

AN EXPERIMENTAL EVALUATION OF THE C¹⁴ METHOD FOR MEASURING PHYTOPLANKTON PRODUCTION, USING CULTURES OF *DUNALIELLA PRIMOLECTA* BUTCHER¹

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ABSTRACT

The photosynthetic uptake of radioactive carbon dioxide was compared with net and gross O₂ production, pH changes, and growth in cultures of *Dunaliella primolecta* Butcher. During the logarithmic growth phase of such cultures, C¹⁴ uptake agreed well with net O₂ production and did not differ greatly from gross O₂ production. The logarithmic growth rate of mass cultures (measured by increases in cell concentration, optical density, dry weight, organic carbon and nitrogen) was generally less than a similar logarithmic rate calculated from organic carbon analyses and measurements of photosynthesis in bottles immersed in the culture. This difference was attributed to greater illuminances in the bottles than in the cultures as a whole. In one experiment, when illumination conditions were similar, the two rates were equivalent.

It is commonly accepted that in the open sea the production of all forms of animal life is ultimately dependent upon the production of organic material by phytoplankton. The phytoplankton produce organic material in excess of their metabolic needs, and this excess or "net production" is then available to other forms of life. This excess consists mainly of protein, carbohydrate, and lipid fractions. This material forms the bulk of the food available for incorporation into the cells and tissues of the zooplankton and larval fishes (secondary producers), and also provides an energy source for their activities. Ideally, to study the dynamic aspects of the transfer of food from phytoplankton to secondary producers, it would be desirable to measure changes in the concentra-

When cultures were incipiently nitrogen-deficient, C¹⁴ uptake was similar to net O₂ production and growth was equivalent to photosynthesis. In one experiment, when nitrogen deficiency was extreme, C¹⁴ uptake was larger than net O₂ production, and agreed more closely with gross O₂ production. In another such experiment, C¹⁴ uptake agreed with net O₂ production. Nitrogen deficiency increased the intracellular C/N ratio, and starch became a major storage product. In one experiment, C¹⁴ uptake in an incipiently phosphorus-deficient culture was intermediate between net and gross O₂ evolution; in a similar experiment, C¹⁴ uptake was less than net O₂ production. C¹⁴ uptake in healthy cultures was equivalent to the uptake of CO₂ as measured by pH changes. These results are discussed in relation to other findings and to oceanographic applications.

tions of protein, carbohydrate, and lipid in the sea, or at least, to measure changes in total phytoplankton biomass. Practically, such measurements are very difficult, because present methods are either quite time-consuming or are too insensitive to measure the small changes that occur in the sea.

Since the three major components of phytoplankton all contain carbon, the development of the C¹⁴ method for measuring the production of organic carbon (Steemann Nielsen, 1952), ap-

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peared to provide a sensitive and easy way of measuring changes in the food supply, if the C^{14} method really measures the excess or net production. Intensive investigations of the spatial and temporal variations of C^{14} assimilation that are now in progress should therefore contribute to our understanding of food production in the oceans, and may provide information of practical importance to fishermen. The quantitative determination of phytoplankton production in the sea may also allow a more precise assessment of the effects of various factors on production and of the manner in which it is controlled. Thus, it is rather important that this method be subjected to a detailed evaluation.

To determine phytoplankton photosynthesis with C^{14} , a sample of water containing phytoplankton is placed in a clear, glass-stoppered bottle to which a standard amount of $Na_2C^{14}O_3$ is added as a tracer. The sample is then incubated for a standard length of time either in situ, under known conditions of illumination and temperature, or under conditions which simulate field conditions. Following incubation, the algae are collected on a membrane filter and dried carefully. Their assimilated C^{14} is then counted. The amount of carbon assimilated during the photosynthetic period is calculated from the measured assimilation of tracer carbon, the amount of tracer added, and the total CO_2 content of the water (cf. formula on page 12). The methodology of using C^{14} has been discussed by Steemann Nielsen (1952), Ryther (1956), and Doty and Oguri (1958); and has been reviewed in great detail by Strickland (1960). Thomas (1963) reviewed the various physiological factors which affect the measurement.

So far, the C^{14} method has been tested mainly by comparison of C^{14} uptake with O_2 evolution (Ryther, 1954; Ryther and Vaccaro, 1954; Ryther, 1956; Steemann Nielsen and Al Kholy, 1956; Ichimura and Saijo, 1958). One comparison of the C^{14} method with increases in algal biomass (growth) has been made (McAllister, Parsons, Stephens, and Strickland, 1961). This comparison utilized a natural population enclosed in a plastic bag.

Growth is a net process; for the biomass of algae to increase, material which is produced must be conserved in excess of respiratory requirements. Herbivores utilize the algal material directly for

food. Algal growth measurements provide a direct assay of the increase in available food. Thus, ideally, the C^{14} method should be compared with growth.

The present paper reports comparisons of C^{14} measurements with increases of phytoplankton solids and of organic carbon in rapidly growing cultures of *Dunaliella primolecta* Butcher and also in incipiently nitrogen- and phosphorus-deficient cultures. Further comparisons of O_2 evolution and C^{14} uptake under varying conditions of intracellular nutrient status are presented. CO_2 assimilation by healthy cultures, as measured by pH changes, is also compared with C^{14} uptake.

MATERIALS AND METHODS ²

THE ALGA

The organism used in these studies was *D. primolecta* Butcher, which was obtained from Beatrice Sweeney of the Scripps Institution of Oceanography. According to Luigi Provasoli (personal communication) and to Butcher (1959), this alga was originally isolated by Gross, and is now number 81 in the Culture Collection of the Marine Biological Association at Plymouth, England. According to Robert Guillard (personal communication), *D. euchlora*, which was used by Ryther (1954, 1956), is a separate species.

CULTURE PROCEDURES

Stock cultures were maintained on agar slants made with a medium similar to that of Sweeney and Hastings (1957), which consisted of 75 percent sea water, 0.1 percent Na_2EDTA ,³ 2 percent soil extract, 2,000 μg -at. $KNO_3/1.$, and 200 μg -at. $K_2HPO_4/1.$ Subcultures used in each experiment consisted of 50-ml. liquid cultures of the same medium. Stock cultures and the liquid subcultures were grown routinely at 500 foot-candles (ft.-c.) and 21° C. Culture methods used in the individual experiments are described below.

Growth of cultures in most experiments was measured by cell counting. A 10-ml. aliquot of the suspension was first treated with a few drops of Lugol's iodine solution to kill and stain the cells. Subsamples from this 10-ml. aliquot were then placed in the chambers of a hemocytometer, and the cells were counted until a total of at least

² Reference to a company or product throughout this manuscript does not imply approval by the U.S. Bureau of Commercial Fisheries.

³ Na_2EDTA is the sodium salt of ethylene diamine tetraacetic acid.

200 cells had been counted. The standard error of the mean count of these subsamples ranged from ± 2 to ± 33 percent. The average standard error was ± 13 percent.

In some experiments, growth was also measured by determinations of dry weight and optical density. For dry-weight determinations, aliquots of the cell suspension were filtered through tared HA Millipore® filters (pore size 0.45μ), which were dried at room temperature in a vacuum desiccator. Optical density determinations were made at 600 and 650 $m\mu$ with a Bausch and Lomb Spectronic 20® Spectrophotometer and 1-inch absorption tubes.

Growth is expressed in terms of logarithmic growth rate constants using the formula (Krauss and Thomas, 1954):

$$K_2 = \frac{\log_2 C_2 - \log_2 C_1}{t_2 - t_1}$$

where C_1 and C_2 are cell numbers, dry weights, optical densities, or other growth measurements at times t_1 and t_2 . The reciprocal of K_2 is the time taken for the population to double and is designated the "generation time" in this paper.

CARBON¹⁴ METHODS

The C¹⁴ method used is similar to that described by Steemann Nielsen (1952), Ryther (1956), and others. The radioactive solution was prepared by acidifying BaC¹⁴O₃ in vacuo and absorbing the resulting C¹⁴O₂ in NaOH solution. This solution was then diluted, and the pH was adjusted to pH 9.5–10.0. The diluted solution contained 1 $\mu c./ml.$ and was packaged in ampoules, which were then sterilized in the autoclave.

The solution was standardized by precipitating the C¹⁴ as BaC¹⁴O₃ and filtering portions of the resulting suspension through HA Millipore® filters (pore size 0.45μ). With the standardization procedure described by Steemann Nielsen (1952), it was difficult to obtain reproducible radioactivities at low thickness of precipitate ($<0.5 \text{ mg./cm.}^2$), although thicker preparations were quite reproducible. Therefore, the following modifications were introduced to make thin samples more reproducible: 1) the amount of carrier sodium carbonate suggested by Steemann Nielsen was reduced by one-half; 2) the filters were washed with 0.001 molar BaCl₂ to avoid losses of precipitate; 3) the BaC¹⁴O₃ suspension was stirred with a magnetic

stirrer during precipitation and sampling; and 4) the filter was shaken during filtration to distribute the precipitate uniformly on the surface of the filter.

Self-absorption curves obtained using these modifications are shown in figure 1. For window-

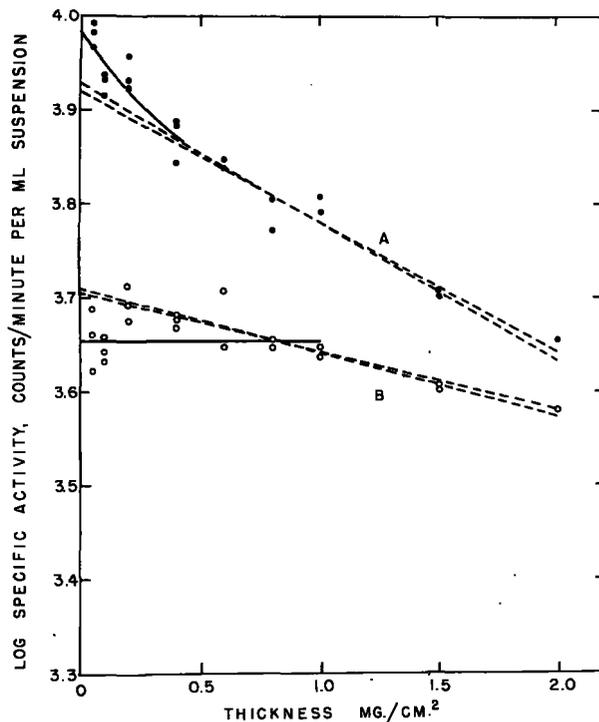


FIGURE 1.—BaC¹⁴O₃ self-absorption curves: (A) Samples counted with a windowless flow chamber; and (B) samples counted with a chamber fitted with a Mylar window of approximately 1 mg./cm.^2 thickness. Broken lines show the extrapolation to zero thickness using thick samples ($\geq 0.4 \text{ mg./cm.}^2$ in curve A; $\geq 0.2 \text{ mg./cm.}^2$ in curve B). Solid lines show the empirical extrapolations that were actually used to determine zero-thickness activity.

less counting, the values were empirically extrapolated to zero thickness (solid line in curve A, fig. 1) and the error of this value was approximately ± 5 percent. Thicknesses of BaC¹⁴O₃ as low as 0.05 mg./cm.^2 were used in the extrapolation. Jitts and Scott (1961) have criticized on various grounds our extrapolation from thicknesses below 0.4 mg./cm.^2 . Though their criticisms may be valid, an empirical extrapolation to zero thickness activity using low thicknesses ($<0.4 \text{ mg./cm.}^2$) results in a value that is about 13 percent higher than that found by using thick samples alone (dashed lines in curve A, fig. 1): Their

liquid scintillation method results in a zero-thickness value that is 17–26 percent higher than that obtained by extrapolation from thick pads. Thus, our empirical method of extrapolation gives zero-thickness activities which do not differ greatly from the liquid scintillation values obtained by Jitts and Scott.

Using a counting chamber fitted with a Mylar window, we found a slight decrease in specific activity (cpm/ml.) as the thickness approached zero (open circles, fig. 1). This decrease was observed in three separate standardizations and may be due to a decrease in "self-scattering" of the beta radiation, as suggested by Steinberg and Udenfriend (1957). Since this effect does not occur when the same pads are counted with a windowless chamber, it is probably related to scattering of those beta particles having energies great enough to allow them to pass the window. Despite the downward trend of specific activity at very low thicknesses, at thicknesses below 1 mg./cm.² these specific activities do not differ significantly from each other. Thus, a mean of specific activities obtained at thicknesses below 1 mg./cm.² was taken to be that added to the experimental bottles. This mean had a standard error of ± 4 percent and is shown by the solid line of curve B, figure 1. It was about 11 percent less than that obtained by extrapolation from thick samples (dashed lines in curve B).

For C¹⁴ measurements of photosynthesis in laboratory cultures, 50-ml. portions of the cell suspension were placed in 67-ml. bottles. In some experiments where the cell suspension was very dense, 10-ml. portions of the suspension were diluted to 50 ml. with sterile sea water. The bottles were then inoculated with 1 ml. of C¹⁴ solution. They were prepared in duplicate and incubated for periods not exceeding 6 hours (generally 1–2 hours). Darkened bottles and illuminated bottles were always incubated together. Bottles were darkened by wrapping them with aluminum foil.

Following incubation, the contents of the bottles were filtered through membrane filters (pore size 0.45 μ), and the filters were washed four times with 10- to 20-ml. portions of nonradioactive sea water. The filters were then dried in a vacuum desiccator at room temperature.

The radioactivity on the filters was measured with a proportional flow counter using either a

windowless chamber or a similar chamber having a Mylar window interposed between the center wire and the filter. Using the windowless chamber, we observed a progressive decrease in sample counting rates. This decrease in the counting rate was avoided by counting the sample for 1 minute, removing it from the chamber, and counting it again at least one-half hour later. This decrease was apparently due to electrostatic charging of the nonconducting filter pads and was a troublesome problem in the dry atmosphere of California. Similar difficulties in counting samples on membrane filters in a windowless chamber were reported by Setter, Hagee, and Straub (1958). The Mylar window completely prevented this charge effect, and reduced the count of a standard C¹⁴ source by only one-half.

The determinations of algal activity were corrected to zero-thickness by means of empirical algal self-absorption curves. Different volumes of suspensions of radioactive *Dunaliella* cells were filtered to obtain varying thicknesses of algae. These samples were then counted, and the specific activity plotted against thickness (fig. 2). With

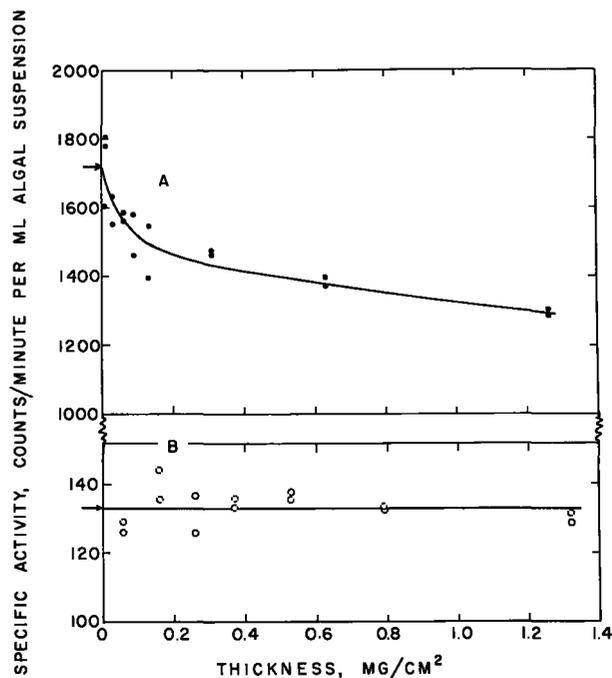


FIGURE 2.—Algal self-absorption curves: (A) Samples counted with a windowless flow chamber; (B) samples counted with a chamber fitted with a Mylar window of approximately 1 mg./cm.² thickness. Solid lines show the empirical extrapolations to zero-thickness activity.

the windowless chamber (curve A), considerable difficulty was experienced in extrapolating from very thin pads (<0.2 mg./cm.²) to zero thickness, and the estimated error of corrections made from curve A is ± 6 percent. With the Mylar window, as is shown in curve B, self-absorption corrections appeared to be unnecessary at thicknesses below 1.3 mg./cm.² The standard deviation of the mean of all values for specific activity was ± 3 percent. In the experiments reported below, algal thicknesses were never this large.

The radioactivity used in calculating production by the C¹⁴ method was the mean value obtained by filtering duplicate light bottles minus the mean value from duplicate darkened bottles. The overall precision of the incubation, filtration, and counting procedure was determined by incubating 10 replicate cultures under uniform conditions of illumination and temperature. The standard error of the mean radioactivity taken up in these bottles was ± 7 percent.

Total CO₂ in the experimental C¹⁴ bottles was measured by the excess acid method of Anderson and Robinson (1946), and the equations and factors given by Harvey (1955). A pH meter having a reproducibility of ± 0.02 pH unit was used for this determination. An error of ± 0.02 units in measuring the final pH after the addition of standard acid results in an error in the total CO₂ concentration of approximately ± 6 percent.

Production was calculated from the following equation:

$$\text{Photosynthesis} = \frac{\text{cpm (light)} - \text{cpm (dark)}}{\text{cpm added}}$$

$$\times 1.06 \times \text{total CO}_2 / \text{time}$$

The factor 1.06 corrected for the isotope discrimination effect (Steemann Nielsen, 1952). No corrections were made for the respiration of labeled carbon during the incubation nor for excretion of labeled organic matter.

The total experimental error of the C¹⁴ method in our hands was estimated by pooling all the various sources of error. This was accomplished by taking the square root of the sum of the squares of each error. For windowless counting, the error in the amount of activity added was ± 5 percent; the error for incubation, filtration, and counting of activity taken up in replicate bottles was ± 7 percent for both light and dark bottles; the error for algal self-absorption was ± 6 percent; and the

error in total CO₂ was ± 6 percent. The pooled total error was thus ± 14 percent. With Mylar window counting, some of these individual errors were decreased and the pooled total error was ± 13 percent. The windowless counter was used in experiments 1, 2, 4, and 5; the counting chamber fitted with the Mylar window was used in experiments 3, 6, 7, 8, and 9.

OXYGEN METHODS

Oxygen production was measured by filling 67-ml. bottles with the cell suspension. The initial amount of oxygen was measured in one pair of bottles, another pair was darkened with aluminum foil, and the third pair was illuminated. Light and dark bottles were incubated under the same conditions as the C¹⁴ bottles for periods not exceeding 6 hours. Dissolved oxygen was measured with the Winkler technique using 0.01 normal sodium thiosulfate for the final titration.

A test of the precision of the technique in our hands was made by filling 22 such bottles, measuring the oxygen content in 6 initial bottles, and incubating 6 dark bottles and 10 light bottles. The determinations and their standard errors were as follows: initial oxygen, 5.35 ± 0.03 ml. O₂/l.; dark bottles, 5.08 ± 0.07 ml. O₂/l.; light bottles, 11.51 ± 0.42 ml. O₂/l. The relative errors of each measurement are small, ± 1 percent for the initial and dark bottles and ± 4 percent for the light bottles. This test showed the relative experimental errors that might be expected when photosynthesis was intense, i.e., using cultures that were still in the logarithmic period of growth and incubating the bottles at high illuminances. Since the amounts of oxygen produced or consumed varied with the conditions prevailing during each experiment, the mean of duplicate bottles was used to determine production and the experimental error of each measurement was determined from the deviation from this mean.

The production of oxygen was converted to carbon production by use of a photosynthetic quotient (O₂/CO₂) of 1.25 as suggested by Ryther (1956). The problem of choosing a correct quotient has been reviewed by Ryther. Under the conditions of our experiments—cells grown with nitrate as an N-source, cells grown at high illuminances, N-deficient cells, etc.—the correct quotient probably lies within ± 12 percent of this value. This error is pooled with the experimental

error for each oxygen measurement. Pooling of these errors was done in the same manner as with the C¹⁴ measurements.

ORGANIC CARBON DETERMINATIONS

In experiments 1 and 2, the method of Kay (1954) was used for determining organic carbon in aliquots of the culture suspension. While the combustion mixture, glassware, and the train were similar to those of Kay, absorption of the resulting CO₂ was carried out in the sidearm of a 125-ml. Erlenmeyer flask containing the Ba(OH)₂ absorbing solution. This modification was suggested by George Bien of the Scripps Institution of Oceanography. Our mean recovery of 0.5–2.0 mg. of carbon in the form of glucose, methionine, or tryptophane by this method was 98 ± 8 percent. Similar amounts of algal carbon were measured.

In experiment 3, a much less complex method (Strickland and Parsons, 1960) was used to determine algal carbon. Aliquots of the culture suspension containing 1–2 mg. of carbon were filtered on fiber glass filter papers covered with 40 mg. of MgCO₃ powder. The filters were then frozen until the analyses could be done. The filters were placed in 30-ml. beakers, and 1 ml. concentrated phosphoric acid was added, followed by 1 ml. of distilled water. The beakers were heated in a boiling water bath for 30 minutes to remove residual chloride ion, and then were cooled. Two ml. distilled water was added, followed by 10 ml. of a dichromate-sulfuric acid oxidizing reagent. After the beakers were heated for an additional 60 minutes and cooled, the solution was decanted into 1-cm. absorption cells. The optical density was measured at 650 m μ against a distilled water blank which was carried through the whole procedure. This procedure differed slightly from that of Strickland and Parsons; instead of measuring the disappearance of dichromate ion (at 440 m μ), the procedure measured the formation of trivalent chromium ion (at 650 m μ). Furthermore, no final dilution was made; thus, a maximum extinction would be measured.

The method was calibrated by analyzing a set of glucose standards with each set of unknown samples. Recovery of tryptophane was 100 percent, but that of methionine was only 50 percent. Thus, some part of the algal carbon may not be completely determined by this method, but this fraction is believed to be quite small (Strickland

and Parsons, 1960). The extinction of the samples was corrected by subtracting a blank value obtained by taking a glass filter with MgCO₃ through the whole procedure.

The correspondence between the two methods for measuring organic carbon was tested by comparing values for the mean carbon per cell as measured by each method during the logarithmic phase of growth. In experiment 2, with Kay's method, the mean carbon per cell was 35.8 ± 6.7 μ g.C/cell; in experiment 3, with the Strickland and Parsons method, it was 41.2 ± 7.9 μ g.C/cell. The difference between these experiments was not statistically significant, and a mean value of 39.0 μ g.C/cell was used in converting cell concentration to organic carbon in experiments in which organic carbon was not measured directly.

ORGANIC NITROGEN DETERMINATIONS

In experiments 3 and 4, algal nitrogen was determined by a micro-Kjeldahl method. The cell sample was digested for four hours with a salicylic acid-sulfuric acid mixture (Ranker, 1927) and a copper sulfate-selenium catalyst. Ammonia in the digest was then distilled into boric acid solution and titrated with 0.01 normal sulfuric acid.

LIGHT MEASUREMENTS

Incident illumination was measured with an International Rectifier Corporation B2M selenium photocell. This cell had a spectral sensitivity of 300 to 760 m μ with a peak at 580 m μ . It was calibrated against a Weston foot-candle meter with the same type of light source (a bank of daylight fluorescent tubes) that was used for illuminating the cultures and photosynthetic bottles.

STATISTICAL METHODS

"Student's" parametric *t* test was used to determine statistically significant differences between means of various measurements (Snedecor, 1956). Where sample numbers were small, the nonparametric Mann-Whitney *U* test was also used (Siegel, 1956).

RESULTS

COMPARISONS OF C¹⁴ UPTAKE, O₂ EVOLUTION, AND GROWTH DURING THE LOGARITHMIC GROWTH PHASE

Experiment 1

In an initial experiment, several 1,500-ml. cultures of *Dunaliella* were grown at 21° C. and

550 ft.-c. in sea water enriched with 500 $\mu\text{g.}$ -at. $\text{NO}_3\text{N/l.}$, 50 $\mu\text{g.}$ -at. $\text{PO}_4\text{-P/l.}$ and 0.25 p.p.m. Fe (as the citrate, Rodhe, 1948). These cultures were not aerated or shaken. When the cultures were visibly green, aliquots were transferred to sterile 67-ml. bottles for C^{14} and O_2 measurements of photosynthesis. Photosynthesis bottles were incubated under the same conditions as the cells were grown. The remainder of the cell suspension was harvested for organic carbon and other growth measurements. The measurements were repeated on 4 successive days. At the end of this period, the pH had risen from 8.0 to 9.2 and growth had ceased.

Table 1 reports corresponding measurements of photosynthesis and increase in organic carbon during this 4-day period. During the first 3 days, C^{14} uptake agreed with net O_2 evolution within the experimental error. This was not the case on the fourth day when C^{14} uptake was probably limited by a decrease in the amount of free CO_2 in solution. The increase in organic carbon was calculated by dividing the difference in organic carbon by the time between samplings. Thus the increase was measured for a longer experimental period than the photosynthetic measurements. This increase was similar to photosynthesis, but was not exactly equivalent to it.

TABLE 1.—Comparative photosynthesis and organic carbon measurements of cultures of *Dunaliella primolecta*

[Experiment 1]

Days	C^{14} uptake	Net O_2 evolution	Gross O_2 evolution	Increase in organic carbon
	$\mu\text{g. C/l.}/\text{hour}$	$\mu\text{g. C/l.}/\text{hour}$	$\mu\text{g. C/l.}/\text{hour}$	$\mu\text{g. C/l.}/\text{hour}$
1.....	18.1 \pm 2.5	23.4 \pm 9.6	24.0 \pm 9.6	
2.....	158 \pm 22	182 \pm 24	212 \pm 26	208.0
3.....	124 \pm 17	125 \pm 15	187 \pm 22	47.9
4.....	36.2 \pm 5.1	72.2 \pm 8.8	107 \pm 20	

Visual observations of these flask cultures showed that they did not all grow to the same extent, i.e., that they were not exactly parallel cultures. Differences between flasks were probably the result of uneven illumination at subsaturating light intensities. In the next experiment a 40-liter mass culture was used instead of several flasks, so that many samples could be taken for an extended period of time from the same culture, and so that CO_2 limitations could be avoided by stirring and aeration.

EXPERIMENTAL EVALUATION OF C^{14} METHOD

Experiment 2

For this experiment, twenty 2-liter cultures, containing sea water enriched as in the previous experiment, were inoculated with *Dunaliella*. After 4 days of growth in the culture room at an illuminance of 550 ft.-c. and a temperature of 21° C., during which the cell concentration increased from 3,000 cells/ml. to 118,000 cells/ml., the cell suspension from all twenty cultures was mixed in a plastic-lined aquarium. The aquarium culture was placed in a water bath thermostated at 26° C. and was illuminated from below at an illuminance of 800 ft.-c. The culture was stirred with a glass stirrer coupled to a motor mounted above the aquarium, and 5 percent CO_2 -in-air was supplied to the culture through a fritted glass dispersion tube at approximately 300 ml./min. The aquarium was covered with plate glass to reduce evaporation from the culture and contamination by laboratory dust.

After incubation for 24 hours, growth and photosynthesis measurements were started. Seven such sets of measurements were made during the following 48 hours. Photosynthesis bottles were incubated on the bottom of the aquarium and within the culture itself. During the 48-hour period, the pH of the culture was 7.06 to 7.30 and the total CO_2 concentration was 40.2 to 49.7 mg. $\text{CO}_2\text{-C/l.}$

Table 2 gives comparative C^{14} uptake and O_2 evolution measurements for this culture. During the first 29 hours of this experiment O_2 evolution (gross and net) generally agreed with C^{14} uptake within experimental error. At zero time, however, C^{14} uptake was somewhat lower than O_2 production. At 35 hours and 48 hours, both net and gross O_2 production were much less than C^{14} uptake. Photosynthesis was probably so intense that the water in the light bottles became supersaturated with O_2 to such an extent that the

TABLE 2.—Comparative photosynthesis measurements in a 40-liter mass culture of *Dunaliella primolecta*

[Experiment 2]

Time	C^{14} uptake	Net O_2 evolution	Gross O_2 evolution
	$\mu\text{g. C/l.}/\text{hour}$	$\mu\text{g. C/l.}/\text{hour}$	$\mu\text{g. C/l.}/\text{hour}$
0.....	558 \pm 78	789 \pm 100	862 \pm 100
5.....	847 \pm 120	1,090 \pm 130	1,200 \pm 150
10.....	1,440 \pm 200	1,500 \pm 190	1,630 \pm 210
24.....	2,160 \pm 300	1,820 \pm 220	1,980 \pm 240
29.....	2,160 \pm 300	1,930 \pm 230	2,170 \pm 260
35.....	3,610 \pm 510	2,600 \pm 240	2,330 \pm 280
48.....	5,200 \pm 730	1,820 \pm 240	2,220 \pm 280

production could not be measured as a change in dissolved O₂.

The growth of this culture was measured by increases in cell concentration, dry weight, and organic carbon, and was plotted on a logarithmic scale (fig. 3). From these data, mean logarithmic

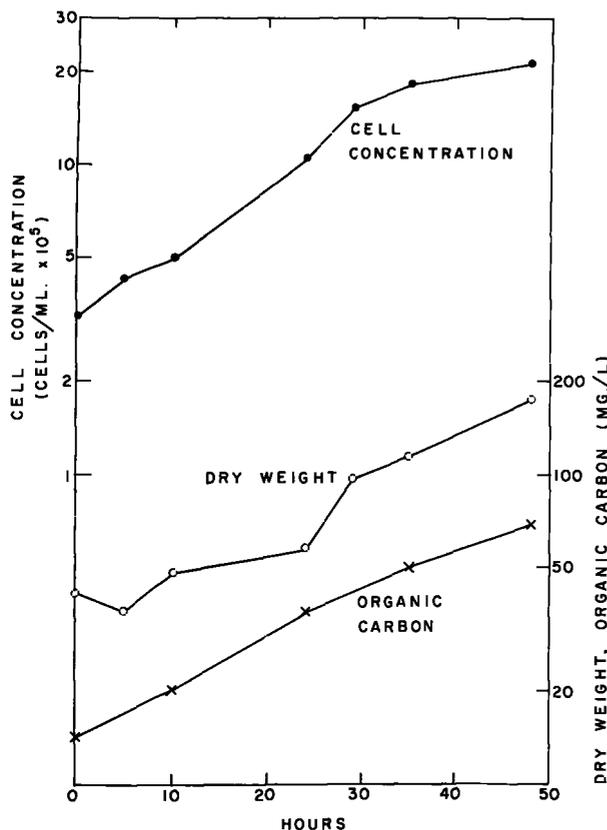


FIGURE 3.—Growth of a mass culture of *Dunaliella primolecta*.

growth constants (K_2) for each type of measurement were calculated by averaging K_2 values obtained between each sampling time.

In experiment 1, linear increases in organic carbon were compared with photosynthesis determinations. Obviously, a more meaningful comparison could be made if photosynthesis measurements were converted to logarithmic K_2 values. In the present experiment, the organic carbon measurements provided an initial concentration (C_1) from which photosynthetic K_2 values could be calculated. A sample calculation of a photosynthetic K_2 value is shown in table 3.

TABLE 3.—Sample calculation of a photosynthetic K_2 value¹

$$\begin{aligned}
 C_1 &= 20.5 \text{ mg. C/l.} && \text{Photosynthesis (C}^{14}\text{)} = 1.4 \text{ mg. C/l./hour} \\
 C_2 &= 20.5 + 1.4 = 21.9 \text{ mg. C/l.} \\
 t_1 &= 0 \\
 t_2 &= 1 \\
 K_2 &= \frac{3.32 \log_{10} 21.9 - 3.32 \log_{10} 20.5}{1} \\
 &= \frac{3.32 (1.34044 - 1.31175)}{1} \\
 &= 0.09525 \text{ hours}^{-1} \\
 \text{Generation time} &= 1/0.09525 = 10.5 \text{ hours}
 \end{aligned}$$

¹ Data at 10-hour sampling time, experiment 2.

TABLE 4.—Mean K_2 values and generation times calculated from growth and photosynthesis measurements in a mass culture of *Dunaliella primolecta*

[Experiment 2]

Type of measurement	Mean K_2	Generation time
Growth:		
Cell concentration.....	Hours ⁻¹ 0.0613	Hours 16.3
Dry weight.....	.0492	20.3
Organic carbon concentration.....	.0470	21.3
Mean of growth values.....	.0532	18.8
Photosynthesis:		
C ¹⁴ uptake.....	.0866	11.5
Net O ₂ production.....	.0665	15.0
Mean of photosynthesis values.....	.0765	13.1

Table 4 gives mean K_2 values and generation times for the three types of growth measurements and two types of photosynthesis measurements in experiment 2. Statistical analysis showed that the K_2 values for the three types of growth measurements did not differ significantly; similarly K_2 values for C¹⁴ uptake did not differ significantly from those calculated from net O₂ measurements. Also given in table 4 are the overall means for growth and for photosynthesis. This mean growth rate was about 70 percent of the mean photosynthetic rate, and the difference was highly significant ($p < 0.01$). Thus, growth in the culture was significantly less than photosynthesis in bottles placed at the bottom of the culture.

Further comparisons of C¹⁴ uptake, O₂ evolution, and growth were made during the initial logarithmic phases of mass cultures in which growth was eventually limited by nitrogen deficiency (experiments 3 and 4) and by phosphorus deficiency (experiment 6).

EFFECTS OF NITROGEN DEFICIENCY

Experiment 3

This experiment was similar to the previous one, except that the amount of added nitrate was halved, so that growth would be limited by

nitrogen deficiency, and the light intensity incident to the bottom of the aquarium culture was increased to alleviate possible light limitations. Two-liter cultures were incubated for 2 days in the culture room at 550 ft.-c. and 21° C. Then they were mixed in the aquarium, supplied with air for 10 hours, and with 5 percent CO₂-in-air thereafter, at a rate of 300 ml./min. Growth and photosynthesis were measured every 10 hours for the next 100 hours and also at 120 hours. Growth was measured by determinations of cell concentration, organic carbon, organic nitrogen, dry weight, and optical density. C¹⁴ uptake and O₂ evolution were determined as in the previous experiment by placing the photosynthesis bottles in the bottom of the aquarium. At the beginning of these measurements, the pH of the culture was 8.68, at 10 hours it was 6.55, and then increased to a maximum of 7.82 at 120 hours. The total CO₂ concentration was 20.5 mg. CO₂-C/l. at the beginning of the experiment, and thereafter it was 33.1 to 37.9 mg. CO₂-C/l.

Photosynthesis and cell composition measurements are given in table 5; and growth is shown in figure 4. During the first 50 hours, growth in the culture proceeded in a logarithmic fashion

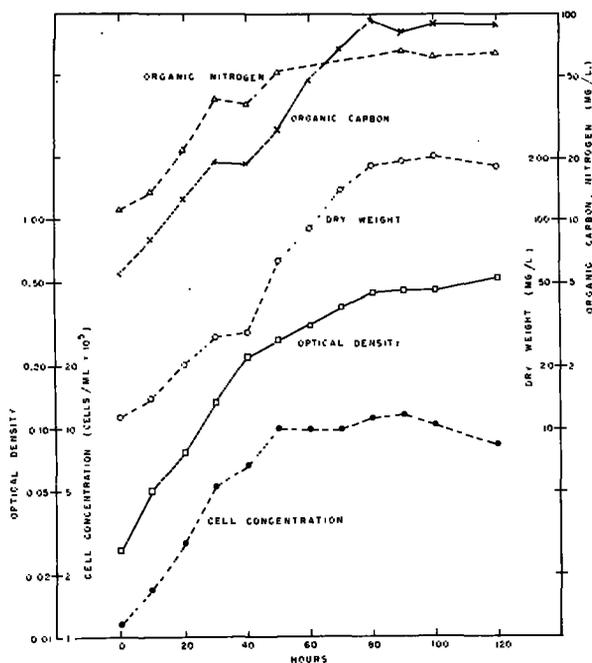


FIGURE 4.—Growth of a mass culture of *Dunaliella primolecta* containing a limiting concentration of nitrogen.

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and the concentration of nitrogen in the cells was higher than that found in the last three measurements. The C/N ratio was lower. During this period of logarithmic growth, C¹⁴ uptake agreed well with both net and gross O₂ production.

Mean K₂ values and generation times for the first 50 hours were calculated from the five types of growth measurements (table 6); also reported are the K₂ values and generation times calculated from photosynthesis measurements (C¹⁴ uptake and net O₂ evolution) and the measured amounts of organic carbon (table 6). The mean growth rate is about 65 percent of the mean K₂ value calculated from photosynthesis measurements. This difference is statistically highly significant (p < 0.01). As in the previous experiment, growth in the culture was significantly less than photosynthesis in the bottles.

TABLE 5.—Comparative photosynthesis and cell composition measurements in a mass culture of *Dunaliella primolecta* [Experiment 3]

Time	Photosynthesis			Cell composition	
	C ¹⁴ uptake	Net O ₂ evolution	Gross O ₂ evolution	N	C/N ratio
<i>Hours</i>	<i>µg. C/l./hour</i>	<i>µg. C/l./hour</i>	<i>µg. C/l./hour</i>	<i>Percent</i>	<i>Atoms</i>
0.....	447±58	399±48	447±54	10.00	5.4
10.....	541±70	564±70	644±80	9.79	6.8
20.....	772±100	818±100	956±120	10.83	6.8
30.....	873±110	1,030±130	1,190±140	11.90	5.8
40.....	1,320±170	1,230±150	1,460±180	9.44	5.9
50.....	2,830±370	1,870±230	2,380±290	8.20	8.3
60.....	3,240±420	2,620±330	3,150±390	-----	-----
70.....	2,160±280	2,290±270	2,840±340	-----	-----
80.....	1,900±250	2,020±240	2,580±320	-----	-----
90.....	1,550±200	1,580±190	2,210±260	3.36	14.4
100.....	1,220±160	1,390±180	1,700±240	3.05	16.8
120.....	338±44	429±51	880±110	3.64	15.4
123.....	(2,000 µg.-at./l. N added)				
144.....	1,350±180	1,200±160	1,670±220	9.48	6.2

TABLE 6.—Mean K₂ values and generation times during the logarithmic growth phase as calculated from growth and photosynthesis measurements in a mass culture of *Dunaliella primolecta*

[Experiment 3]		
Type of measurement	Mean K ₂	Generation time
Growth:	<i>Hours⁻¹</i>	<i>Hours</i>
Cell concentration.....	0.0629	15.9
Dry weight.....	.0490	20.4
Organic carbon concentration.....	.0583	17.1
Organic nitrogen concentration.....	.0430	23.3
Optical density.....	.0657	15.2
Mean of growth values.....	.0558	17.9
Photosynthesis:		
C ¹⁴ uptake.....	.0877	11.4
Net O ₂ production.....	.0841	11.9
Mean of photosynthesis values.....	.0859	11.6

In the present experiment, optical densities and illuminances at the bottom of the culture were measured. From these measurements, rough calculations could be made of the illuminance at the midheight in the bottles and at midheight in the culture (table 7). The calculations indicate that the culture as a whole received less light than photosynthesis bottles placed on the bottom of the culture. The values given in table 7 refer to measurements made in the center of the aquarium. Although no detailed study was made of the horizontal distribution of light in the aquarium, a few measurements taken near the ends were less than those taken in the center. Thus the culture as a whole may have received even less light than is indicated in table 7.

TABLE 7.—Illuminance at the bottom of a mass culture of *Dunaliella primolecta*; illuminance at the midheight of 67-ml. photosynthesis bottles placed on the bottom of the culture; and illuminance at the midheight of the culture itself

[Experiment 3]

Time	Illuminance		
	At culture bottom	At bottle midheight	At culture midheight
<i>Hours</i>	<i>Ft.-c.</i>	<i>Ft.-c.</i>	<i>Ft.-c.</i>
0.....	2,070	1,785	1,650
10.....	1,850	1,500	1,220
20.....	1,850	1,530	1,280
30.....	1,750	1,170	720
40.....	1,620	900	350
50.....	1,750	880	300
60.....	1,800	795	310
70.....	1,850	700	426
80.....	1,900	610	220
90.....	1,850	590	200
100.....	1,850	560	200
120.....	1,850	495	155

If growth and photosynthesis are proceeding at subsaturating illuminances, the rates of these processes will be dependent upon the light received. Photosynthesis in bottles containing about 10^5 cells per ml. is saturated at an incident illuminance of about 1,200 ft.-c. at 26° C., the temperature of the culture (Thomas, unpublished data). This cell concentration approximates that at zero time in experiment 3. Values close to or less than 1,200 ft.-c. were found in the culture after 10 hours and in the bottles at 30 hours and thereafter (table 7). Thus light probably limited the rate of growth in the culture during most of the initial 50-hour period. At least it seems certain that the culture received less light than the bottles and that this is the reason that K_2 values for growth were less than those for photosynthesis. Probably growth and photosynthesis would have agreed very well if

illumination conditions were exactly the same for both types of measurements (see experiment 6).

After 50 hours, cell division ceased in the culture. The amount of nitrogen added to the culture was only half the amount added in the previous experiment, and only about half as many cells were produced. Cells taken at the next three sampling times (60, 70, and 80 hours) were probably in the beginning stages of nitrogen deficiency. Unfortunately, the samples for organic nitrogen taken at these times were lost, but it is unlikely that the organic nitrogen concentration was greater than that found at 70 hours and thereafter.

During this period of incipient deficiency, organic carbon continued to accumulate and the dry weight also increased (fig. 4). Photosynthesis reached a maximum at 60 hours and decreased thereafter (table 5). At 60 hours both gross and net O_2 evolution were less than C^{14} uptake, probably because of supersaturation of the water in the O_2 bottles. At 70 and 80 hours, C^{14} uptake agreed most closely with net O_2 production. During this 60- to 90-hour period, K_2 values for growth, as calculated from organic carbon, optical density, and dry-weight measurements, did not differ significantly from K_2 values calculated from C^{14} uptake and net O_2 production and the measured organic carbon concentration. The mean K_2 value for the growth measurements was 0.0123 hours⁻¹ (generation time=24.6 hours), while that for the photosynthesis measurements was 0.0196 hours⁻¹ (generation time=15.4 hours).

At 90 hours and thereafter, the effects of nitrogen deficiency were quite marked. The nitrogen in the cells was only 3 to 3.5 percent of the dry weight, the C/N ratio was high, and growth had ceased in the culture (table 5 and figure 4). Photosynthesis continued to decrease and C^{14} uptake agreed well with net O_2 evolution. At 120 hours net O_2 evolution was only half of the gross O_2 evolution, and C^{14} uptake was close to the net O_2 value.

At 123 hours, the culture was brought up to a volume of 28 l. and KNO_3 was added to bring the N concentration to 2,000 $\mu\text{g.}-\text{at.}/\text{l.}$ At 144 hours, growth and photosynthesis measurements were made. During this 21-hour period of recovery from N deficiency, the cell nitrogen increased and the C/N ratio decreased (see table 5). The culture took up nitrogen at a high rate; the K_2 value for increase in organic N was 0.0926 hours⁻¹.

The cell concentration did not increase, but K_2 values for increases in organic carbon, dry weight, and optical density were 0.0303 hours⁻¹, 0.0268 hours⁻¹, and 0.0122 hours⁻¹, respectively. All photosynthesis values were over twice the amounts recorded at 120 hours, even though the culture had been diluted. Net O₂ evolution was 72 percent of gross O₂ evolution. C¹⁴ uptake was intermediate between net and gross O₂ evolution. K_2 values calculated from organic carbon concentration at 144 hours and photosynthesis measurements at that time were 0.0329 hours⁻¹ (C¹⁴ uptake) and 0.0294 hours⁻¹ (net O₂ evolution).

Experiment 4

In a further investigation of the effects of nitrogen deficiency on photosynthesis, two 20-liter bottles, each containing 6 liters of sea water medium, were inoculated with *Dunaliella*. One bottle was enriched with 2,000 μg.-at.N/l., 200 μg.-at. P/l. and 0.5 p.p.m.Fe, and served as a control culture for comparison with the other bottle, which contained sea water enriched with the same amount of P and Fe, but an amount of N (250 μg.-at./l.) that would limit the final population. Also efforts were made to keep the cultures bacteria-free and to keep the volume of medium in the bottles more constant than in previous experiments. Each bottle was fitted with an air inlet tube extending below the surface of the culture, through which 5 percent CO₂-in-air was supplied at 500 ml./min. The gas was filtered through cotton wool to avoid bacterial contamination. Each bottle was also fitted with a tube for adding fresh sterile medium after each sampling, and with a siphon tube for taking the sample. A final tube was added for the egress of air.

After inoculation, the bottles were placed side by side on a reciprocating shaker which agitated them gently throughout the experiment. The cultures were continuously illuminated from below at an incident illumination of 1,500 ft.-c, and they were incubated at 22° C. in an air-conditioned room. In the control culture (+N), the pH was 6.30 to 6.95, and the total CO₂ concentration was 43.9 to 59.9 mg.CO₂-C/l. In the deficient culture, the pH was 6.28 to 6.69 and the total CO₂ concentration was 39.5 to 51.1 mg.CO₂-C/l.

Samples were taken for growth and photosynthesis measurements at intervals over a period of 8 days. Growth was measured only by cell

counts and is expressed in K_2 units and generation times as previously described. Samples for cell nitrogen analyses were centrifuged at 4,400 g. for 20 minutes. The cells were resuspended in distilled water and recentrifuged; they were then transferred to tared glass cups and dried in vacuo at 50° C. Measurement of growth by dry-weight determinations was attempted from these samples, but unavoidable losses of cell material occurred during centrifugation and resuspension. Thus, these samples could only be used to determine the percent N in the cells. After each sample was taken from the cultures, equivalent volumes of fresh media were added, but no nitrogen was added to the N-deficient culture until after 138 hours of incubation.

Photosynthesis bottles were incubated separately from the cultures at 1,250 ft.-c. illuminance and 22° C. These measurements were carried out with portions of the undiluted culture during the first seven samplings; 10-ml. aliquots were diluted with sterile sea water for the last three measurements. Thus, in these latter determinations, light limitations due to mutual shading of the cells were avoided, as were errors due to supersaturation of the suspension with oxygen.

The results of this experiment are given in tables 8 and 9. To compare data from different sampling times, photosynthesis is expressed as the photosynthetic capacity or photosynthesis per cell, as well as in terms of photosynthesis per liter. The condition of the cells in relation to nitrogen is shown by the figures for percent nitrogen in the cells. These nitrogen values are less than those reported in the previous experiment for cells in the same condition. Probably some N was lost during the washing procedure.

During the first 42 hours, mean growth constants, generation times, and photosynthetic capacities were similar in both cultures, but there was considerable variation in these values from sampling time to sampling time in each culture. Mean photosynthesis per cell, from all determinations in both cultures was 2.10 μμ g.C/cell/hour, for C¹⁴ uptake; 2.02 μμ g.C/hour, for net O₂ production; and 2.88 μμ g.C/cell/hour, for gross O₂ production.

The mean growth constant (K_2) of the -N culture was 0.1058 hours⁻¹ (9.5 hours generation time) and 0.1354 hours⁻¹ (7.4 hours generation time) in the +N culture. These mean K_2 values

TABLE 8.—Growth and comparative photosynthesis measurements of *Dunaliella primolecta* cells containing sufficient nitrogen

[Experiment 4]

Time, (hours)	Growth		Photosynthesis			Photo-synthetic capacity			Nitrogen
	Growth constant between samples K_2 (hours ⁻¹)	Generation time (hours)	C ¹⁴ uptake $\mu\text{g. C/l./hr.}$	Net O ₂ production $\mu\text{g. C/l./hr.}$	Gross O ₂ production $\mu\text{g. C/l./hr.}$	C ¹⁴ uptake $\mu\text{g. C/cell/hr.}$	Net O ₂ production $\mu\text{g. C/cell/hr.}$	Gross O ₂ production $\mu\text{g. C/cell/hr.}$	
0	0.1281	7.8	347±49	343±61	622±90	2.99	2.95	5.36	6.50
8	.1203	8.3	565±79			2.90			8.15
24	.0912	11.0	1,400±200	1,480±100	1,730±220	2.48	2.61	3.04	9.47
32	.2021	4.9	991±140	1,570±220	2,620±310	1.24	1.97	3.28	11.13
42	.0411	24.3	1,900±270	2,950±440	3,120±270	1.38	2.15	2.27	9.23
66	.0423	23.6	2,620±370	2,980±390	3,400±490	1.05	1.19	1.36	
90	.0443	22.6	2,410±340	2,770±360	3,510±460	0.82	0.94	1.19	6.12
114	.0053	188.0	918±130	931±150	980±150	1.34	1.35	1.43	8.87
138	.0031	328.0	697±98	727±90	779±97	1.28	1.34	1.43	8.55
163			583±82	686±96	774±100	1.31	1.44	1.62	6.78

TABLE 9.—Growth and comparative photosynthesis measurements in nitrogen-deficient cells of *Dunaliella primolecta*

[Experiment 4]

Time, (hours)	Growth		Photosynthesis			Photo-synthetic capacity			Nitrogen
	Growth constant between samples K_2 (hours ⁻¹)	Generation time (hours)	C ¹⁴ uptake $\mu\text{g. C/l./hr.}$	Net O ₂ production $\mu\text{g. C/l./hr.}$	Gross O ₂ production $\mu\text{g. C/l./hr.}$	C ¹⁴ uptake $\mu\text{g. C/cell/hr.}$	Net O ₂ production $\mu\text{g. C/cell/hr.}$	Gross O ₂ production $\mu\text{g. C/cell/hr.}$	
0	0.0716	14.0	429±60	333±80	554±99	2.12	1.64	2.74	7.75
8	.1330	7.5	404±57	601±87	787±110	1.62	2.40	3.07	8.77
24	.1247	8.0	2,410±340	1,740±220	2,080±260	2.84	2.05	2.45	9.47
32	.0937	10.7	3,390±490	2,530±320	3,810±460	2.34	1.75	2.63	3.86
42	.0307	32.6	1,210±170	797±110	1,190±160	1.06	0.69	1.04	
66	.0077	130.4	383±54	472±74	891±130	0.21	0.27	0.51	1.77
90	.0107	63.3	138±19	171±21	353±42	0.12	0.13	0.28	1.79
114	.0317	31.6	40.4±5.6	16.4±5.7	8.2±8.5	0.28	-0.11	0.06	2.22
138 ¹	.0249	40.2	6.3±0.9	7.9±3.6	15.9±2.2	0.08	-0.11	0.21	1.65
163			69.3±9.7	66.3±10.3	91±11	1.66	1.59	2.16	6.86

¹ 2,000 $\mu\text{g. at. N/l}$ added after samples were taken.

did not differ statistically. Photosynthesis bottles were incubated at an illuminance of 1,250 ft.-c., whereas the cultures were incubated at 1,500 ft.-c. Thus, K_2 values calculated from photosynthesis

and organic carbon estimations would not be exactly comparable. However, such K_2 values were calculated. In these calculations, the initial organic carbon (C_1) was estimated by multiplying the mean value for carbon per cell (obtained from the logarithmic phases of experiments 2 and 3) by the cell concentration, and C^{14} uptake or net O_2 evolution values were added to the initial organic carbon estimate to obtain C_2 . For cells from the -N culture, the mean of such photosynthetic K_2 values were 0.0800 hours⁻¹ (C^{14}) and 0.0706 hours⁻¹ (net O_2); respective generation times were 12.5 and 14.1 hours. Cells from the +N culture gave mean K_2 values of 0.0872 hours⁻¹ (C^{14}) and 0.0897 hours⁻¹ (net O_2), and the generation times were 11.5 and 11.1 hours, respectively. These mean values did not differ statistically. The mean growth constant for both cultures was 0.1200 hours⁻¹; the mean photosynthetic K_2 value calculated from all photosynthesis measurements and organic carbon estimates in both cultures was 0.0810 hours⁻¹. The growth rate was 48 percent greater than the photosynthetic rate and this difference was statistically significant. This difference may be attributed to different illuminances in the culture and in the photosynthesis bottles.

In the control (+N) culture, after 42 hours, photosynthetic capacities were somewhat reduced. C^{14} uptake agreed most closely with net O_2 production, but neither C^{14} nor net O_2 production differed from gross O_2 production by an amount greater than the experimental error of the measurements. Although the growth rate decreased and growth eventually ceased, photosynthesis and photosynthetic capacity were not strongly inhibited. The reduction in growth rates and in photosynthetic capacities was probably due to light limitations in the very dense culture. The culture retained its bright green appearance, and the concentration of N in the cells did not change greatly.

After 42 hours, N deficiency was very marked in the -N culture, which was pale green in color. Much less nitrogen was found in the cells. During the next 48 hours, growth continued at a reduced rate and the photosynthetic capacity was greatly reduced. Net oxygen production fell from the usual 70 to 90 percent of gross production to 50 percent of gross production. C^{14} uptake agreed with net production during this period. N-deficient cells stained more heavily with Lugol's

iodine solution and more of them were nonmotile than cells in the +N culture.

After 90 hours, growth constants were negative in the -N culture. Net photosynthesis was negative, and C¹⁴ uptake agreed more closely with gross O₂ evolution.

After 138 hours, 2,000 µg.-at.N/1. were added to the -N culture. Although the cell concentration did not increase during the next 23 hours, the photosynthetic capacity increased greatly. C¹⁴ uptake agreed closely with net O₂ production. No measurements were made beyond this point, but the culture was very much more green and dense on the following day.

In this experiment on nitrogen deficiency, aseptic technique was employed to keep the culture bottles bacteria-free. This point was checked at each sampling time by adding a portion of the sample to a tube of sea water enriched with 1 percent dextrose and 5 percent peptone. These tubes became turbid with bacterial growth in 3 days at room temperature. Thus, bacteria were undoubtedly present, although contamination was probably not great, since turbidity did not develop in less than 3 days.

Experiment 5

To study the effects of N deficiency on photosynthesis in the absence of bacteria, small-scale, bacteria-free cultures containing limiting and sufficient amounts of nitrogen were inoculated with *Dunaliella*. One-liter Erlenmeyer flasks containing 700 ml. of the same +N and -N media used in experiment 4 were incubated on the reciprocating shaker at 1,000 ft.-c. illuminance and 22° C. No aeration was provided, and after 8 days the pH had risen from 8.1 to 9.2 in both cultures, and growth had ceased. Then 15 millimoles of sodium bicarbonate were added aseptically to each culture. After 3 more days, the pH had risen again to 9.1. The -N culture was pale green, while the +N culture was bright green in appearance. For photosynthesis measurements, 10-ml. portions of each culture were then added to 50 ml. sterile sea water containing 0.2 ml. 0.5 molar NaHCO₃ in 67-ml. bottles. Bottles for O₂ measurements were filled with sterile sea water, and C¹⁴ bottles received 1 ml. of C¹⁴ solution. The pH in these bottles was 8.6. The bottles were incubated on the shaker under the same conditions of illuminance and temperature that were used to grow the cells.

EXPERIMENTAL EVALUATION OF C¹⁴ METHOD

Peptone-dextrose sterility test tubes showed that bacteria were absent from these cultures. The results of these photosynthesis measurements are shown in table 10.

TABLE 10.—Photosynthesis in N-deficient and N-sufficient bacteria-free cultures of *Dunaliella primolecta*

[Experiment 5]

Measurements	Culture	
	-N	+N
Photosynthesis:		
C ¹⁴ uptake (µg.C/1./hour).....	300±42	834±120
Net O ₂ production (µg.C/1./hour).....	309±37	933±110
Gross O ₂ production (µg.C/1./hour).....	380±46	1,010±120
Photosynthetic capacity:		
C ¹⁴ uptake (µµg.C/cell/hour).....	1.33	2.77
Net O ₂ production (µµg.C/cell/hour).....	1.37	3.09
Gross O ₂ production (µµg.C/cell/hour).....	1.68	3.35

The ratio of net O₂ evolution to gross O₂ evolution in the N-deficient culture was 81 percent, while that in the control (+N) culture was 91 percent. Thus the effects of deficiency were not as pronounced as those shown by measurements taken after 40 hours in the previous experiment. In both cultures, C¹⁴ uptake agreed most closely with net O₂ production.

EFFECTS OF PHOSPHORUS DEFICIENCY

Experiment 6

To study the effects of phosphorus deficiency on photosynthesis and growth, the mass culture apparatus (aquarium) was again used. Forty liters of sterilized sea water were placed in the aquarium and enriched with 2,000 µg.-at. N/1., 1 p.p.m. Fe, and a limiting concentration of P (20 µg.-at./1.). The culture was inoculated with *Dunaliella* and incubated for 19 hours before growth and photosynthesis measurements were begun. During this period no CO₂ was supplied, but the culture was stirred with a glass stirrer. The aquarium was incubated at 26° C. and continuously illuminated from below at an illuminance of 800 ft.-c.

After the 19-hour preliminary period, 5 percent CO₂-in-air was supplied at 300 ml./min. for the rest of the experiment. Temperature, illumination, and stirring conditions were maintained at the level previously set. When the initial sample was taken, the pH of the culture was 8.05 and varied from 6.22 to 6.89 thereafter. Similarly, the initial CO₂ concentration was 23.0 mg. CO₂-C/1. and varied from 33.7 to 44.6 mg. CO₂-C/1. for

the rest of the experiment. Growth was measured by cell concentration and by optical density measurements. Photosynthesis was measured as previously described by placing the bottles on the bottom of the culture.

The growth of the culture is depicted in figure 5

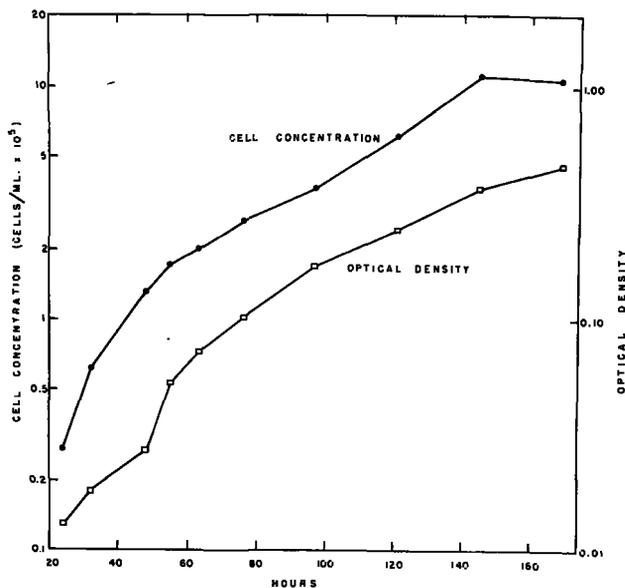


FIGURE 5.—Growth of a mass culture of *Dunaliella primolecta* containing a limiting concentration of phosphorus.

and photosynthesis measurements are given in table 11. The initial cell concentration and optical density were too low to measure. Initial photosynthesis rates were correspondingly low; C^{14} uptake agreed with net O_2 evolution within the experimental error at this low phytoplankton concentration.

TABLE 11.—Comparative photosynthetic measurements at 800 ft.-c. during the development of phosphorus deficiency in a mass culture of *Dunaliella primolecta*

[Experiment 6]

Time (hours)	Photosynthesis			Photosynthetic capacity		
	C^{14} uptake $\mu\text{g. C/l./hour}$	Net O_2 production $\mu\text{g. C/l./hour}$	Gross O_2 production $\mu\text{g. C/l./hour}$	C^{14} uptake $\mu\text{g. C/cell/hour}$	Net O_2 production $\mu\text{g. C/cell/hour}$	Gross O_2 production $\mu\text{g. C/cell/hour}$
0						
24	11±1	19±8				
32	112±15	135±27	154±20	4.06	4.88	5.58
48	126±16	157±20	188±24	2.07	2.56	3.06
55	231±30	279±36	423±51	1.77	2.13	3.24
63	371±48	367±60	449±67	2.18	2.16	2.64
76	475±62	541±70	678±82	2.41	2.73	3.42
97	764±99	667±110	864±130	2.88	2.50	3.24
121	1,350±180	1,060±160	1,210±180	3.69	2.88	3.31
145	1,580±210	1,470±160	1,640±200	2.58	2.41	2.67
145	1,320±170	1,090±150	1,740±230	1.19	0.98	1.56

During the period from 24 to 55 hours, rapid logarithmic growth occurred in the culture. C^{14} uptake agreed most closely with net O_2 production. The mean growth rates (K_2) were 0.0884 hours⁻¹ and 0.0781 hours⁻¹, as determined by cell concentrations and optical density measurements, respectively. Cell concentrations were converted to an estimated organic carbon concentration which was used in calculating photosynthetic K_2 values from C^{14} uptake and net O_2 production measurements. These mean photosynthetic K_2 values were 0.0843 hours⁻¹ and 0.1048 hours⁻¹, as calculated from C^{14} uptake and net O_2 production respectively. The overall mean K_2 for growth was 0.0835 hours⁻¹, while that for photosynthesis was 0.0945 hours⁻¹. Statistical tests showed that these mean K_2 values did not differ significantly. Rough calculations of illuminances in the bottles and at midheight in the culture showed that at 24, 32, and 48 hours these illuminances did not differ by more than 10 percent, and at 55 hours, illuminance in the bottles was 25 percent greater than illuminance at the midheight of the culture. Thus during this period before the culture became too dense, illumination conditions were nearly equivalent in the culture and in photosynthesis bottles placed on the bottom of the culture, and photosynthesis was equivalent to growth.

The growth rate of the culture was much reduced during the period from 55 to 145 hours. The mean growth constant K_2 , as calculated from increases in cell concentration, was 0.0291 hours⁻¹; that calculated from increases in optical density was 0.0344 hours⁻¹. K_2 values calculated from photosynthesis determinations and estimated organic carbon concentrations were 0.0892 hours⁻¹ (C^{14}) and 0.0761 hours⁻¹ (net O_2). The overall mean photosynthetic constant was 0.0827 hours⁻¹; that for growth was 0.0317 hours⁻¹. Statistical tests showed that this difference was significant. Growth in the culture proceeded at only about 40 percent of the rate of photosynthesis in bottles placed on the bottom of the culture. Rough calculations of light intensities showed that the intensity at midheight in the culture was only 45–75 percent of that in the bottles.

During this 90-hour period, C^{14} uptake corresponded to both net or gross O_2 evolution, which did not differ greatly from each other. Further indications that light was limiting in the culture, were found by incubating a duplicate set of photo-

synthesis bottles at 1,800 ft.-c. These photosynthesis measurements are shown in table 12. Photosynthetic capacities were greatly increased by incubation at this light intensity, and C^{14} uptake was similar to both net and gross O_2 production.

TABLE 12.—Comparative photosynthesis determinations at 1,800 ft.-c. during the development of phosphorus deficiency in *Dunaliella primolecta*

[Experiment 6]

Time (hours)	Photosynthesis			Photosynthetic capacity		
	C^{14} uptake $\mu\text{g. C/l./hour}$	Net O_2 production $\mu\text{g. C/l./hour}$	Gross O_2 production $\mu\text{g. C/l./hour}$	C^{14} uptake $\mu\text{g. C/cell/hour}$	Net O_2 production $\mu\text{g. C/cell/hour}$	Gross O_2 production $\mu\text{g. C/cell/hour}$
76.....	1,440±190	1,170±140	1,370±170	5.44	4.39	5.13
97.....	2,010±260	1,810±230	1,970±240	5.48	4.93	5.36
121.....	2,330±300	2,360±290	2,520±310	3.82	3.85	4.12
145.....	2,310±300	1,880±230	2,520±310	2.08	1.68	2.26

At 145 hours, cell division ceased, but optical density continued to increase for an additional 24 hours. To test for phosphorus deficiency, 50-ml. aliquots of the cell suspension were placed in four 125-ml. flasks at 153 hours. Two flasks were enriched with phosphorus at a concentration of 20 $\mu\text{g. at. P/l.}$ Two others received no addition. The flasks were incubated for 16 hours at 1,500 ft.-c and 22° C. on a reciprocal shaker. The initial optical density was 0.409; after 24 hours the optical density of cultures to which phosphate was added was 0.561, while that of the control (–P) cultures was 0.481. Thus, the increase in optical density was greater in cultures which contained added phosphate than in control cultures. The cells were undoubtedly phosphorus-deficient, but only in the beginning stages of deficiency, since some growth (increase in optical density) occurred in the culture and in the flasks to which no phosphorus was added. At 145 hours, for bottles placed in the culture and incubated at an incident illuminance of 800 ft.-c, net O_2 production was 62 percent of the gross value. These net gross ratios are generally less than those found earlier in this experiment, and are also indicative of the beginning stages of phosphorus deficiency. At 145 hours, C^{14} uptake was intermediate between, but did not differ significantly from, net and gross O_2 evolution. Photosynthesis was not measured at 169 hours.

EXPERIMENTAL EVALUATION OF C^{14} METHOD

Experiment 7

Another experiment on the effects of phosphorus deficiency was performed with small-scale, bacteria-free cultures containing limiting and sufficient amounts of phosphate. One-liter Erlenmeyer flasks containing 700 ml. of media were inoculated with *Dunaliella* and incubated on the reciprocal shaker at 1,600 ft.-c. and 26° C. The complete (+P) medium consisted of sea water enriched with 2,000 $\mu\text{g. at. NO}_3\text{-N/l.}$, 200 $\mu\text{g. at. PO}_4\text{-P/l.}$ and 1 p.p.m. Fe. In the phosphorus-deficient culture the sea water was enriched with only 20 $\mu\text{g. at. PO}_4\text{-P/l.}$

After 188 hours, the –P culture contained 7,700,000 cells/ml., while the +P culture contained 9,500,000 cells/ml. Sixty-seven-ml. bottles containing 50 ml. sea water and 0.1 millimole of NaHCO_3 were inoculated with 10-ml. portions of each culture. Bottles for O_2 measurements were then filled with sterile sea water, those for C^{14} measurements received 1 ml. C^{14} solution. They were incubated at 1,400 ft.-c. and 25° C.

Table 13 shows the results of this experiment with bacteria-free cultures. In the control (+P) culture, C^{14} uptake agreed quite well with net O_2 production. In the phosphorus deficient culture, it was much less than either net O_2 production or gross O_2 production.

TABLE 13.—Photosynthesis in P-deficient and P-sufficient bacteria-free, cultures of *Dunaliella primolecta*

[Experiment 7]

Measurements	–P Culture	+P Culture
Photosynthesis:		
C^{14} uptake ($\mu\text{g. C/l./hour}$).....	165±21	511±60
Net O_2 production ($\mu\text{g. C/l./hour}$).....	272±33	504±61
Gross O_2 production ($\mu\text{g. C/l./hour}$).....	318±39	542±66
Photosynthetic Capacity:		
C^{14} uptake ($\mu\text{g. C/cell/hour}$).....	1.07	2.69
Net O_2 production ($\mu\text{g. C/cell/hour}$).....	1.77	2.65
Gross O_2 production ($\mu\text{g. C/cell/hour}$).....	2.06	2.85

COMPARATIVE MEASUREMENTS OF PHOTOSYNTHESIS BY pH CHANGES, C^{14} UPTAKE, AND O_2 EVOLUTION

Experiments 8 and 9

In previous experiments, C^{14} uptake was compared with O_2 evolution and with growth. It appeared desirable to compare C^{14} uptake with measurements of CO_2 assimilation that were more direct than determinations of O_2 evolution and growth. By using cultures where the rate of photosynthesis was high (cultures in the logarithmic growth phase, and incubated at high illuminances), it was feasible to measure CO_2

assimilation directly from pH changes and to compare CO₂ assimilation with C¹⁴ uptake and O₂ evolution.

Cells for these comparisons were grown in sea water enriched with 2,000 µg.-at. NO₃-N/l., 200 µg.-at. PO₄-P/l. and 1 p.p.m. Fe. After 6 days of growth at 500 ft.-c. and 26° C., 25 ml. of the cell suspension was added to 25 ml. of sterile sea water containing 0.1 ml. of 0.5 molar NaHCO₃ in 67-ml. bottles. Bottles for O₂ and pH measurements were filled with sterile sea water; those for C¹⁴ measurements received 1-ml. C¹⁴ solution. All bottles were incubated at 23° C. and 1,450 ft.-c.

The results of these experiments are shown in table 14. The pH changed in the first experiment from 8.66 to 8.88 during ¾ hours of incubation; in the second experiment it changed from 8.15 to 8.98 during four hours. From these changes and the carbonate alkalinity, the amount of CO₂ taken up was calculated. The total (pooled) error of the pH method was ±8.5 percent—±6 percent for each measurement, beginning and final. In both experiments CO₂ assimilation as measured by pH changes agreed with that measured by C¹⁴. O₂ measurements were also equivalent to both CO₂ measurements within the experimental error.

TABLE 14.—*Photosynthesis in Dunaliella primolecta as measured by pH changes, C¹⁴ uptake, and O₂ evolution*

Type of measurement	Experiment 8	Experiment 9
pH change (µg. C/l./hour).....	1,380±120	2,770±240
C ¹⁴ uptake (µg. C/l./hour).....	1,720±220	2,670±350
Net O ₂ production (µg. C/l./hour).....	1,300±160	2,230±280
Gross O ₂ production (µg. C/l./hour).....		2,350±280

DISCUSSION

Under conditions of adequate nutrition and illuminance, the difference between total or "gross" O₂ evolution and net O₂ evolution is not large. Commonly the difference is only 5–20 percent. To compare such measurements with C¹⁴ uptake it is necessary to calculate the equivalent carbon production using a photosynthetic quotient (O₂/CO₂) which may vary nearly as much (±12 percent, Ryther, 1956) as the experimental error of the C¹⁴ method. The present results show that, under these ideal conditions, C¹⁴ uptake agrees well with net O₂ evolution, but does not differ greatly (and often not significantly) from gross O₂ evolution either. This was shown

by experiments using cells taken from the logarithmic phases of growth in the aquarium cultures (experiments 1, 2, 3, and 6) and in control cultures (+N and +P) of the experiments on N and P deficiency (experiments 4, 5, and 7). These results agreed with those of other investigators who have used healthy cultures to compare O₂ evolution and C¹⁴ uptake (Ryther, 1954; Ryther and Vaccaro, 1954; Steemann Nielsen and Al Kholly, 1956; Ichimura and Saijo, 1958).

The growth of an algal culture is also a net process, and such growth is more representative of an increase in the food which would be available to zooplankton, for instance, than is net O₂ evolution. In the present experiments, growth was measured in several ways and logarithmic growth constants were calculated from these growth measurements. Organic carbon determinations provided a way of converting photosynthesis measurements to similar K₂ values, so that photosynthesis could be compared with growth.

In experiments 2 and 3, growth was somewhat less than photosynthesis as measured by C¹⁴ uptake and net O₂ evolution. This difference was attributed to differences in the illumination of cells in photosynthesis bottles placed within the culture and that reaching the culture itself. At low cell densities (experiment 6), when illuminance in the bottles was similar to that in the culture, growth and photosynthesis did not differ. In experiment 4, when the illuminance in the culture was greater than that in the bottles, growth was greater than photosynthesis. These results indicate that during the logarithmic phase of growth, if conditions are the same for the two measurements, growth and photosynthesis are similar, and that C¹⁴ measurements are equivalent to increase in algal biomass. It would have been more desirable to compare C¹⁴ uptake and growth in the same vessel, but a frequent sampling routine could not have been maintained. Furthermore, glass-stoppered bottles are used as containers for photosynthesizing cells in field C¹⁴ measurements, and it was desired to approximate field conditions as much as possible.

Using a natural phytoplankton population enclosed in a large plastic bag, McAllister et al. (1961) have recently compared several methods of measuring production. During the logarithmic phase of growth in the bag, measurements

made with the C^{14} method were approximately equivalent to those calculated from O_2 changes, pH changes, increases in organic carbon, and increases in cell volume (see their fig. 7). This equivalence between growth and photosynthesis was confirmed by our results with *Dunaliella* cultures.

In the sea, rapid logarithmic phytoplankton growth is probably not common. It occurs mainly during the spring outburst in temperate waters, and during the development of "red tides." In most tropical and subtropical seas, at least, incipient nutrient deficiency is probably a more common condition, a condition under which phytoplankton grow and produce organic matter at a rate which is limited by the rates of such processes as the remineralization of nutrients in the surface layers or slow diffusion from subsurface supplies.

Incipient nitrogen deficiency in *Dunaliella* was studied in experiments 3 and 4. Under such conditions, C^{14} uptake was similar to net O_2 evolution. During the period of incipient deficiency, K_2 values for increases in organic carbon calculated from photosynthesis measurements did not differ significantly from those calculated for growth.

It is also interesting to note that incipient nitrogen deficiency resulted in a change in metabolism—a "shunt" in which cell material is formed which is low in nitrogen. During logarithmic growth, some 8 to 10 percent of the dry weight was nitrogen. During incipient deficiency, nitrogen made up only 3 percent of the dry weight and carbon continued to accumulate. Thus, the C/N ratio shifted from approximately six during the logarithmic growth period to approximately 15 when the cells became deficient (see table 5). The main product of this shunt metabolism was probably starch, since deficient cells were stained more heavily by iodine. Similar shifts in metabolism from predominantly protein formation to carbohydrate or lipid formation have been reported for other green algae when nitrogen was deficient (Spoehr and Milner, 1949; and others).

Extreme nutrient deficiency is probably of more academic than oceanographic significance. When deficiency becomes extreme, net O_2 production falls below 50 percent of gross O_2 production. Steemann Nielsen and Al Kholy (1956) pointed out

that a phytoplankton population would not persist in nature under such conditions. During the night, respiration would presumably continue at the same rate as during the day, and, assuming a 12-hour day-length, gross photosynthesis must be at least twice the amount of respiration. This point was reiterated by Strickland (1960). Extreme nitrogen deficiency was achieved in one of the present experiments (experiment 4); C^{14} uptake agreed most closely with gross O_2 production under this condition. At this time, the photosynthetic capacity was much reduced; and growth constants and net O_2 evolution were negative.

Ryther (1954), using a related alga, *D. euclyora*, presented data on the effects of nutrient deficiency on C^{14} and O_2 measurements. He gave no details about the culture conditions used in growing the alga, so that one cannot tell whether his results were due to nitrogen or to phosphorus deficiency. After 15 days of growth, however, net O_2 production was 50 percent of gross O_2 production; after 30 days, net production was only about 5 percent of gross production. In both cases, C^{14} uptake agreed well with net O_2 production. On the other hand, Steemann Nielsen and Al Kholy (1956), using the fresh-water green alga, *Chlorella pyrenoidosa*, showed that C^{14} uptake in nitrogen- and phosphorus-deficient cultures did not differ greatly from gross O_2 production. The conditions of their cultures appear to approach those of Ryther after 30 days of growth, since net O_2 production was only a small fraction of gross O_2 production.

Results given in experiments 3 and 4 indicate that C^{14} uptake agrees closely with net O_2 production during a period of incipient nitrogen deficiency. These data agree with those obtained by Ryther in 15-day cultures. Furthermore, the agreement between growth and photosynthesis under these conditions also suggests that the C^{14} method measures a net increase in algal biomass.

The effects of extreme nitrogen deficiency were less clear. In the latter stages of experiment 4, when the cells were extremely deficient, net O_2 evolution was negative, and C^{14} uptake corresponded more closely with gross O_2 production. This result is more in agreement with the comparisons of Steemann Nielsen and Al Kholy than those of Ryther. However, the low values for net O_2 evolution in experiment 4 may be erroneous

due to bacterial contamination. Cells from an older, bacteria-free culture (experiment 5), which may also have been extremely nitrogen-deficient, assimilated C^{14} at a rate which corresponded to net O_2 evolution. Obviously, further studies of the effects of extreme nitrogen deficiency in bacteria-free culture would be of interest but would be less significant than investigations of incipient deficiency.

Phosphorus deficiency was not studied in as great detail as nitrogen deficiency. In the latter stage of experiment 6 (tables 11 and 12), with cells that were probably in a stage of incipient deficiency, C^{14} uptake did not differ significantly from either net or gross O_2 production, and was intermediate between the two O_2 values. In another phosphorus-deficient culture (experiment 7), which was also bacteria-free and was older than that of experiment 6, C^{14} uptake was much less than net O_2 evolution. Phosphorus deficiency in this experiment may have been more extreme than in experiment 6, and, under such conditions, soluble, C^{14} -labelled, organic matter might have been excreted by *Dunaliella* cells. Such material would not have been collected on the membrane filter and included in the measurement of C^{14} uptake.

In another test of the general validity of the C^{14} method, C^{14} uptake was shown to be equivalent to the uptake of CO_2 as measured by pH changes. These experiments (8 and 9) were carried out with healthy cells.

To relate studies with laboratory cultures to C^{14} measurements carried out in the open sea, it would be necessary to use cultures of representative oceanic phytoplankton species in experiments such as these. *D. primolecta* may only be representative of the phytoplankton of British coastal waters, from which it was isolated (Butcher, 1959), and caution is necessary in applying the results of the present experiments to measurements of oceanic photosynthesis.

To the extent that *Dunaliella* is a representative alga, however, these experiments indicate that the C^{14} method provides a reliable measure of net photosynthesis and of increase in phytoplankton biomass, under good conditions for phytoplankton growth and under conditions of incipient nitrogen deficiency. Fortunately, cultures of representative oceanic phytoplankton are becoming available

(Thomas, 1959), and could be used for further studies of the C^{14} method.

SUMMARY

1. During the logarithmic phase of growth of cultures of *D. primolecta*, photosynthetic C^{14} uptake agreed well with net O_2 production, but also did not differ greatly from gross O_2 production.

2. During this growth phase, the growth rate of mass cultures was generally less than the photosynthetic rate (C^{14} uptake and net O_2 production) in bottles of the cell suspension placed within the culture. This difference was attributed to different illumination conditions between the bottles and the culture. In one experiment, when illumination conditions were similar, growth in the culture was equivalent to photosynthesis in the bottles.

3. In cultures that were incipiently nitrogen-deficient, C^{14} uptake was similar to net O_2 production and growth was equivalent to photosynthesis.

4. In one experiment, under conditions of extreme nitrogen deficiency, C^{14} uptake was greater than net O_2 evolution, and more closely approximated gross O_2 evolution. In another such experiment, C^{14} uptake agreed well with net O_2 evolution.

5. Nitrogen deficiency in *Dunaliella* suppresses the formation of protein and a carbohydrate, probably starch becomes a major component of the cells.

6. In one experiment, C^{14} uptake in an incipiently phosphorus-deficient culture was intermediate between net and gross O_2 evolution; in another experiment, C^{14} uptake was less than net O_2 production in a phosphorus-deficient culture.

7. C^{14} uptake in healthy cultures of *Dunaliella* was equivalent to the uptake of CO_2 as measured by pH changes.

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