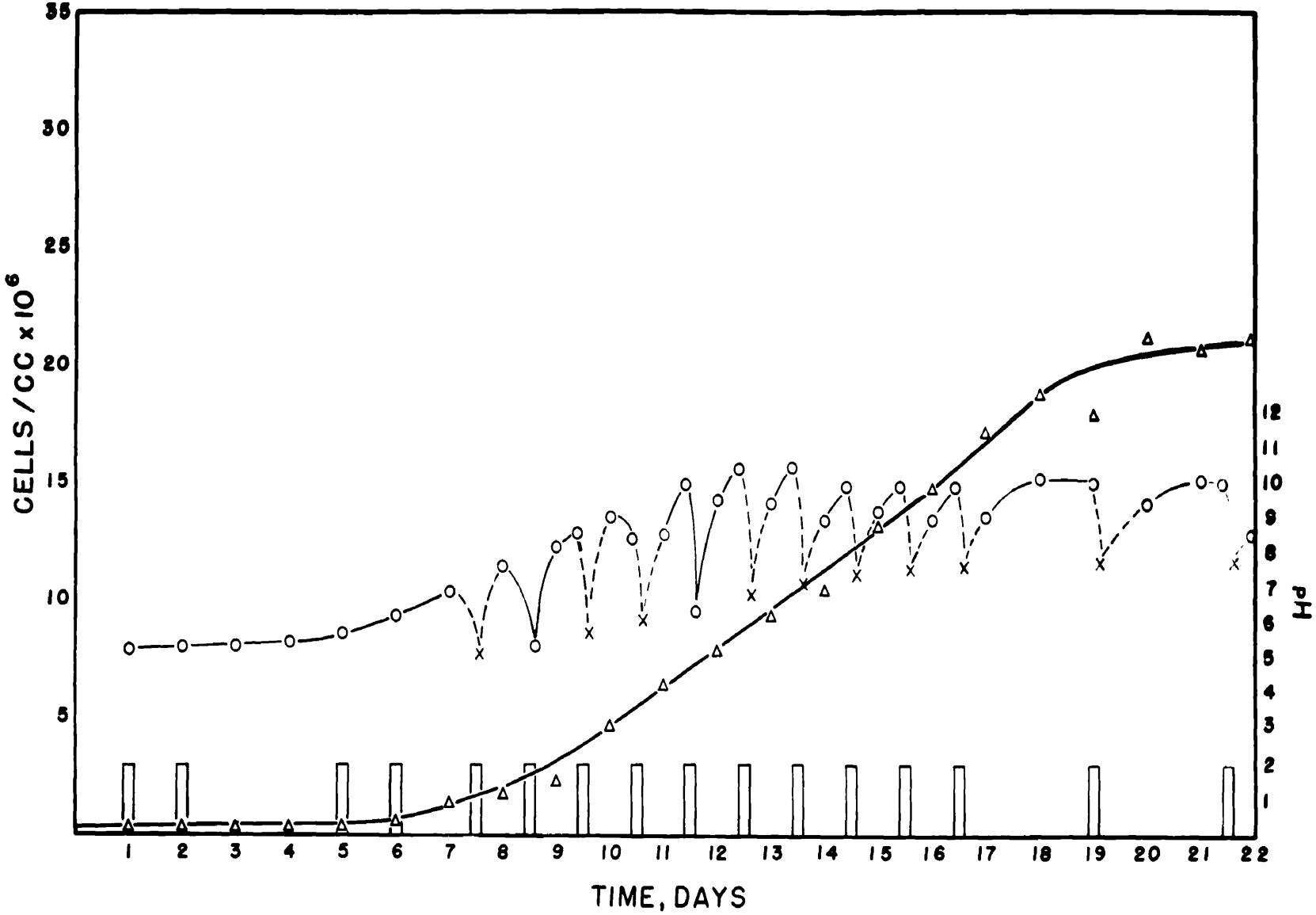
CELL COUNTS, TRANSMISSION, pH, AND ADDITION OF PURE CARBON DIOXIDE FOR CULTURE ONE.*

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	Hours CO_2 Added	pH
16	17.1	16.0	0.0	9.0
17	18.5	15.0	0.0	10.3
18	17.6	15.0	.5	10.3
	17.6	15.0		8.3
19	21.0	14.0	0.0	9.2
20	20.0	13.0		10.0
		11.0	1.0	9.9
21	21.0	33.0[a]		8.5
		35.0[a]	1.0	9.1
22	21.0	35.0[a]	0.0	8.8

*Where two readings are recorded, the first is at 9:30 A. M.; the second at 9:30 P. M.

[a]Reading taken from one part of culture sample diluted with one part of water.

Fig. 3. The growth curve and pH changes in Culture One. Bars represent additions of pure CO_2 for a period of usually one hour (see table III). Solid lines and circled points plot observed pH changes. Dotted lines connecting points represented by "X" were plotted from information taken from subsequent cultures.


CELLS/CC x 10^6
35
30
25
20
15
10
5
pH
12
11
10
9
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
TIME, DAYS

Growth is delayed for several days after inoculation. During this period the cells, almost all of which exist singly, are observed to increase in size from as small as 3 X 14 microns to reach a size of 8 X 19 microns. This increase in size was observed in all cultures prior to the first division and in a lesser degree prior to subsequent divisions. The normal course in cell growth was for a single cell to grow in volume and then to divide into four autospores which, growing in size, ruptured the mother cell wall and formed a coenobe of four united cells. These remained united in colony form until each had attained an intermediate size, after which the colony separated and the cells lived singly until division occured again. As the cultures aged, a greater percentage of cells was found in the coenobe form. After the lag phase in growth the increase in population was extremely rapid. It was not until Cultures Four and Five that the precise contour of the rate curve was established.

The mechanism of the common lag period prior to a rapid rate of division has been investigated by Monod (53) for a considerable number of microorganisms. He has concluded that the duration of the lag period is shortened by increased size of inoculum which, in turn, either allows for colloidal adsorbtion of toxic trace elements or introduces biologically synthesized substances necessary for growth. High light intensity may have had an additional affect in prolonging the lag phase as has been shown by Meyers (54, 56) in experiments with light intensities ranging from 40,000 foot candles down. In additional experiments Meyers (55) points out that photosynthesis may increase until limited by the dark reaction at about 350 foot candles depending on intensity of prior culture but that growth is not stimulated by intensities above 100 foot candles. Intensities above 100 foot candles therefore may cause secretion into

the solution or storage. In order to keep the cultures similar, no effort was made to reduce this lag period by increasing inoculum size or reducing initial light.

Soon after the initiation of division fluctuations were observed in the pH of the solution after addition of the periodic supply of carbon dioxide. By readings taken before and after the application of the gas the curve shown in figure 3 for pH fluctuation was plotted. It was apparent that anions were being much more rapidly removed from solution than cations. The anion thus removed was most likely nitrate as demonstrated by Uhran (92). The effect of removal of anions by the cell was accentuated by the fact that phosphate precipitates at higher pH values. The data confirmed that of Sphoer and Milner (86) showing increase in pH in the solution supplied nitrogen by means of NO_3. Of especial interest is the speed of removal of the carbon dioxide supply. At the higher pH values the carbon dioxide must have existed primarily as bicarbonate which served as a buffer balancing the surplus cations and holding the pH near 7.5. Upon removal of the source of carbon dioxide, however, the algae were especially efficient in active absorption of the carbonate ion even though the pH thereby created was injurious.

Carbon dioxide has been frequently supplied to cultures of algae in the form of bicarbonates which can be utilized effectively for photosynthesis. It was considered possible that the intermittent addition of pure carbon dioxide for limited periods would form sufficient bicarbonates to adequately supply the cells during the periods when the gas was not being delivered. The growth curve for the first culture (fig. 3) suggests that this method is unsatisfactory unless the carbon dioxide is delivered at intervals which are frequent enough to hold the pH below 7.4.

This observation corroborates the findings of Emerson and Green (22) that at pH values above 8.0 the carbon dioxide concentration is so low that photosynthesis is drastically reduced unless exceptionally large amounts of carbonates and bicarbonates are added.

From the data of Emerson and Green (22) and this investigation, it is clear that if carbon dioxide were continuously supplied in sufficient concentrations there would be enough bicarbonate ions produced to replace the anions absorbed from the nutrient solution thus supplying a buffer to hold the pH within a range suitable for efficient photosynthesis. Therefore, a pH of below 8.0 would indicate that ample bicarbonate was being formed in the solution and that sufficient free carbon dioxide was present to give a maximum rate of photosynthesis.

Considerable debate, nevertheless, has centered upon the unavailability of iron at higher pH values. Wann and Hopkins (94) have presented curves showing an immediate fall-off in growth of Chlorella due to iron-precipitation at any pH above 7.3. Emerson and Green (22) and Emerson and Lewis (23) have generally agreed with these findings. Österlind (62), on the other hand, found no reduction in growth in Scenedesmus between pH 7 and 10, showing that iron was apparently not a factor in limiting the growth of these organisms. It is possible that organic acids secreted into solutions by certain species, (Aleev, 1), especially under high light intensities, may render the availability of iron less a problem than suspected.

Culture Two. The second culture was continually supplied with carbon dioxide added from a 5% carbon dioxide-in-air mixture in a prepared cylinder. The growth curve is shown in figure 4. The rate was calculated by the

Fig. 4. Comparative growth curves for Cultures One, Two, Three, and Four plotted from hemocytometer counts.

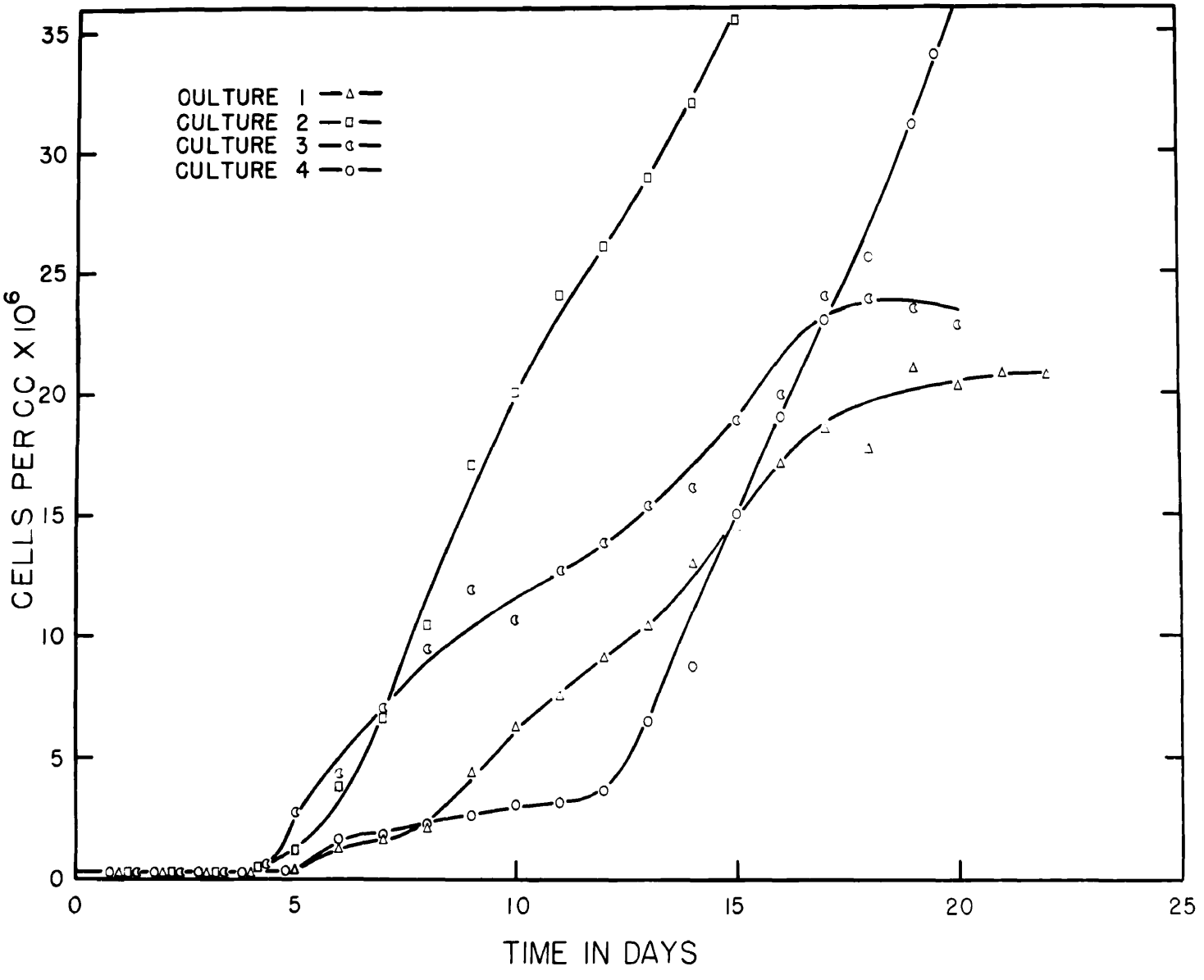

CULTURE 1
CULTURE 2
CULTURE 3
CULTURE 4
CELLS PER CC X 10^6
TIME IN DAYS
0
5
10
15
20
25
30
35

TABLE IV

CELL COUNTS, TRANSMISSION, pH,
CONDUCTIVITY, AND GROWTH RATES FOR CULTURE TWO*

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	pH	Conductivity (K X 10^5) (mho's)	Growth Rate (K)
0	.0	100	5.1	65.0	....
1	.0	100	5.2	65.0	0.0
2	.0	100	5.2	65.0	3.36/
3	.2	99	5.4	65.0	
	.2	98	5.5	59.5	3.36/
4	.2	95	6.0	59.5	
	.8	92	6.7	60.0	2.49
5	1.1	89	6.6	58.7	
	1.2	80	7.5	57.0	1.37/
6	4.9	70	7.6	56.7	
	5.4	62	6.8	57.5	1.37/
7	6.7	55	7.2	57.3	
	8.6	49	7.0	55.5	0.68
8	10.5	44	7.3	54.0	
	14.0	38	7.7	56.5	0.65
9	17.1	29	8.4	49.5	
	18.4	34	7.1	55.0	0.40
10	20.2	30	7.1	54.0	
	25.7	28	7.3	50.7	0.50
11	24.2	24	7.6	50.5	
	25.7	22	7.4	50.5	0.00
12	26.0	19	7.4	49.0	
	27.2	18	7.6	48.0	0.08
13	28.9	15	8.8	43.0	0.09
14	32.6	14	7.6	43.6	0.19
15	35.9	12	8.0	44.0	0.11

*Where two readings are recorded, the first is at 9:30 A. M.; the second at 9:30 P. M.

/Calculated for a period of 48 hours.

formula of Hjort et al.(35) using logs to the base 2.*

$$\log_n \frac{C_2}{C_1} = \bar{K} (T_2 - T_1)$$

$\bar{K}$ was calculated from observed concentrations and not from a smoothed curve. $\bar{K}$ therefore gives the number of times the population doubled during a time interval of 24 hours. The rate showed a remarkable rise after the first four days but then dropped off immediately. The rise was so sudden and so rapid during the first few days that the shape of the early part of the curve was uncertain. This portion was accurately determined in later experiments. The conductivity changes are shown in table IV. If it is assumed that the CO_2 supply is producing enough bicarbonate ions to replace the anions absorbed, the reduction in conductivity should reflect the total removal of cations if the pH is held constant. Any sudden drop in the conductivity should be apparent in a sudden rise in pH. This actually occurred on the ninth day after inoculation when carbon dioxide in the cylinder became exhausted and there was a six hour lapse prior to the installation of a second tank.

The growth curve of Culture Two plotted in figure 4 was considered to be normal and compares favorably with those secured by Ketchum (42) and Osterlind (62).

Culture Three. Information secured from Cultures One and Two suggested that data concerning the mineral nutrition of the organisms would be essential if a steady growth rate were to be maintained for

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*Conversion of common logs to logs to base 2 is achieved by the following:

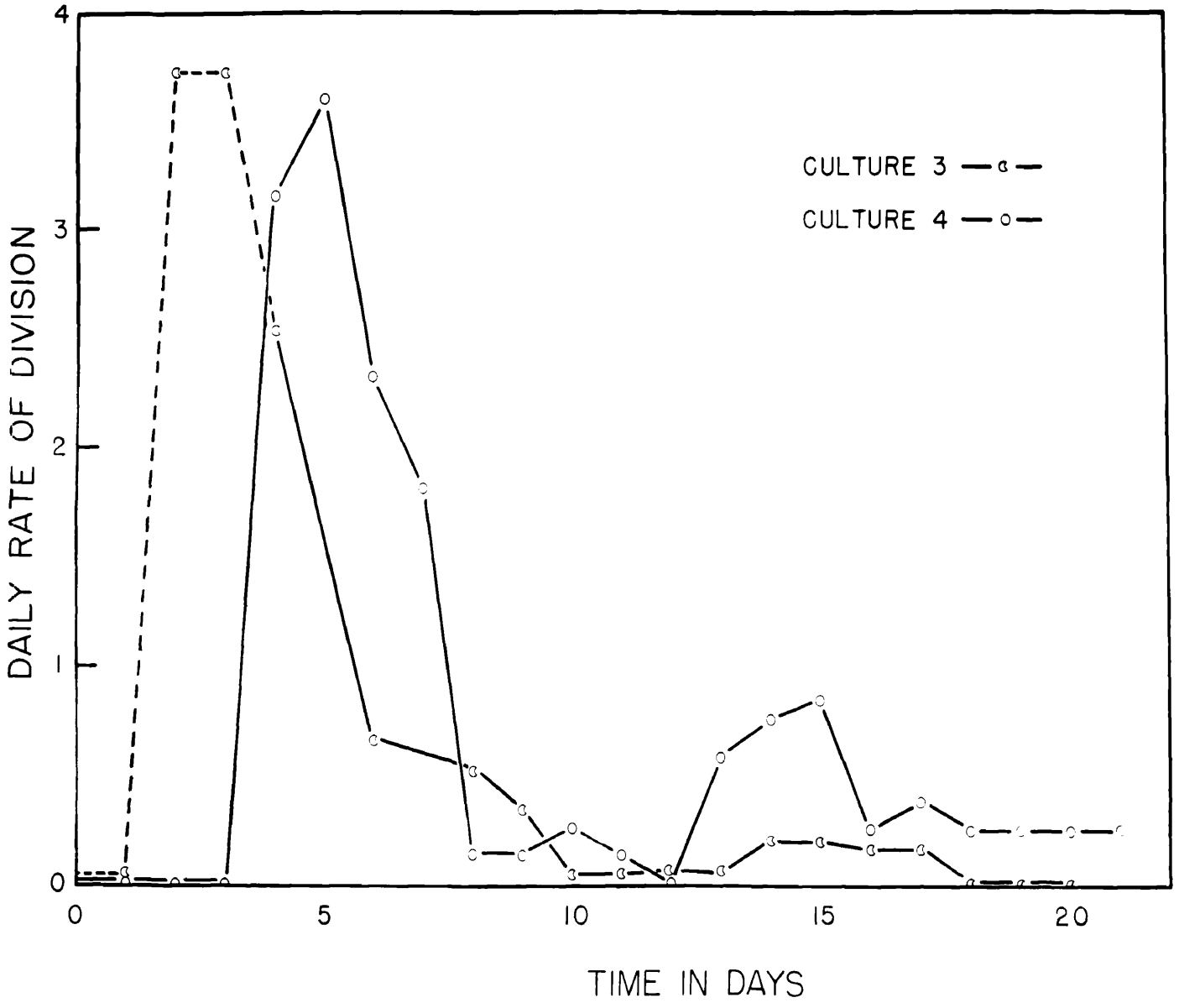
$$\log_2 N = \log_{10} N \times 3.32$$

any considerable period of time. The composition of the nutrient medium could not be permitted to change if a steady state were to be attained. In order to determine the amount and rate of removal of all mineral nutrients from solution an analysis was made to determine progressive removal of all ions except Fe. In order to increase the precision of the analysis it was decided to allow no water showing a resistance of less than 200,000 ohms to reach the tank. In addition, water heated to 70-80° C. was utilized to reduce the amount of foreign microorganismal contamination. Carbon dioxide mixtures were supplied by the compressor system rather than from prepared tanks. Delivery was even and continuous except on the eighth and tenth days when apparent clogging of the diaphragm valve slowed the carbon dioxide supply. The amount of carbon dioxide delivered to the tanks was increased from 5% carbon dioxide-in-air to 10% carbon dioxide-in-air after the fifth day. The growth curve is plotted in figure 4, and the growth rate in figure 5. The growth curve is essentially the same as that for Culture Two except that it is displaced considerably to the right and only about half as many cells were produced at the end of fifteen days by Culture Three. This caused some concern but the difficulty was not resolved until the examination of curves in the early part of Cultures Four and Five.

Values of pH, conductivity, and growth rate are found in table VI showing close similarity to those determined for Culture Two.

The inorganic analyses presented in table V confirm the deductions from the information presented for Cultures One and Two. It was recognized that refinement in analytical technique for subsequent cultures would give more accurate information concerning removal of ions, but

Fig. 5. Growth rates for Cultures Three and Four. The dotted line is an estimated rate for Culture Three during the stages of growth. (The pattern of acceleration during this period and the maximum growth rate were not accurately determined until Cultures Four and Five.)


DAILY RATE OF DIVISION
TIME IN DAYS
CULTURE 3
CULTURE 4
0
1
2
3
4
0
5
10
15
20

[redacted]

TABLE V

INORGANIC ANALYSES OF THE MEDIUM FOR CULTURE TUBES COVERING NINETEEN DAYS

Sample	Potassium		Calcium		Magnesium		Nitrate		Phosphate		Sulfate		Chloride		Bicarbonate		Carbonate		pH
Days after Inoculation	ppm	epm*	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	
Calculated Starting Concentration	67.0	1.72	57.3	2.86	3.27	.68	175.0	2.82	58.0	1.84	32.4	.68	39.6	1.11	....	....	....	....	...
	71.0	1.81	64.0	3.20	6.0	.50	164.0	2.65	57.0	1.80	31.2	.64	42.5	1.20	17.7	.29	0.0	.00	5.1
1	71.0	1.81	65.0	3.24	6.0	.50	170.0	2.74	58.0	1.84	30.3	.63	42.5	1.20	29.3	.48	0.0	.00	5.9
4	71.0	1.81	63.0	3.15	6.0	.50	169.0	2.72	54.5	1.72	36.0	.74	42.5	1.20	29.3	.48	0.0	.00	6.8
6	67.5	1.72	52.0	2.60	7.0	.57	128.0	2.06	9.8	.31	32.4	.67	42.5	1.20	44.0	.72	0.0	.00	7.2
8	67.5	1.72	33.0	1.65	.3	.02	77.5	1.25	1.6	.05	31.7	.65	42.5	1.20	11.6	.19	17.4	.58	9.9
10	65.5	1.67	35.0	1.75	.5	.04	44.5	.72	7.6	.24	21.3	.44	42.5	1.20	79.0	1.30	8.4	.28	8.8
12	63.0	1.61	36.0	1.80	6.0	.49	4.0	.06	12.7	.35	18.8	.39	42.5	1.20	224.0	4.00	0.0	.00	7.4
14	61.0	1.56	41.0	2.02	7.0	.57	0.0	.00	14.3	.45	16.4	.34	42.5	1.20	116.0	2.72	0.0	.00	7.6
16	61.0	1.56	41.0	2.02	7.0	.57	0.0	.00	12.0	.38	21.0	.43	42.5	1.20	166.0	2.72	0.0	.00	7.3
18	59.0	1.51	41.0	2.02	6.0	.49	0.0	.00	12.0	.38	9.7	.20	42.5	1.20	166.0	2.72	0.0	.00	7.5

*"An equivalent per million (e.p.m.) is a unit chemical equivalent weight of solute per million unit weights of solution. Concentration in equivalents per million is calculated by dividing concentration in parts per million (p.p.m.) by the chemical combining weight of the substance or ion. This unit has also been called 'milli-equivalents per liter' and 'milligram equivalents per kilogram.' The latter term is precise, but the former will be in error if the specific gravity of the solution is not exactly 1.0." A.S.T.M. Standards, 1940; part III, page 541.

[redacted]

TABLE VI

CELL COUNTS, TRANSMISSION, pH,
CONDUCTIVITY, AND GROWTH RATES FOR CULTURE THREE

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	pH	Conductivity (K X 10^5) (mho's)	Growth Rate (K)
0	.0	100	5.1	65.0	....
1	.0	100	5.4	64.0	0.0
2	.1	99	5.9	64.0	3.32#
3	.1	99	5.9	64.0	3.32#
4	.5	91	6.8	65.0	2.53
5	2.9	71	8.0	62.0	2.42
6	4.5	67	7.4	57.5	0.66
7	6.7	57	7.2	59.0	0.55
8	9.6	47	9.9	50.5	0.52
9	12.0	38	7.4	52.0	0.38
10	10.6	52[a]	8.8	48.2	0.05#
11	12.8	46[a]	7.4	50.0	0.05#
12	13.8	43[a]	7.4	47.0	0.06#
13	15.2	40[a]	7.5	45.3	0.06#
14	16.1	38[a]	7.6	44.7	0.21
15	19.0	51[b]	7.6	46.5	0.20
16	19.8	53[b]	7.3	47.0	0.17#
17	24.1	51[b]	7.4	45.0	0.17
18	24.0	50[b]	7.5	44.5	0.00
20	22.2	50[b]	7.5	42.5	0.00

#Denotes average K calculated for a period of 48 hours.

[a]Reading from one part of solution diluted with one part of water.

[b]Reading from one part of solution diluted with two parts of water.

general trends are apparent. Contrary to the information obtained by Hoagland and Davis (36) on Nitella, potassium was only slightly absorbed, decreasing 0.3 m.e./L. for the entire run. Calcium and magnesium also showed little reduction except for the considerable dip from the eighth to the twelfth day. This can be readily attributed to the failure of the carbon dioxide supply and rise in pH at this time. Carbonates formed must have been calcium and magnesium carbonates that were essentially insoluble. With the lower pH restored the calcium and magnesium were returned to the solution, as shown by analysis. Nitrate removal was exceptionally rapid, almost all of it being removed after only ten days. It is to be noted that growth continues long after the nitrate supply is exhausted. There apparently is a luxury consumption of nitrogen during the early phases of growth which allowed for continued division when nitrate would otherwise be limiting. Phosphate removal is rapid and abrupt. The decrease, however, cannot be construed to be uptake for, as Buehrer (10) has shown, at the higher pH values most of the phosphate is in the form of insoluble $^{=}HPO_4$ which precipitates as insoluble salts. A better estimate of uptake, given by extrapolating to zero, is presented in later analyses. Sulphate removal is linear throughout the culture. The large figure obtained for the sixteenth day is not in line with the general trend. Analysis on subsequent cultures indicates that this figure is in error. Chloride shows no change in concentration. The test is sensitive and would show even a slight change. The data suggest that chloride is actively excluded from the cells. The carbonate-bicarbonate balance is a function of the pH which in turn is determined by the rate of the carbon dioxide uptake by the cells as well as the rate of delivery of the

carbon dioxide to the solution. Österlind (62) has demonstrated that a concentration of 2-10 micromoles of bicarbonate is sufficient for rapid growth of Scenedesmus. At lower pH values, however, it is not possible to increase the bicarbonate in solution no matter how rapid the delivery of carbon dioxide.

A review of the mineral analyses indicates that removal of all ions except nitrate is so slow that an addition of this ion at the proper point to compensate for its rate of removal would maintain a favorable nutrient background for a considerable period of time unless uptake of the other ions changed with the new balance obtained. From these data it was calculated that the addition of approximately one-half the original nitrate concentration be added when the cell count reaches 10×10^6. The pH would then be expected to drop back to near 5, if readjustment to the original concentration was accomplished.

Cultures Four and Five. In order to follow the leads suggested from Culture Three, two identical tanks were prepared, each to be handled initially in the same way, and to be followed by the same analyses. The addition of the nitrate in the amount calculated was expected to remove the limiting factor caused by uptake of this ion from solution. At least for a limited period, this would leave only one major limiting factor to be dealt with -- the reduction of light available per cell unit caused by the increase in cell concentration. In order to remove the latter limitation it was essential that the cells be taken from solution as fast as they were produced. A point was reached where the division rate multiplied by concentration gave a maximum figure. A sedimentation-elutration system had been tested on earlier cultures but proved to be too slow for removal of the cells.

Instead a chemical, basket centrifuge with a solid stainless steel head in a porcelain, drainage chamber was used. The culture solution passed to the centrifuge through a siphon which could be regulated for rate of delivery. The cleared solution was returned to the vats by means of a centrifugal pump.* Such a system worked exceptionally well showing a removal of approximately 95% of the cells from the solution. Under conditions of low nitrogen supply, however, the system was not nearly as effective for the lower specific gravity of the high lipid cells interfered with the rate of separation. This observation is in agreement with the information procured by Spoehr and Milner (86).

It was also decided that daily determinations of the bacterial count of the cultures should be made. In order to minimize contamination in the tanks prior to inoculation they were irradiated continuously during initial filling with quartz, mercury-arc ultra-violet lamp. The filling of the tanks took approximately 16 hours. They were allowed to stand 24 hours prior to inoculation. However, five days after the cultures were inoculated not only was there no sign of growth, but all of the original inoculation had disappeared. According to Duggar (20) lethal effects of ultra-violet irradiation do not affect the media in any way. He disagrees with the conclusions of Bedford, reviewed by Duggar (20), who has maintained that there

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*The centrifugal pump was made of monel metal which is an alloy of nickel and copper. To check for possibility of contamination by copper, nutrient solution was recirculated through the pump for one-half hour after which it was tested by the micro-method of Center and MacIntosh (13) for copper. No trace of copper was detected.

is considerable lethal action caused by the formation of peroxide in solution. It is recognized that the 16 hours of radiation in a closed chamber is unusual. However, there is no doubt that there was most effective destruction of the cells under the circumstances either from the rapid break-down of O_3 or H_2O_2 in solution under high light intensity. The odor of O_3 was easily discernable at the surface of the water. This seems the more likely source of nascent oxygen probably causing the destruction. The solution was replaced and fresh tanks prepared without the benefit of ultra-violet sterilization.

The growth curve for Culture Four is presented in figure 4 and table VII. The curve for Culture Five is not plotted but is essentially similar to that of Culture Four until the time of centrifugation when dry weight determinations were made instead of cell counts as presented in table IX. The curve for Culture Five was behind that of Culture Four for several days, but the count had become approximately equal before centrifugation.

As the curves were being plotted from daily counts and analyses it was immediately apparent that the growth was progressing at only one-half the rate of Culture Three which in turn was only about one-half that of Culture Two. None of the physical and environmental conditions was different and so the cause was sought in the chemical environment. An apparent dilution factor of one-half with each subsequent culture seemed obvious.

The problem of introducing micronutrients was considered prior to the first culture. Much of the literature (Spoehr and Milner (86), Bold (6), Pringsheim (75), etc.) has recorded success without the use of trace elements. Emerson and Lewis (23), Trelease and Trelease (91),

TABLE VII

CELL COUNTS, TRANSMISSION, pH,
CONDUCTIVITY, AND GROWTH RATES FOR CULTURE FOUR*

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	pH	Conductivity (K X 10^5) (mho's)	Growth Rate (K)
0	.001 .001	100	5.0	61.0	....
1	.001 .002	100	5.1	61.0	0.0
2	.001 .001	100	5.1	65.0	0.0
3	.001 .004	100	5.1	63.0	0.0
4	.009 .310	98	5.4	60.0	3.16
5	.110 .160	94	6.3	62.0	3.60
6	.550 1.550	76	6.9	59.5	2.32
7	1.910 1.850	66	7.1	50.0	1.61
8	2.000 2.270	54	7.2	59.0	0.14
9	2.310 2.480	49	7.3	60.0	0.14
10[c]	2.760 2.910	42	4.8	63.5	0.26
11	3.000 3.020	44	6.2	60.0	0.14
12[d]	3.010 3.720	42	6.7	56.0	0.00
13	4.500 6.420	32	7.1	59.5	0.59

TABLE VII

CELL COUNTS, TRANSMISSION, pH,
CONDUCTIVITY, AND GROWTH RATES FOR CULTURE FOUR*

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	pH	Conductivity (K X 10^5) (mho's)	Growth Rate (K)
14	7.550				0.76
	8.720	28	7.6	57.0	
15	13.240				0.84
	15.200	19	8.1	58.0	
16	16.200				0.26
	19.040	18	7.[illegible]	54.5	
17	20.860				0.38
	23.440	13	7.5	52.5	
18	23.760				0.26
	25.410	12	7.5	48.5	
19	28.920				0.26
	30.720	9	7.9	49.5	
20	33.900				0.26
	41.920	23[a]	8.4	46.0	

*Where two readings are recorded, the first is for 9:30 A. M.; the second for 9:30 P. M.

[a]Reading from one part of solution diluted with one part of water.

[c]1.4 m.e./L. HNO_3 added.

[d]Trace elements added.

TABLE VIII

CELL COUNTS, TRANSMISSION, pH,
CONDUCTIVITY, AND GROWTH RATES FOR CULTURE FIVE*

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	pH	Conductivity (K X 10^5) (mho's)	Growth Rate (K)
0	.001 .002	100	5.0	61.0	....
1	.001 .003	100	5.1	60.2	0.0
2	.001 .001	100	5.1	64.0	0.0
3	.003 .005	100	5.3	61.0	1.58
4	.009 .014	98	5.2	61.5	1.58
5	.040 .070	97	5.6	61.0	2.14
6	.180 .550	90	6.4	58.7	2.16
7	1.390 1.620	73	6.9	58.5	2.94
8[e]	1.920 1.070	67	7.1	59.0	0.49
9[e]	1.480 1.140	78	7.2	59.5	
10[c,e]	1.130 1.090	80	4.2	63.5	
11[e]	1.070 .610	84	5.2	59.0	
12[d,e]	1.010 1.270	78	7.9	54.0	
13[h]	2.020 2.230	76	7.2	58.5	

TABLE VIII

CELL COUNTS, TRANSMISSION, pH,
CONDUCTIVITY, AND GROWTH RATES FOR CULTURE FIVE*

Days after Inoculation	Cells/cc X 10^6	Per Cent Transmission	pH	Conductivity (K X 10^5) (mho's)	Growth Rate (K)
14[f]	3.200				
	1.820	83	7.2	55.0	
15[g]	2.290				
	1.450	87	7.5	56.0	
16[g]	1.680				
	1.030	93	7.2	55.0	
17[f]	1.450				
	1.070	91	7.3	56.5	
18[g]	1.210				
	.820	95	7.4	51.0	
19	.800				
	1.130	87	7.5	52.0	
20[f]	1.420				
	1.070	88	8.0	52.0	

*Where two readings are recorded, the first is at 9:30 A. M.; the second at 9:30 P.M.

[c]1.4 m.e./L. HNO_3 added.

[d]Trace elements added.

[e]Six hours of centrifuging.

[f]Eight hours of centrifuging.

[g]Ten hours of centrifuging.

[h]Twelve hours of centrifuging.

TABLE IX

COMPARATIVE INCREMENTS IN GRAMS
DRY WEIGHT FOR CULTURES FOUR AND FIVE

Days after Inoculation	Total Dry Weight in Grams			
	In Culture Four	In Culture Five	Centrifuged from Culture Five	Total produced by Culture Five*
8	45.9	34.2	14.1316	48.3
9	67.8	37.5	11.5837	63.2
10	61.2	28.2	9.1735	63.2
11	63.2	17.7	8.1159	60.7
12[d]	63.9	11.1	10.1349	62.2
13	95.7	17.7	23.1260	94.1
14	105.6	18.9	32.9764	128.1
15	143.1	20.4	18.3546	148.5
16	141.6	12.3	11.4713	151.8
17	163.5	17.7	5.1312	162.4
18	165.6	14.1	7.3392	166.2
19	202.5	22.5	....	188.7
20	209.7	15.0	11.8411	193.0

*The total produced by Culture Five is a summation of the weight in the tank determined by 100 ml. samples plus the weight removed by all prior centrifugations listed in the fourth column of this table.

[d]Trace elements added.

and Arnon (2), on the other hand, have demonstrated the indispensability of additional micronutrients. Two variables seem to account for the conflicting results in these cases. One is the water supply and the other is the purity of the chemicals. It seemed desirable to forego addition of the elements unless they were demonstrated to be limiting in these experiments. Excellent comparative results with Culture Two indicated that the curves were better than average and that no additional elements were needed. The appearance of the dilution factor, much as observed by Steinberg (87) in fungi, however, made the possibility of deficiency of trace elements highly likely. Prior to decision to add micronutrients the 1.4 m.e. of nitric acid per liter calculated from earlier curves was added. There was no increase in either growth or growth rate. On the twelfth day after inoculation, zinc, manganese, and boron were added in the amounts suggested by Trelease and Trelease (91). The immediate readjustment of the growth curve to conform to that obtained in Culture Two is shown in figure 4. The growth rate shows the reflected increase as plotted in figures 4 and 5 and in tables VII and VIII. Copper was not added at this time because of the indefinite information concerning its limits of toxicity.

The growth curve for Culture Three matches almost exactly that obtained by Ketchum et al. (42). Culture Four even after the suppression during the early part of the curve exceeds that of Ketchum by approximately 10,000,000 cells per cc. Though not directly comparable because of the differences in light intensity there seems little doubt that their curve could be raised to a higher plateau by the addition of the trace elements to the nutrient solution. The

same postulate could certainly be applied to all work done without the addition of the trace elements. Even though water may appear to supply sufficient micronutrients the spectacular effect here obtained would certainly render conclusions based on such cultures open to question.

Mineral analyses for Cultures Four and Five are presented in tables X and XI, and figures 6 and 7 for Culture Four. The general trend for each ion is similar to that in the analysis for Culture Three. More precision was obtained for calcium by the use of the flame photometer. Magnesium analyzed using the Titan Yellow technique of Peech and English (68) is still considered unsatisfactory. The 1.4 m.e. of nitric acid/L. added on the tenth day is reflected in the analysis. This addition was more than enough to replace that taken up by the cells. It is also reflected in the reduction of the pH to 4.2 and 4.8 rather than the initial pH of 5.0. Uptake of the additional nitrogen was extremely rapid in Culture Four but not as rapid in Culture Five. The cell counts and weights showing lower concentrations of cells in Culture Five indicated some injury due to the excess addition of nitrate.

On the eighth day after inoculation, i.e., two days prior to the introduction of the nitrogen, removal of cells by centrifugation was begun in Culture Five. At this point the populations of Cultures Four and Five were almost equal, Culture Four being only slightly smaller. During centrifugation an attempt was made to hold Culture Four at the population of 1.3×10^{-6} cells per cc., where the rate multiplied by the concentration was at a maximum. This quantity was too low for it had been calculated from the curve for Culture Three

TABLE I

INORGANIC ANALYSES OF THE MEDIUM FOR CULTURE POOL COVERING TWENTY-ONE DAYS

Sample	Potassium		Calcium		Magnesium		Nitrate		Phosphate		Sulfate		Chloride		Bicarbonate		Carbonate		pH
Days after Inoculation	ppm	epm*	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	
Calculated Starting Concentration	67	1.72	57.3	2.86	6.27	.68	175.0	2.82	58.0	1.84	32.4	.68	39.6	1.11	....	....	...	...	...
0	68	1.74	56.0	2.80	6.0	.50	164.5	2.65	57.0	1.80	32.1	.67	41.5	1.17	10.4	.17	0.0	0.0	5.0
2	70	1.79	56.0	2.80	6.2	.52	175.5	2.81	57.5	1.82	31.8	.66	42.5	1.20	10.4	.17	0.0	0.0	5.1
4	69	1.76	56.0	2.80	8.0	.67	171.5	2.76	57.0	1.80	33.6	.70	41.5	1.17	8.5	.14	0.0	0.0	5.4
6	69	1.76	53.0	2.65	8.0	.67	141.0	2.27	52.0	1.64	32.1	.67	42.5	1.20	45.8	.75	0.0	0.0	6.9
8	71	1.81	42.0	2.10	9.0	.74	103.0	1.65	52.5	1.66	29.4	.61	42.5	1.20	79.8	1.31	0.0	0.0	7.2
10	69	1.76	51.0	2.55	9.0	.74	176.0	2.84	39.3	1.24	28.4	.59	42.0	1.19	10.3	.17	0.0	0.0	4.8
12	66	1.69	49.0	2.45	8.2	.68	152.0	2.45	36.2	1.14	26.0	.54	42.0	1.18	28.0	.46	0.0	0.0	6.7
14	66	1.69	36.0	1.80	8.8	.73	108.0	1.74	17.8	.56	23.6	.49	42.5	1.20	73.0	1.20	0.0	0.0	7.6
16	63	1.61	47.0	2.35	9.0	.74	70.5	1.14	24.4	.80	15.9	.33	42.0	1.19	117.5	1.93	0.0	0.0	7.3
18	59	1.51	34.0	1.70	8.2	.68	28.6	.46	17.8	.56	14.4	.30	42.0	1.18	152.5	2.50	0.0	0.0	7.5
20	59	1.51	33.0	1.65	8.0	.67	0.0	.00	8.9	.28	13.5	.28	41.5	1.17	183.0	3.00	0.0	0.0	8.4

*"An equivalent per million (e.p.m.) is a unit chemical equivalent weight of solute per million unit weights of solution. Concentration in equivalents per million is calculated by dividing concentration in parts per million (p.p.m.) by the chemical combining weight of the substance or ion. This unit has also been called 'milli-equivalents per liter' and 'milligram equivalents per kilogram.' The latter term is precise, but the former will be in error if the specific gravity of the solution is not exactly 1.0." A.S.T.M. Standards, 1940; part III, page 541.

TABLE X

INORGANIC ANALYSES OF THE MEDIUM FOR CULTURE FOUR COVERING TWENTY-ONE DAYS

Sample	Potassium		Calcium		Magnesium		Nitrate		Phosphate		Sulfate		Chloride		Bicarbonate		Carbonate		pH
Days after Inoculation	ppm	epm*	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	
Calculated Starting Concentration	67	1.72	57.3	2.86	6.27	.68	175.0	2.82	58.0	1.84	32.4	.68	39.6	1.11	....	....	...	...	...
0	68	1.74	56.0	2.80	6.0	.50	164.5	2.65	57.0	1.80	32.1	.67	41.5	1.17	10.4	.17	0.0	0.0	5.0
2	70	1.79	56.0	2.80	6.2	.52	175.5	2.81	57.5	1.82	31.8	.66	42.5	1.20	10.4	.17	0.0	0.0	5.1
4	69	1.76	56.0	2.80	8.0	.67	171.5	2.76	57.0	1.80	33.6	.70	41.5	1.17	8.5	.14	0.0	0.0	5.4
6	69	1.76	53.0	2.65	8.0	.67	141.0	2.27	52.0	1.64	32.1	.67	42.5	1.20	45.8	.75	0.0	0.0	6.9
8	71	1.81	42.0	2.10	9.0	.74	103.0	1.65	52.5	1.66	29.4	.61	42.5	1.20	79.8	1.31	0.0	0.0	7.2
10	69	1.76	51.0	2.55	9.0	.74	176.0	2.84	39.3	1.24	28.4	.59	42.0	1.19	10.3	.17	0.0	0.0	4.8
12	66	1.69	49.0	2.45	8.2	.68	152.0	2.45	36.2	1.14	26.0	.54	42.0	1.18	28.0	.46	0.0	0.0	6.7
14	66	1.69	36.0	1.80	8.8	.73	108.0	1.74	17.8	.56	23.6	.49	42.5	1.20	73.0	1.20	0.0	0.0	7.6
16	63	1.61	47.0	2.35	9.0	.74	73.5	1.18	24.4	.80	15.9	.33	42.0	1.19	117.5	1.93	0.0	0.0	7.3
18	59	1.51	34.0	1.70	8.2	.68	28.6	.46	17.8	.58	14.4	.30	42.0	1.18	152.5	2.50	0.0	0.0	7.5
20	59	1.51	33.0	1.65	8.0	.67	0.0	.00	8.9	.28	13.5	.28	41.5	1.17	183.0	3.00	0.0	0.0	8.4

*"An equivalent per million (e.p.m.) is a unit chemical equivalent weight of solute per million unit weights of solution. Concentration in equivalents per million is calculated by dividing concentration in parts per million (p.p.m.) by the chemical combining weight of the substance or ion. This unit has also been called 'milli-equivalents per liter' and 'milligram equivalents per kilogram.' The latter term is precise, but the former will be in error if the specific gravity of the solution is not exactly 1.0." A.S.T.M. Standards, 1940; part III, page 541.

[redacted]

TABLE XI

INORGANIC ANALYSES OF THE MEDIUM FOR CULTURE FIVE COVERING TWENTY-ONE DAYS

Sample	Potassium		Calcium		Magnesium		Nitrate		Phosphate		Sulfate		Chloride		Bicarbonate		Carbonate		pH
Days after Inoculation	ppm	epm*	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	ppm	epm	
Calculated Starting Concentration	67	1.72	57.3	2.86	8.27	.68	175.0	2.82	58.0	1.84	32.4	.68	39.6	1.11	....	....	...	...	...
0	68	1.74	56.0	2.80	6.0	.50	166.0	2.68	57.0	1.80	32.8	.68	41.5	1.17	10.4	.17	0.0	0.0	5.0
2	69	1.76	54.0	2.70	5.9	.49	174.0	2.80	57.0	1.80	31.8	.66	41.5	1.17	10.4	.17	0.0	0.0	5.1
4	68	1.74	53.0	2.65	5.9	.49	171.0	2.76	54.5	1.72	32.8	.68	41.5	1.18	9.8	.16	0.0	0.0	5.2
6	68	1.74	53.0	2.65	7.4	.62	150.0	2.42	48.0	1.52	30.8	.64	42.5	1.20	23.8	.39	0.0	0.0	6.4
8	64	1.64	52.0	2.60	9.0	.74	113.0	1.82	48.0	1.52	31.2	.65	42.5	1.20	67.8	1.11	0.0	0.0	7.1
10	64	1.64	52.0	2.60	6.6	.55	189.0	3.05	41.0	1.30	29.8	.62	42.0	1.19	4.3	.07	0.0	0.0	4.2
12	62	1.59	50.0	2.50	9.0	.74	151.0	2.44	33.0	1.04	26.9	.56	41.0	1.16	3.2	.52	0.0	0.0	7.9
14	59	1.51	47.0	2.35	8.8	.73	84.5	1.36	23.4	.74	22.2	.46	41.5	1.17	96.5	1.58	0.0	0.0	7.2
16	59	1.51	47.0	2.35	8.2	.68	67.0	1.08	19.0	.60	23.0	.48	42.0	1.18	121.2	1.99	0.0	0.0	7.2
18	57	1.46	46.0	2.30	8.0	.67	50.5	.82	17.8	.56	19.7	.41	41.0	1.15	135.0	2.22	0.0	0.0	7.4
20	53	1.36	46.0	2.30	8.0	.67	30.0	.48	12.7	.40	16.3	.34	41.0	1.15	146.1	2.40	0.0	0.0	8.0

*"An equivalent per million (e.p.m.) is a unit chemical equivalent weight of solute per million unit weights of solution. Concentration in equivalents per million is calculated by dividing concentration in parts per million (p.p.m.) by the chemical combining weight of the substance or ion. This unit has also been called 'milli-equivalents per liter' and 'milligram equivalents per kilogram.' The latter term is precise, but the former will be in error if the specific gravity of the solution is not exactly 1.0." A.S.T.M. Standards, 1940; part III, page 541.

[redacted]

Fig. 6. Levels of principal ions in solution after the first, ninth, and twenty-first days of Culture Four.

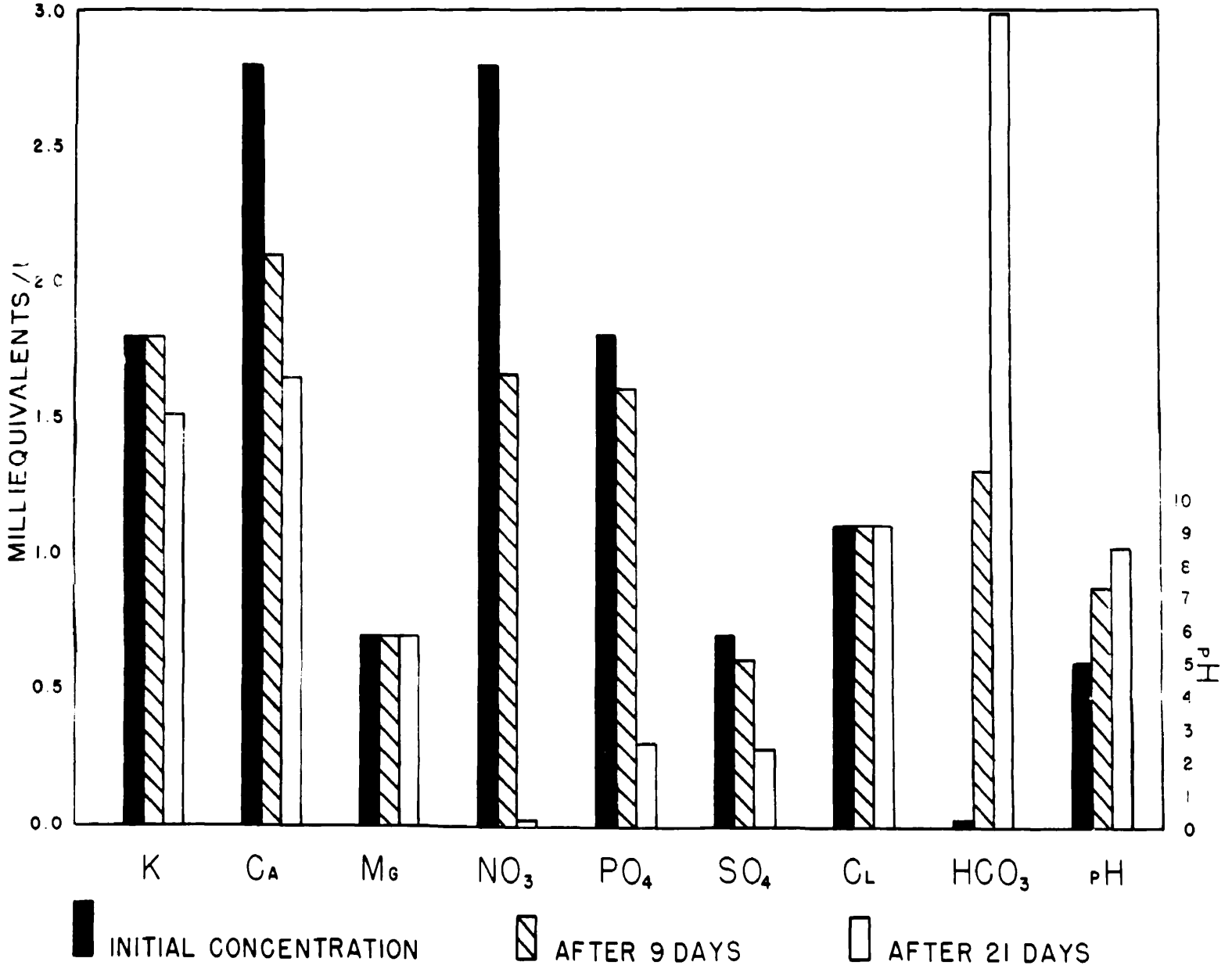
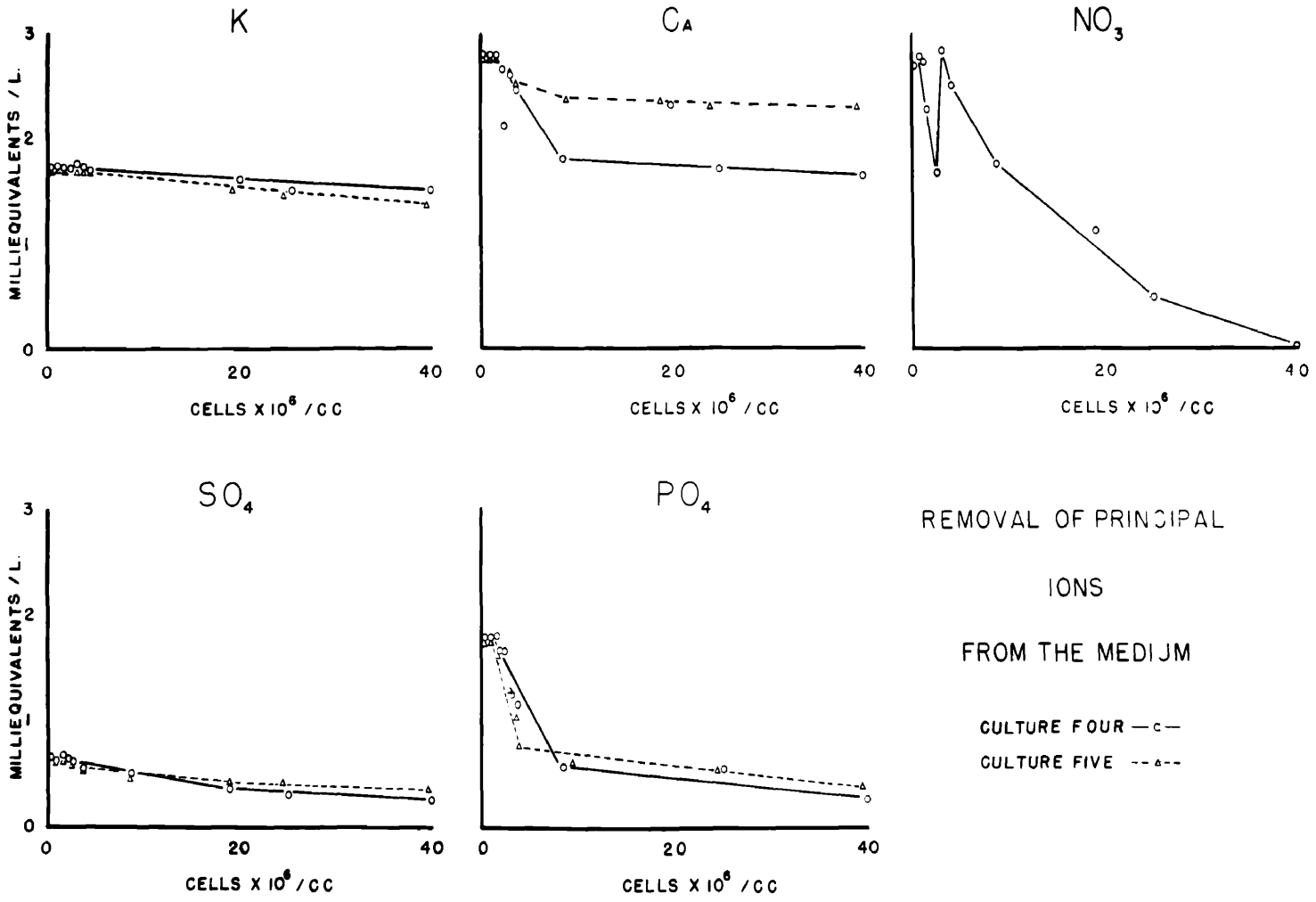

3.0
2.5
2.0
1.5
1.0
0.5
0.0
MILLIEQUIVALENTS/L
10
9
8
7
6
5
4
3
2
1
0
pH
K
CA
MG
NO3
PO4
SO4
CL
HCO3
pH
INITIAL CONCENTRATION
AFTER 9 DAYS
AFTER 21 DAYS

Fig. 7. Removal of principal ions from solution during Cultures Four and Five. Solid line is for Culture Four; dotted line is for Culture Five. The line plotted for Culture Five is approximated from dry weight determination where actual cell counts are unavailable. No line for NO_3 removal is plotted in Culture Five for slight error in approximation would cause considerable error in the curve.



REMOVAL OF PRINCIPAL

IONS

FROM THE MEDIUM

CULTURE FOUR —○—

CULTURE FIVE --△--

which was abnormal due to the absence of trace elements. Growth after the beginning of centrifugation can best be followed by reference to figure 4. Prior to the addition of the trace elements the rate of growth in both tanks was essentially the same even though the population in Culture Five became inadvertently reduced below the rate calculated for best efficiency. After addition of the trace elements growth in both tanks was accelerated but the yield in Culture Five exceeded that of Four for four to five days, as was expected, after which it fell behind. Apparently the reduction of the cell count below that necessary for optimum yield prevented continued maximum growth.

Table XII gives the bacterial count followed for Cultures Four and Five. No attempt was made to identify the bacteria represented. It was observed that 75% or more of the bacterial colonies were of uniform size and shape and bright orange in color. Examination of these colonies showed that this organism was a non-motile, non-spore forming, gram negative, non-colonial, rod-shaped organism. The characteristics are those of common saprophytic water bacteria of the genus Flavobacterium. The count increased gradually until the twelfth day when the minor elements were added after which it fell suddenly. The immediate drop in bacterial count could have been caused by two factors. First, one of the ions added may have been toxic to the bacteria. As mentioned earlier boron is toxic to bacteria at relatively low concentrations. Secondly, the algae themselves may have synthesized a substance toxic to the bacteria after the introduction of the trace elements. The bacterial count did not again show a major rise above that observed on the fourth day.

TABLE XII

BACTERIAL COUNT PER DAY FOR CULTURES FOUR AND FIVE

Days after Inoculation	Culture Four Cells/cc X 10^6	Culture Five Cells/cc X 10^6
1	...	...
2	...	...
3	...	...
4	9	2
5	9	5
6	15	13
7	92	54
8	27	49
9	37	104
10	139	85
11	195	89
12	120	50
13	11	6
14	13	11
15	12	8
16	6	9
17	4	4
18	4	5
19	6	2
20	17	8

DISCUSSION

The progressive adjustment of the physical and chemical environment in mass culture has shown the feasibility of growth in an open system under high light intensity. Yields obtained are higher than those of Osterlind (62) and Ketchum (42) plotted for Scenedesmus in closed systems. They compare favorably with the maximum yields of Cook (9) using Chlorella. The maximum growth obtained in this manner was roughly one-tenth of that obtained by Cook (9). Though no direct comparison can be made, Chlorella vulgaris has been shown to have a much higher growth capability. Winokur (97) has obtained a Chlorella culture of 1,000 X 10^6 cells in the same period of time necessary for a culture of 40 X 10^6 cells for Scenedesmus in this experiment. It is probable that the efficiency attained here could be greatly improved within the genetic limits of the species. Only about 0.5% of the total radiation is trapped as chemical energy during the period of maximum yield. This approximation is based on the estimate that all the dry weight is carbohydrate or equivalent in reduction. The rapid rate of division obtained at the beginning of the cultures in each of the experiments, if maintained or even approached for the entire time of active growth, would greatly increase this yield. The increase in the growth rate after the addition of the micronutrients in Culture Four to near one with a cell concentration of 15 x 10^6 cells per cc. establishes a much greater capability for division at higher concentrations than had been suspected. The sudden drop-off in the rate curve after attaining a peak would be replaced by a more gradual slope with the proper concentration of micronutrients from the beginning. It cannot be established at this time what relation

the micronutrients bear to the rate of growth and photosynthesis at high light intensities experienced during the early part of each culture when the cell concentration is low. Whether the remarkable upsweep in growth on the fourth day is all that can be expected or whether it too can be accelerated must be determined in future work.

A major concern prior to the study was the deleterious effect which might be expected from contaminants. Serious reduction in growth had been reported in earlier investigations and cultures are often discarded if contamination is suspected. The cultures were carefully observed, therefore, to determine what contamination occurred and what effect it might have. Contamination by fungi was very slight, being evident in only a few of the early plates taken for the bacterial count. The bacterial count as shown in table XII is fairly high but shows certain possibilities for control by maintaining the proper trace element background.*

The bacteria that did appear in the media were primarily of a saprophytic variety that existed on material from dead cells or perhaps on compounds secreted from the algae. Though no final

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*In tentative studies aimed at finding other compounds capable of limiting the growth of bacteria without injuring the algae p-aminobenzenesulfonylamide was tested in concentrations from .0001 M to .02 M. Growth of the algae was halted completely at all concentrations. The cells were killed at concentrations above .004 M but were still green and somewhat enlarged in concentrations at and below .004 M -- though there was no multiplication. The controls grew rapidly. It was obvious that this compound could not be used as a selective bacteriacide or bacteriastatic. However, the inhibition of the growth of the green plant by an analogue of para-amino-benzoic acid, which has been demonstrated as specific for this growth factor (Stephenson (88), was of especial interest. It strongly suggests the presence of para-amino-benzoic acid or folic acid metabolism in the green plant -- an identification of a vitamin not previously recorded.

statement can be made, the recorded growth of the algae strongly suggests that bacterial contamination may not be a major deterent to this type of system.

Of greater concern might well be infestation by other species of algae. No algal contamination was observed until late in the growth of several cultures -- after the majority of the analyses were recorded. In all cases the incidence of contaminant was very slight, at most 1 to 1000. There was no doubt that the characteristics recorded were those of a Scenedesmus culture. In all cases but one these contaminants were diatoms. In a single instance, the last three days of Culture Five, Chlamydomonas cells were observed. Animal contaminants were limited to one infusorian which occurred to a limited degree at the end of Culture Three.

There is a distinct possibility that a system may be discovered which would allow for the selective growth of the algae or even possibly a species of algae in open system. Either a selective chemical background or a rapid removal of the algae at an optimum concentration seems promising to achieve this end. The centrifuge system can be expected to remove the cell walls discarded upon liberation of the autospores which are aplanosporic in formation. These empty walls are observed in the sediment and are the major source of free organic matter in the media.

In order to allow for maximum efficiency, it would be necessary to determine rate of return of elements in the solution to compensate for those removed. All other environmental factors can be kept at a steady state with little difficulty. Scott (81), Myers (57), and others have achieved this end by removing both the medium and the cells

regularly and adding enough fresh nutrient solution to replace the loss. This method uses considerable amounts of water but is excellent for many studies. It is recognized that scarcely enough data exist to calculate a master formula for replacement of nutrients. However, the following values have been calculated for the mineral analyses presented and give the replacement in m.e./L. necessary for the production of a concentration of 100 X 10^6 cells per cc. starting with a minor inoculation.*

Potassium -- .05 m.e./L.
Calcium -- .00 m.e./L.
Magnesium -- .05 m.e./L.
Nitrate -- 1.05 m.e./L.
Phosphate -- .08 m.e./L.
Sulphate -- .10 m.e./L.
Chloride -- .00 m.e./L.

These requirements will vary with conditions of culture. By way of example the nitrogen removal has been plotted against both time and cell concentration for both Culture Three and Culture Four in figure 8. Culture Three with more minor elements available showed a more rapid rate of growth but absorbed less nitrogen per unit cell. Culture Four, however, absorbed less nitrogen per unit time but absorbed far more nitrogen per unit cell produced. The effect was as though the efficiency of one or more of the minor trace elements increased the cell's ability to absorb nitrogen but reduced the rate of growth. Investigation of this phenomenon should prove fruitful.

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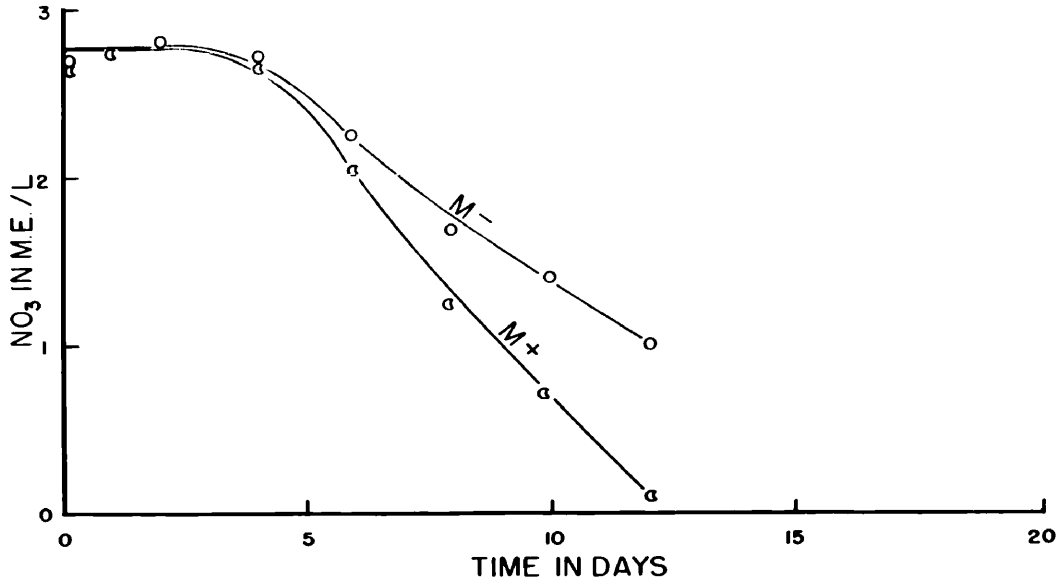
*The determination for PO_4 and Ca was made from extrapolating the curve toward the ordinate. This gives a more accurate picture of the rate of removal at the pH normal to the solution.

Fig. 8. The rate of removal of nitrate from the nutrient media during Cultures Three and Four, showing the effect of micronutrient deficiency.

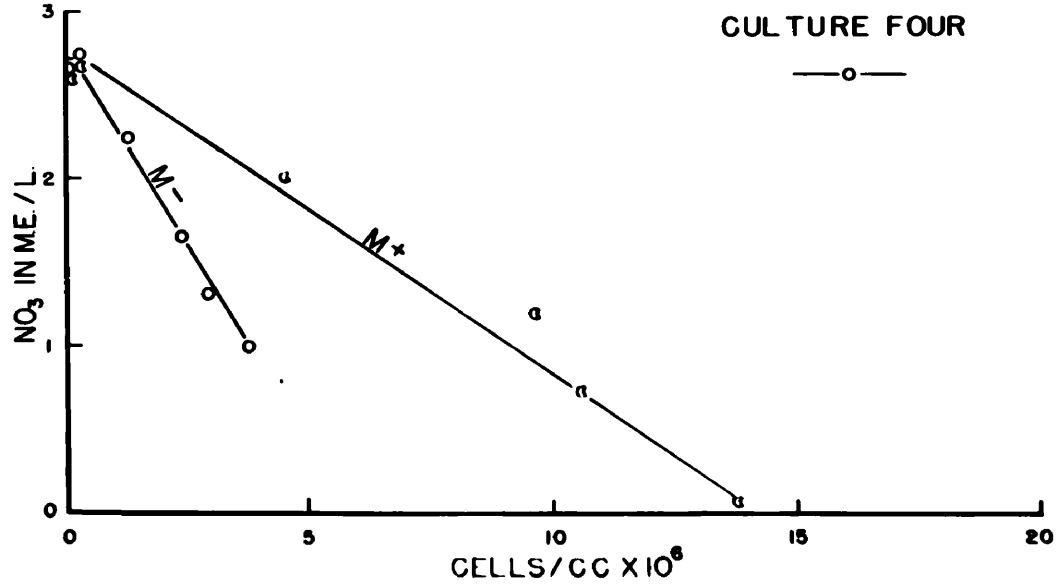


CULTURE THREE

—a—

CULTURE FOUR

—o—



INFLUENCE OF MICRONUTRIENTS ON NITROGEN UPTAKE

SUMMARY AND CONCLUSIONS

1. Scenedesmus obliquus (Turp.) Kützz. was maintained in mass culture in an open system in a controlled environment chamber providing light intensity of 2000 foot candles, near the maximum for prolonged artificial light, by utilizing a combination of incandescent and fluorescent lamps.

2. Growth was followed by means of cell counts, light transmission, and dry weight determinations. Both physical and chemical environmental factors were adjusted in each subsequent culture to produce higher yields.

3. Analyses of culture media showed the progressive removal of each of the major ions. Nitrate was removed from the media at a rapid rate in contrast to the other ions. Evidence suggests that there may be a luxury consumption of nitrogen when available in sufficient quantities. A tentative formula is presented for replacement of ions during normal growth.

4. Micronutrient deficiencies act to reduce growth but accelerate nitrogen uptake per cell. Correction of the deficiencies is immediately reflected by rapid increase in the growth rate.

5. A continuous carbon dioxide supply is shown to provide a bicarbonate buffer system by replacing the nitrate anions removed, thereby maintaining an electrostatically balanced system. In the absence of carbon dioxide the cells rapidly remove most of the available bicarbonate causing a rise in p^H to inhibiting levels.

6. The growth of the bacterial population was followed and methods for reduction of the bacterial count are discussed. Efficient growth

of algae in an open system is deemed possible.

7. Evidence is presented for the existence of the para-amino-benzoic acid -- folic acid vitamin system in green plants.

8. A centrifuge system for maintaining the concentration of cells at a level for maximum yield by continual harvest is introduced. Such a system should provide for maximum efficiency as optimum environmental conditions are approached.

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