THE GROWTH OF *CHLORELLA PYRENOIDOSA* UNDER VARIOUS CULTURE CONDITIONS[1]

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(WITH FIVE FIGURES)

The unicellular algae are being used with increasing frequency in studies on the nature of the photosynthetic mechanism. These algae are excellent experimental material. Their measurable gas exchange is not subject to certain of the complexities of higher plants, *e.g.*, stomatal behavior. They can be used in thick suspensions to obtain almost total light absorption or in such thin suspensions that the variation in light intensity within the sample becomes negligible. They are adapted to the comparatively simple and accurate manometric measurement of gas exchange. Furthermore, effects of individual variation among samples from any one batch of cells are minimized since any one sample usually contains billions of individuals.

The procedure of growing algae introduced by WARBURG (**16**) and described in detail by GAFFRON (**4**) has been commonly followed. Certain strains of the alga Chlorella have been most widely used. In one sense the choice of this form is not particularly fortunate since it reproduces by formation of a variable number of autospores within the parent cell. This means that Chlorella cells may be subject to wider variation in size than might be expected of a form such as Stichococcus which multiplies by binary fission. Cultures are commonly grown in a mineral salt solution in 250- to 500-ml. flasks, with a current of air fortified with carbon dioxide ($\sim$ 3 to 5 per cent.) bubbled through, at a temperature in the range of 20 to 25° C., and illuminated by a tungsten filament lamp giving an incident intensity in the range of 50 to 500 foot candles. This technique has proved so convenient and reliable that no comprehensive study of growth conditions has been undertaken. A few workers have chosen other algae or followed other procedures of culture, but the study of growth conditions or comparison between various algae has been limited.

In one sense the standardization of procedure in culture is fortunate since comparisons between data of different laboratories are facilitated. But by the same token the photosynthetic mechanism has been given an appearance of stability which may not be warranted. It would seem that a point has been reached at which the relations between culture conditions and the development of the photosynthetic mechanism can profitably be explored. It is the thesis of this and succeeding papers of the series that one approach to the photosynthesis problem lies in a study of photosynthetic behavior in relation to conditions of culture. A step in this direction has been taken by SARGENT (**12**) in studying certain effects of light intensity on photosynthetic behavior.

[1] This is the first of a series of papers on: Culture conditions and the development of photosynthetic mechanism.

Experimental procedure

Considered in this paper are certain features of the growth curve for *Chlorella pyrenoidosa*. The strain used was obtained from DR. ROBERT EMERSON and its photosynthetic characteristics have been described by him (**3**). The Knop's solution used as the basic medium contained 0.010 M $MgSO_4$, 0.012 M KNO_3, 0.009 M KH_2PO_4, 0.003 M $Ca(NO_3)_2$, 0.00001 M $Fe_2(SO_4)_3$. In later experiments the calcium nitrate was omitted and the microelements boron, copper, manganese, molybdenum, and zinc were provided by the addition of the "A5" solution of ARNON (**1**). Unless otherwise stated, the cultures were grown in 100 ml. of medium in 250-ml. Pyrex Erlenmeyer flasks with air or mixtures of carbon dioxide and air bubbled through. Air-carbon dioxide mixtures were obtained compressed in cylinders and their composition checked by analysis with a Haldane apparatus. Before entering a culture flask the gas was first humidified by bubbling through a flask of Knop's solution held in the same bath.

The culture flasks were immersed in a glass-bottomed water bath thermostated at 24° ± 0.1° C., illuminated from below by a circle of six 100-watt tungsten-filament bulbs, and continuously agitated by a mechanical drive to the holding rack. This is the apparatus described by MEIER (**7**). Light intensities were measured by a Weston photronic cell contained in a 250-ml. Erlenmeyer flask so that it could be inserted in place of any one of the culture flasks. The cell and microameter used were calibrated against a Model 603 Weston Foot-Candle Meter.

Cultures were inoculated from suspensions grown in cotton-stoppered flasks for one to two weeks in a north window. Sterile precautions were maintained up to the beginning of each growth experiment. The nature of the apparatus and daily withdrawal of samples precluded continued observance of sterile precautions. Some bacteria do develop in such cultures and, though the amount of their protoplasm compared to that of the algae is negligibly small, the possibility of effects on growth must be admitted. Growth was estimated by daily hemocytometer counts, at least 450 cells being counted in each of two drops. The statistical errors involved in hemocytometer counts have been discussed by STUDENT (**13**) and by PEARSALL and LOOSE (**9**). An experimental check made by counting about 10,000 cells of a single suspension has shown that in our hands the standard deviation of the mean for thousand-cell counts is less than 5 per cent. but somewhat greater than the 3 per cent. predicted from theory on the basis of errors of random sampling. pH of the medium was determined by the Leeds and Northrup glass electrode. Nitrate concentration of the medium was determined in several experiments by alkaline reduction and Nesslerization according to YOE (**17**) after centrifuging or filtering off the cells on an asbestos mat.

Cultures were grown in duplicate. Except for occasional unexplained discrepancies, counts of duplicate cultures made at the same time agreed within about 5 per cent. In the growth curves to be presented each point

represents the average of the cell counts on duplicate cultures. An exception is figure 3 in which counts from each of duplicate cultures are plotted.

Results

Growth studies

Illustrated in figure 1 are the data of one of our earlier experiments in comparison of two light intensities. The usual Knop's solution was used and 3.5 per cent. carbon dioxide in air bubbled through the flasks. The density of population has been plotted on a logarithmic scale in order to emphasize the comparatively long period of logarithmic growth. Such a feature is a distinct advantage in culturing cells for physiological study

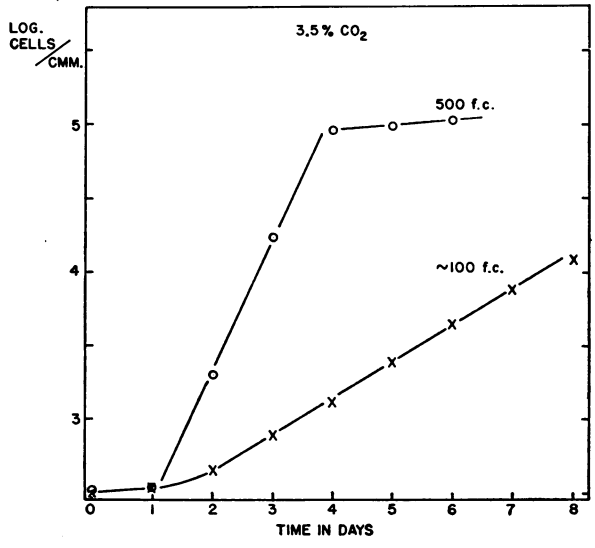

Fig. 1. Growth of *Chlorella pyrenoidosa* at two light intensities.

since the "cross-sectional age" during this period will be constant. As might be expected, the rate of growth during the logarithmic phase is smaller under lower light intensity.

It has seemed best to first concentrate on the later features of the growth curve since those conditions which limit growth might do so through their effects on photosynthesis. Theoretically, any one of four conditions might cause the falling off or limitation of growth for algal cells cultured in this way: (1), the production of some toxic substance by the cells; (2), a limited rate of provision of carbon dioxide; (3), a change or deficiency of some component of the mineral salt solution; or (4), an actual energy limitation. The production in algal cultures of a toxic substance which inhibits growth and reproduction has been demonstrated by Pratt and Fong (**11**). No attempt has been made here to study such substances; but consideration of the data to follow makes it seem unlikely that inhibitors can be the principal factor causing limitation of the various growth curves obtained.

Changes in the media

The possible contributions of changes and deficiencies within the media have been considered. Fresh stock solutions were made up in pyrex containers from a new batch of salts. The calcium nitrate was omitted from the Knop's solution since it was found that at the higher pH values which always are found during the later stages of growth a precipitate, apparently of calcium phosphate, develops. It has been shown that growth is at least equally as good when calcium is omitted (**5, 10, 14**). With the particular batches of salts used a pronounced effect of microelements could be demon-

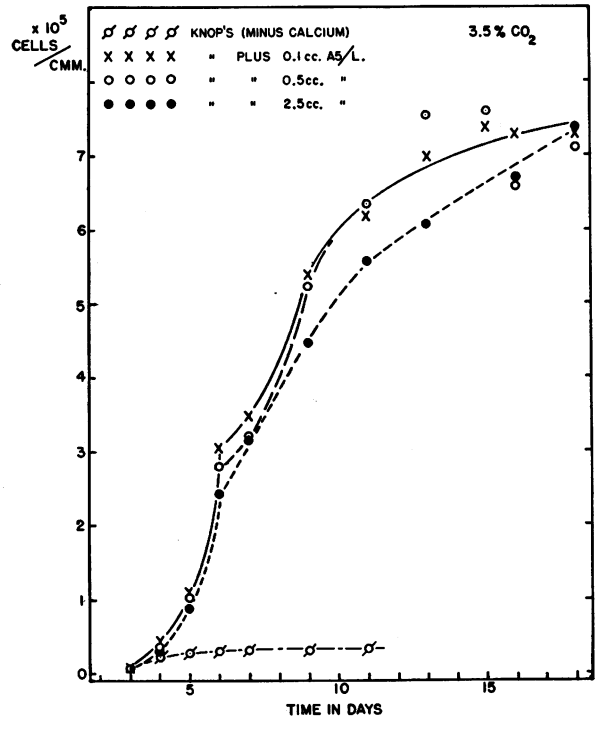

FIG. 2. The effects of varying concentration of microelements on growth using the A5 solution of ARNON (1). Light intensity, 500 fc. Culture medium: Knop's solution (minus calcium) with added A5 as indicated.

strated, even though the major salts were subjected to no special purification. Figure 2 illustrates an experiment in which parallel cultures received varying amounts of the A5 solution. There is a pronounced limitation of cell number in the absence of added microelements. The upper curves show the effect of various increments of the A5 solution. Populations of 700,000 cells/cmm. were attained. Fortunately, there seems to be a rather wide range of tolerance to the A5 solution. For further work a supplement of 0.2 ml./liter was used. The particular microelements required under our conditions have not been investigated. Further effects of other micro-ele-

ments are possible but seem improbable in view of the lack of any special purification of the major salts.

An apparent inflection in the curves of figure 2 has been drawn in since it appeared in all these and later curves obtained under similar conditions. Its meaning is not yet clear. Perhaps it occurs only because of our use of cell number as an estimate of growth. Any sudden increase in cell size might cause exactly such an effect.

Measurements of pH made with the glass electrode indicated marked changes during the growth cycle. This point has been investigated in more detail for cultures grown in duplicate in air and in 3.5 per cent. carbon dioxide under 500 fc intensity. The results are indicated in figure 3. In

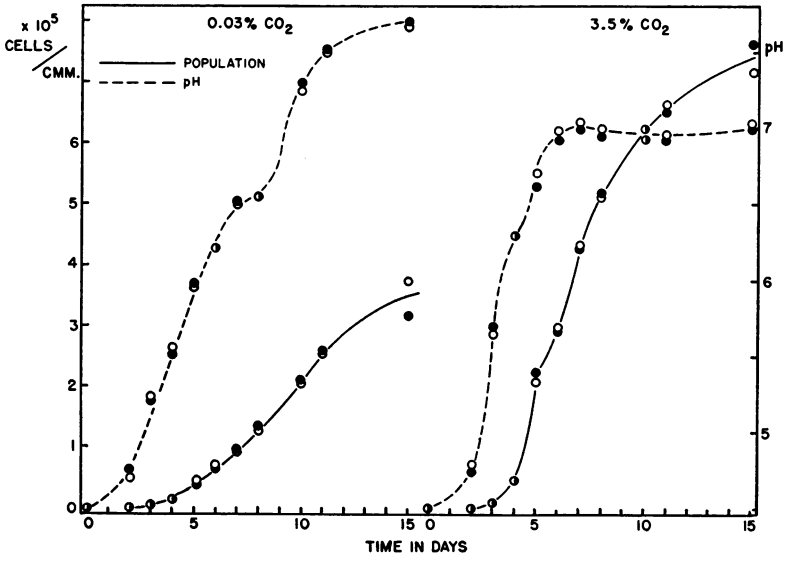

FIG. 3. Changes in pH of the medium accompanying the growth curve. For 0.03 and 3.5 per cent. carbon dioxide. Culture medium: Knop's (minus calcium) plus 0.2 ml./l. of A5 solution. Light intensity, 500 fc. Solid and open circles represent data of duplicate cultures.

air the increase in cell number falls off at about 350,000 cells/cmm. while the pH follows a course which suggests the titration curve of potassium acid phosphate. In high carbon dioxide the cell number attains a much higher limit of about 700,000 cells/cmm., but the pH becomes stabilized at about 7.0.

The difference in final pH between cultures grown in air and those grown in 3.5 or 5 per cent. carbon dioxide is due principally to the higher carbon dioxide concentration in the latter case. The pH was determined so rapidly that the sample may be considered as in equilibrium with the gas mixture bubbled through. Carbon dioxide is not appreciably ionized at pH 4.5; but it is 50 per cent. ionized at a pH of about 6.5 and about 75 per cent. ionized at pH 7.0. In fresh Knop's solution (pH $\sim$ 4.5) the pH is not appreciably influenced by the carbon dioxide concentration of the air bubbled through;

but in the region of pH 7.0 the pH is noticeably depressed by concentrations of carbon dioxide of 3.5 to 5 per cent.

The absorption of nitrogen from culture media has frequently been observed to be associated with changes of pH. The assimilation of nitrate from Knop's solution has been studied by making analyses of the culture media. In the usual Knop's solution KNO_3 is 0.0125 M. Growth curves have been followed under 500 fc illumination using this initial concentration of nitrogen (N/1), one-third the usual concentration (N/3), and three times the usual concentration (3 N). The initial concentrations of all other components of the Knop's solution were the same throughout. Parallel series have been obtained for air and for 5 per cent. carbon dioxide and the data are presented in figure 4.

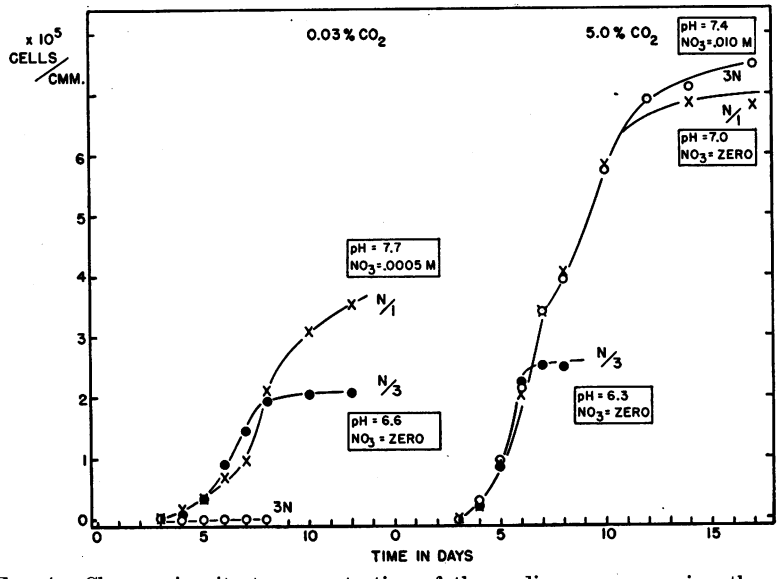

FIG. 4. Changes in nitrate concentration of the medium accompanying the growth curve. For 0.03 per cent. (left) and 5.0 per cent. (right) carbon dioxide. Light intensity, 500 fc. Knop's solution (minus calcium) plus 0.2 ml./l. A5 and with three different initial nitrate concentrations: N/1, 0.0125 M; N/3, 0.0042 M; 3 N, 0.0375 M.

In the high carbon dioxide, with one-third the usual amount of nitrate, the increase in cell number is soon brought to a halt. Analyses made at the last point shown failed to detect any nitrate (or NH_4^+) in the nutrient medium; and for the last three days the pH remained constant at 6.3. Even with the usual amount of nitrate (N/1) there is again no detectable nitrate left in the medium at the last point studied on the curve. With three times the usual nitrate concentration (3 N) growth continues to a somewhat higher level, the pH rises as high as 7.4, and about 1/4 of the initial nitrate is still present in the medium at the last point studied.

In air also, nitrate deficiency clearly limits the growth when the initial nitrate concentration is low (N/3). For the usual Knop's solution (N/1)

the nitrate is reduced to 1/25 of the original concentration. With three times the usual nitrate concentration (3 N) no appreciable growth took place. On the seventh day 5 per cent. carbon dioxide was substituted for air and a vigorous growth followed. Such behavior is interesting but entirely unexpected. This point will be checked in future work; at present confirmatory data are lacking.

It is clear from the data of figure 4 that nitrate absorption is rapid and that a limitation of this ion may be at least partially responsible for the falling off of the growth rate even for the nitrate concentrations commonly used. A similar conclusion may be drawn from the results of VAN HILLE (**15**) although his algae were cultured under quite different conditions.

CARBON DIOXIDE PROVISION

Study of the effects of carbon dioxide on the growth curve has been attempted. Table I presents the data of one experiment carried out under

TABLE I

EFFECT OF MODE OF PROVISION OF CARBON DIOXIDE ON GROWTH IN PARALLEL CULTURES. GROWTH IS EXPRESSED IN TERMS OF CELLS PER CMM. LIGHT INTENSITY = 100 FC

DAY	CULT. NO. 57, 3.5% CO_2	CULT. NO. 59, AIR	CULT. NO. 61, COTTON PLUG
0	300	300	300
15	132,000	47,000	.....
23	254,000	127,000	39,000
34	472,000	453,000	.....
37	.....	.....	63,000

a light intensity of 100 fc. In parallel cultures carbon dioxide was provided by aeration with 3.5 per cent. carbon dioxide, aeration with air, and diffusion through a cotton stopper from room air. The culture obtaining its carbon dioxide by diffusion through the cotton plug was clearly limited in its rate of growth. A still greater relative limitation might be expected at higher light intensities.

Unfortunately, the interpretation of the effects of carbon dioxide provision is complex. The only carbon dioxide pressure of significance is that existing within the liquid medium and available to the cells. During photosynthesis this carbon dioxide pressure at the surface of the algal cell can never be as great as that in equilibrium with the gas phase since a diffusion gradient must always exist. There is no simple means of determining the carbon dioxide pressure within the liquid media; but it can be made to approach that of the gas mixture by rapid aeration, agitation, and a large liquid-gas interface. The data of table I indicate much more rapid growth with 3.5 per cent. carbon dioxide aeration than in the cotton-stoppered flask. Yet it is conceivable that 3.5 per cent. carbon dioxide could be bubbled through so slowly that rate of provision of carbon dioxide would be slower even than that afforded by diffusion from air through a cotton stopper.

It is clear that the carbon dioxide pressure within the medium must be a complex function of the partial pressure in the gas phase, the area of the liquid-gas interface, the rate of aeration, and the rate of uptake of carbon dioxide by the algae. Even in any one culture in which the first three of the above factors are held constant, the multiplication of the algae must result in increasing rates of uptake of carbon dioxide, thus continuously decreasing the pressure of carbon dioxide within the media. The effective carbon dioxide concentration must fall off continuously during the growth curve.

With high concentrations (3 to 5 per cent.) of carbon dioxide in the gas phase (and adequate rate of aeration) it seems likely that the concentration in the liquid media will never fall below the level of carbon dioxide saturation of photosynthesis (~0.1 per cent.). With low carbon dioxide concen-

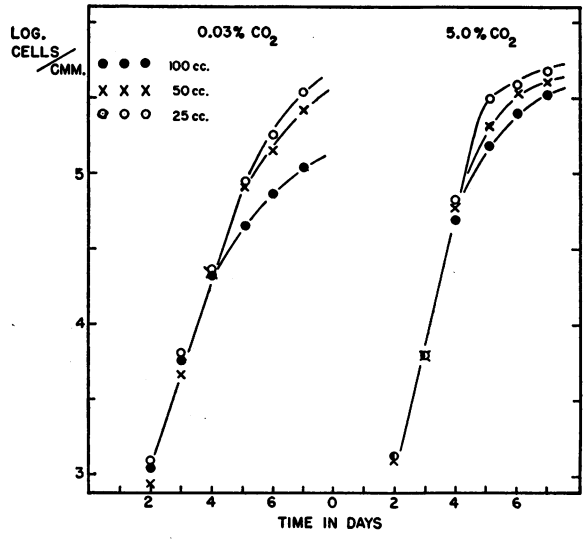

FIG. 5. The effect of thickness of the layer of suspension on growth. The volumes 100, 50, and 25 in 250-cc. Erlenmeyer flasks correspond approximately to thicknesses of 2.0, 1.0, and 0.5 cm., respectively. Light intensity, 500 fc.

trations in the gas phase (~0.03 per cent. as in air) it may be quite possible that a limitation of growth due to *rate of provision* can develop. Such a limitation could be expected to give rise to a growth curve with linear slope, since rate of growth would depend upon a factor provided at a constant rate. This is exactly what happened in culture no. 61 of table I in which rate of provision of carbon dioxide was limited by diffusion through a cotton plug. Similar phenomena of linear growth have appeared in other experiments to be reported in subsequent papers. When air of low carbon dioxide concentration (~0.03 per cent.) is rapidly (~5 ml./sec.) bubbled through a culture, the resulting conditions in regard to carbon dioxide probably vary significantly during the growth curve. An adequate *rate of provision* seems possible, at least during the early part of the growth curve; note the occurrence of logarithmic growth in figure 5. Calculations based on estimated

rates of photosynthesis per cell, however, suggest that *rate of provision* of carbon dioxide may become limiting during the latter part of the growth curve.

Changes in rate of energy absorption

The fraction of the incident light absorbed by a culture increases continually during the growth curve until 100 per cent. absorption is approached. Likewise, the illumination of the "average" cell of the culture continuously decreases as the increasing number of cells mutually shade each other. With high incident intensities considerable growth can take place before a significant number of cells are so shaded as to receive less than a light-saturating intensity. During this period no light limitation can occur. However, as soon as the mutual shading of cells reduces the intensity on a significant fraction of the cells to a light-limiting value for photosynthesis, then rate of photosynthesis and hence rate of growth must fall off. Theoretically such a falling off in growth rate due to a light limitation should occur at greater densities of population in those cultures which have the thinnest layers of suspension. This point has been investigated in the experiment presented in figure 5. A batch of Knop's solution was inoculated to give a density of population of 300 cells/cmm. Aliquots of 100, 50, and 25 ml. of this suspension were placed in three flasks and illuminated side by side in the same bath. The depths or thicknesses of these suspensions were about 2.0, 1.0, and 0.5 cm., respectively. The series with air bubbled through were made up in the usual Knop's solution (nitrate = 0.0125 M). The series in 5 per cent. carbon dioxide were made up in Knop's solution with three times the usual nitrate concentration. There is some degree of uncertainty in drawing the curves. But in any case it is clear that, as predicted on theoretical grounds, the rate of growth falls off more quickly in thicker suspensions. (In the experiments in air the effects of thickness of suspension may relate to both light and carbon dioxide provision.) By limiting the thickness of suspension to 1.0 cm. or less (50 ml. in a 250-ml. Erlenmeyer flask) it has been possible to attain populations well over one million cells per cubic millimeter.

In some cases it is possible to demonstrate a period of logarithmic growth under low light intensities [fig. 1 and Bristol-Roach (**2**)]. This can mean only that here the increase of mutual shading is not rapid enough to be significant during the logarithmic growth period.

Discussion

The data of this paper are regarded only as exploratory. No attempt has yet been made to relate the described culture conditions to photosynthetic behavior. Numerous improvements in the physical apparatus for culturing algae have since been made and more detailed study of various conditions will be reported in subsequent papers.

Any study of the relations between culture conditions and the development of the photosynthetic mechanism is beset with a number of difficulties.

For the most part these arise from the numerous changes in conditions which accompany the growth curve. As the population of a culture increases, the effective light intensity, carbon dioxide concentration, and nitrate concentration are diminished; simultaneously the pH rises. Other variables may also be expected, *e.g.,* the spectral distribution of the effective light (**12**) and the availability of iron as affected by pH (**5**). Furthermore, the way in which all of these variables relate to each other and to the population may differ for different culture conditions.

For instance, the writer is particularly interested in the effect of carbon dioxide concentration on the development of the photosynthetic mechanism. It has been shown (**6, 8**) that cells from a culture grown under high carbon dioxide (5 per cent.) show certain differences in photosynthetic behavior from those of a culture grown under low concentration (air) of this gas. But the experiments described above make clear that we cannot simply relate photosynthetic behavior to carbon dioxide pressure since other conditions (*e.g.,* pH) may differ between the cultures and change in any one culture with time.

Summary

1. The growth of *Chlorella pyrenoidosa* has been studied under various culture conditions by means of hemocytometer counts.

2. During the growth of a culture at least two important changes occur in the nutrient media: the nitrate and hydrogen ion concentrations are continually diminished.

3. It has been shown on theoretical grounds and confirmed experimentally that rate of provision of carbon dioxide may limit growth in such a way as to give rise to a linear rate of growth.

4. Higher populations and longer periods of logarithmic growth may be attained by exposing thinner layers of the algal suspension to the incident illumination.

5. The changes in growth conditions within any one culture are manifold and hence it will be difficult to relate any one of these conditions to effects on the photosynthetic mechanism.

The experimental work of this paper was done in the Division of Radiation and Organisms of the Smithsonian Institution when the author was a National Research Council Fellow. It is a pleasure to acknowledge indebtedness to DR. C. G. ABBOT, DR. E. S. JOHNSTON, and DR. E. D. MCALISTER for their interest in and support of this work.

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