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C^{14} IN PHOTOSYNTHESIS

by

A. A. Benson, M. Calvin, V. A. Haas, S. Aronoff,
A. G. Hall, J. A. Bassham and J. W. Weigl

May 28, 1948

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Iowa State College	43
Kellex Corporation	44-45
Los Alamos	46-48
Massachusetts Institute Of Technology	49
Monsanto Chemical Company, Dayton	50-51
National Bureau Of Standards	52-53
Naval Radiological Defense Laboratory	54
NEPA	55
New York Directed Operations	56-57
Oak Ridge National Laboratory	58-69
Patent Advisor, Washington	70
Technical Information Division, ORDO	71-85
UCLA Medical Research Laboratory (Warren)	86
University of California, Radiation Laboratory	
Information Division	87-90
Chemistry, Bldg 4	91
M. Calvin	92-141
University of Rochester	142-143
Western Reserve University (Friedell)	144
Office of Chicago Directed Operations	145
Declassification Procedure	146-155

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Radiation Laboratory
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Berkeley, California

C^{14} IN PHOTOSYNTHESIS

by

A. A. Benson, M. Calvin, V. A. Haas, S. Aronoff
A. G. Hall, J. A. Bassham and J. W. Weigl

Radiation Laboratory and Department of Chemistry, University of California, Berkeley, California.*

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May 28, 1948

Abstract

The general methods for using $C^{14}O_2$ in studying the mechanism of photosynthesis are described. The results tabulated give relative distributions of radioactive carbon in the photosynthetic intermediates which have been isolated and characterized. This paper is a compilation of the essential results, most of which have been published in separate papers which have been found in this laboratory.

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* This paper is based on work performed under Contract No. W-7405-Eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley, California.

To be declassified for publication in A.A.A.S. Symposium on Photosynthesis.

TABLE OF CONTENTS

INTRODUCTION

EXPERIMENTAL

1. Continuous Cultivation of Chlorella pyrenoidosa and Scenedesmus D-3
2. Barley Seedlings
3. Preparation of $Na_2C^{14}O_3$
4. Determination of Radioactivity
5. Effect of Preillumination on Dark Fixation Rates
6. Effect of Preillumination on CO_2 Reducing Power
7. Effect of Replacing Nitrate in the Nutrient by Ammonium Ion on Preillumination Curves
8. Photosynthetic $C^{14}O_2$ Fixation by Algae
9. Isolation of Radioactive Products from Algae
10. Photosynthesis by Barley Seedlings
11. Identification of Products
    a. Fractionation of Ether-Soluble Carboxylic Acids (I)
    b. Isolation of Radioactive Succinic Acid from Chlorella
    c. Alanine Determination
    d. Qualitative Identification of Radioactive Amino Acids
    e. Identification of 3-Phosphoglyceric Acid
    f. Identification Tests on Compounds Appearing in Fraction III-A
    g. Identification of Neutral Substances
12. Degradation of Products
    a. Succinic Acid
    b. Malic Acid
    c. Alanine
    d. Aspartic Acid

e. Glucose

RESULTS

Table I. Fractionation of 5-Minute Dark Fixation Products of Chlorella with varying times of Preillumination.

Table II. Fractionation of the 1-Minute Dark Fixation and the 30-Second Photosynthesis of Both Chlorella and Scenedesmus.

Table III. Fractionation of Several Photosynthetic Barleys.

Table IV. Degradation Data of Photosynthetic Products.

DISCUSSION

C^{14} IN PHOTOSYNTHESIS

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28 May 1948

INTRODUCTION

A number of tracer elements have already been used in an attack on the problem of photosynthesis. These include oxygen 18 (1), tritium (2), deuterium,

(1) Ruben, S., Randall, M., Kamen, M., Hyde, J. L., J. Am. Chem. Soc., 63, 877 (1941)
Vinogradov, A. P. and Teis, R. B., Compt. rend. acad. Sci. U.R.S.S., 33, 490 (1941)
Vinogradov., A. P., Bull. acad. Sci. U.R.S.S. Ser. Biol. 1947
French, C. S. and Holt, A. S., Reported at A.A.A.S. Symposium on Radioactive Iostopes in Plant Physiology Research, December, 1947

(2) Norris, T. H., Ruben, S., Allen, M. B., J. Am. Chem. Soc., 64, 3037 (1942)

phosphorus 32 (3) and carbon 11 (4). The importance of the carbon isotopes for

(3) Aronoff, S., and Calvin, M., Plant Physiol. (1948)

(4) Ruben, S., Hassid, W. Z., Kamen, M., J. Am. Chem. Soc., 61, 661 (1939)

* This paper is based on work performed under Contract No. W-7405-Eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley, California.

such a study is, of course, obvious and with the ready availability of the long-lived carbon 14 since 1945, the study of the path of carbon in photosynthesis has been undertaken in a large number of laboratories in this country.

The method of attack is a straightforward one. First, the plants must be fed labeled carbon dioxide under as wide a variety of conditions as seems feasible, ranging from dark feeding after suitable pre-treatments to increasingly long periods of photosynthesis in the presence of radioactive carbon. Included also should be a variation in the dark time following the adminsitration of labeled carbon dioxide in the light. During the course of these experiments, the kinetics of the total incorporation of the radioactive carbon dioxide should be studied under each set of conditions, following which an analysis of the plant substance is made in order to identify the compounds or substances into which the radioactive carbon has been incorporated. After these have been identified, the distribution of the radioactive atoms within each compound is to be determined.

With these data at hand, it becomes possible to make hypotheses describing the sequence of intermediates through which the carbon passes on its way from carbon dioxide to the various plant constituents. From the effect of the several variables on the nature of the compounds into which the tracer is incorporated and the rate at which they appear, it should be possible to determine the relative importance of the proposed steps. It should then be possible to test these hypotheses by the usual methods of biochemistry, such as the administration of synthetically prepared labeled intermediates, the use of various poisons, and, finally, the attempted isolation of the enzymatic and photochemical units from the organized plant cells, leading ultimately, perhaps, to the possibility of the reconstruction of the whole sequence of

reactions, each separated from the other.

The bare results of the beginnings of such an investigation have already been reported (5, 6). It is the purpose of this paper to describe the experimental methods that were used to achieve these results, together with a discussion of them. In view of the fact that the preparation of the biolo-

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(5). Benson, A. A., and Calvin, M., Science, 105, 648 (1947)

(6). Calvin, M, and Benson, A. A., Science, 107, 476 (1948)

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gical materials (algae), the radioactive material and its measurement and the chemical isolations, identifications, and degradations were very much the same for each experiment in which the method of administration and the immediate pre-treatment of the algae were varied. These items will be discussed first in general terms. The specific results for each type of experiment will then be presented, followed by a hypothesis based upon these results.

EXPERIMENTAL

Continuous Cultivation of Chlorella pyrenoidosa and Scenedesmus D_3 (*).- The

(*) Obtained through the courtesy of Professor H. Gaffron, Department of Chemistry, University of Chicago.

algae were grown in continuous culture flasks as pictured in Figure 1. The flasks were mechanically shaken and a stream of air (1700 m./min.) and CO_2 (190 ml/min.) drawn through the flasks. The flasks were illuminated from below by two 100 watt white (4500°K) fluorescent lights (intensity approximately 5000 lux). The temperature varied from 20-27° C. The culture solution using doubly-distilled (glass) water, had the following composition.

KNO_3	0.005M	0.506 g/liter
KH_2PO_4	0.001M	0.136 g/liter
$MgSO_4 \cdot 7\ H_2O$	0.002M	0.493 g/liter
$Ca(NO_3)_2$	0.00025M	0.041 g/liter
H_3BO_3	0.000028M	1.43 mg/liter
$MnSO_4 \cdot H_2O$	4.5×10^{-6}M	1.05 mg/liter
$ZnCl_2$	3.7×10^{-7}M	0.05 mg/liter
$CuSO_4 \cdot 5\ H_2O$	1.6×10^{-7}M	0.04 mg/liter
$H_2MoO_4 \cdot H_2O$	5.6×10^{-8}M	0.01 mg/liter
$Fe(NH_4)_2SO_4 \cdot 6\ H_2O$	1.38×10^{-5}M	6.2 mg/liter

pH 4.5

All constituents except the $Fe(NH_4)_2SO_4$ were mixed (designated as solution A) and autoclaved for 20 minutes at 15 lbs. The $Fe(NH_4)_2SO_4$ solution

(designated as solution B) was prepared by adding 5.6 g. of $Fe(NH_4)_2SO_4$ to one liter of boiling water previously acidified to pH 2 with 6N H_2SO_4 and autoclaved immediately. The acid pH and removal of oxygen prevented the oxidation of ferrous ion during autoclaving. One cc. of this solution was added to 899 cc. of solution A each day to make the final culture solution.

Stock cultures of Chlorella pyrenoidosa and Scenedesmus D_3 were kept on 2% agar slants of solution A and B plus 0.2% glucose. After growth, the slants were stored at 5°C until used.

Chlorella pyrenoidosa were harvested every 24 hours by withdrawing 900 cc. of culture from the flask. Approximately 1.5 cc. of packed cells (20 minutes at 530 G) were obtained at each harvesting. A refrigerated centrifuge was used to prevent heating of the cells during the centrifugation. When centrifuged without cooling the cells were heated enough to greatly impair their rate of photosynthesis. Scenedesmus D_3 were harvested every 48 hours, and approximately 1 cc. of packed cells was obtained per 900 cc. of medium. The growth of the cultures may be determined by placing a calibrated photronic cell in series with a microammeter at the surface of the culture flask to measure the light absorbed by the organisms.

After harvesting, one cc. of solution B and 899 cc. of solution A were added to the culture flask. The 100 cc. of culture medium remaining in the flask served as an inoculum for this solution.

The average rate of photosynthesis of cells grown under these conditions was 0.47 $mm^3 O_2/min/mm^3$ for Chlorella pyrenoidosa and 0.43 $O_2/min/mm^3$ for Scenedesmus D_3.

One cc. of packed Chlorella cells contained 4.4 mg. of chlorophyll and 0.155 gms. of dry matter. One cc. of packed Scenedesmus cells contained 5.5 mg. of chlorophyll and 0.127 gms. of dry matter.

Continuous cultures of Chlorella pyrenoidosa have been grown for 2½ months before the production of mucilaginous material by the organisms caused the cells to adhere to one another and to the surface of the culture flask. These cells settled rapidly and showed a decreased rate of carbon dioxide fixation. Therefore, the culture was abandoned and a new continuous culture started with a fresh inoculum of Chlorella pyrenoidosa. Scenedesmus D_3 cultures started to clump after about one month of continuous culture. At this time, a new culture was started using as an inoculum Scenedesmus D_3 from an agar slant.

When the cells were centrifuged a thin layer of white material appeared on the surface of the packed cells. This material, when examined microscopically, appeared to be debris from dead cells and no bacteria were seen in this white layer.

When solution A was used to prepare the culture medium, the initial pH was 4.5. At the time of harvesting, the culture of Chlorella pyrenoidosa has a pH of 7.3 and the culture of Scenedesmus had a pH of 6.5. When $(NH_4)_2SO_4$ replaced KNO_3 in solution A and $CaCl_2$ was used instead of $Ca(NO_3)_2$ no growth of Chlorella pyrenoidosa was obtained. However, when the pH of this medium was changed from the initial value of 4.5 to pH 8.7-9.0 with 1 N NaOH, growth was as rapid as in the corresponding nitrate medium at pH 4.5. During growth in an ammonium medium the pH decreased so that in 24 hours the pH of the

culture was between 6.5-7.0. After 48 hours the pH was 3.0 and growth had ceased. Cells transferred from the culture medium at pH 3 to fresh medium at pH 8.7 were unable to grow. When growing continuous cultures of Chlorella pyrenoidosa in an ammonium medium the pH of the culture was determined by drawing a portion of the liquid into a chamber attached to the culture flask which contained micro pH electrodes (see Figure 2). The culture was kept between pH 9.0-7.0 by adding 1 N NaOH at approximately 12 hour intervals.

Barley Seedlings. - Barley seedlings, grown in the green house in Hoagland's nutrient solution, were freshly cut for experiments when three inches long(*).

(*) We are indebted to the Division of Plant Nutrition for the culture of barley seedlings used in these experiments.

Preparation of $Na_2C^{14}O_3$. - Barium carbonate containing approximately 4% C^{14} was converted to carbon dioxide by adding concentrated sulfuric acid from a by-passed dropping funnel to the dry powder *in vacuo*. The evolved gas was collected in a glass spiral trap of 20-60 ml. volume, immersed in liquid nitrogen, through which the CO_2 generating apparatus is connected to the high-vacuum line. At pressures of 10^{-4} mm. Hg the quantitative transfer of CO_2 is practically instantaneous. The CO_2 generating assembly was then removed from the spiral trap and replaced by a 10 ml. volumetric flask containing 1-2% excess carbonate-free sodium hydroxide (*) dissolved in a

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(*) Prepared by filtering 20.5 N NaOH with Celite filter aid.

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few ml. of water. The alkali solution was frozen in liquid nitrogen and the flask evacuated, whereupon the CO_2 contained in the spiral (a by-passed

spiral is most convenient) was transferred to the flask. After melting, the alkali solution was diluted with water to the given volume. It was found convenient to prepare one to two millicuries of sodium carbonate per 10 ml. solution. For determination of dark fixation rates, the stock solution was diluted 1:25.

Determination of Radioactivity. - In all chemical work radioactivity was determined using large diameter (65 mm.) helium-filled atmospheric pressure Scott Geiger-Müller tubes operation at approximately 2000 volts. The mica window thickness varied from 0.79 to 1.3 mg/cm^2. The efficiency of such tubes is 5-7 disintegrations per count. Very weak samples, 0-20 c.p.m. (counts per minute), were measured using an automatic background-sample alternator developed in this laboratory which served to reduce errors due to background fluctuations (35-45 c.p.m.). In this way, samples could be compared with background in 8 minute cycles as long as necessary. The use of a windowless methane-filled counter such as the Nucleometer will further simplify the counting of weak samples.

Samples of 11.5 cm^2 area were mounted on aluminum and glass (1/32 inch thick) disks described elsewhere (7). Carbonate was usually counted as

(7). Calvin, Heidelberger, Reid, Tolbert, and Yankwich, "Isotopic Carbon" John Wiley and Sons, 1948, in press.

$BaCO_3$ and gave reproducible and reliable results. Organic samples were counted on both types of plates depending on acidity and surface tension properties involved. It has been found that self-absorption corrections are negligible for sample thicknesses less than 0.2 mg/cm^2, and such corrections

are unreliable for thicker layers. Hence, in all quantitative work, sample thicknesses were restricted to 0.2 mg./cm^2 for uniformly smooth layers of non-crystalline substances. Samples of crystalline substances must be very thin since the thickness of the individual crystals will determine the self-absorption of the sample. Such samples give reproducible ($\pm$ 3%) activities, and the results with widely differing materials may be safely and accurately compared as shown by the activity balances involved in Table II of this paper.

In degradations and co-crystallizations, specific activities must be accurately determined. Pure crystalline compounds are weighed on the aluminum disks after which the sample (0.50 to 2.50 mg.) is dissolved on the disk in an appropriate solvent and spread on a rotating turntable under a hot air dryer to give a uniform circular film.

Effect of Preillumination on Dark Fixation Rates. - The illumination vessel (Figure 3) was a water-jacketed circular vessel one centimeter thick of 50 ml. volume. Algal suspensions of 1.0-1.5 cc. packed cells in 50 ml. fresh nutrient solution were prepared from fresh, day-old cultures of Chlorella and two-day old Scenedesmus cultures. Illumination from both sides of the illumination vessel with 300 watt reflector spot lights using adequate water-cooled glass infra-red absorbing filtres was found satisfactory for such cell concentrations (Figure 4). By means of an aliquot bulb equipped with appropriate stopcocks at the bottom of the illumination vessel, a reproducible sample of 1.8 ml. could rapidly be withdrawn and transferred to an evacuated 30 ml. blackened flask containing 0.100 ml. of $Na_2C^{14}O_3$ solution (0.8 μc.). The stopcock attached to the black flask was closed and the sample removed from the illumination vessel. For five-minute

dark fixations, shaking was done mechanically while the one-minute fixations were shaken manually. The algae were killed rapidly by adding 0.500 ml. of acetic acid-concentrated hydrochloric acid solution (4:1) to the ground joint above the stopcock and turning the stopcock at the desired killing time. The algal aliquot had been flushed into the black flask with less than one atmosphere of nitrogen pressure leaving sufficient vacuum to accommodate the acetic acid solution. The fixed non-volatile radioactivity was determined on samples prepared by evaporating 200 μl. of the suspension on glass disks.

Curve A, Figure 5, was obtained by preparing a series of samples from Scenedesmus suspension which had been in the dark in 4% CO_2 in nitrogen for one hour followed by rapid flushing with helium for 20 minutes. The samples were killed after appropriate times of shaking with the labeled carbonate. Curve B was obtained by taking a series of preilluminated samples from the same Scenedesmus suspension after illumination(constant helium flushing) for 10 minutes. The initial slope corresponds to a dark fixation rate of 0.1 $mm^3 CO_2/mm^3$ cells/min. at a CO_2 partial pressure of 0.18 mm. (8).

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(8). Effect of CO_2 pressure on the rate of dark fixation by algae. Calvin, M. and Benson, A. A., to be published.

Effect of Preillumination upon CO_2-Reducing Power. - Chlorella and Scenedesmus cultures used for these experiments are those described above. Fresh cells were rapidly centrifuged and resuspended in fresh nutrient and placed in the illumination vessel as with dark fixation rate experiments.

In order to obtain a reliable starting point for the experiments, the illumination vessel was darkened, and 4% CO_2 in nitrogen was bubbled through the cell suspension for one hour after which it was removed with a vigorous helium stream for 20 minutes. To a large number of black 30 mo. flasks equipped with stopcocks and ground joints was added 100 μl. (0.82 μc.) of the diluted $Na_2C^{14}O_3$ solution. To each of these flasks was added an aliquot (1.68 ml.) of the algal suspension at suitable times during the experiment. The operation of filling the aliquot bulb and flushing the sample into the black flask with 0.8 atm. of nitrogen pressure required less than two seconds, during which time the sample was in the aliquot bulb. Several dark samples were always taken to ascertain that the CO_2-fixing power of the algae was constant. These results give the initial straight line in Figures 6 and 7.

Two 300 watt reflector spot lights were then turned on the illumination vessel using appropriate water-cooled glass infra-red absorbing filters. Circulation of tap water through the water-jacket of the illumination vessel maintained a temperature in the algae suspension at 20°C. The curve shown in Figure 6 shows the results with Chlorella in which the aliquot of algae is shaken for 5.0 minutes in the dark with $C^{14}O_2$. The curves in Figures 5 and 7 were obtained with Dark $C^{14}O_2$-fixation times of one minute.

Experiments in which CO_2-free air was used instead of helium during preillumination gave similar curves. The rate of growth of reducing power was unaffected, but the maximum attained and its stability with time appeared to be diminished appreciably.

<u>Effect of Replacing Nitrate in the Nutrient by Ammonium Ion on Preillumination Curves</u>. - Chlorella cells grown in nutrient containing nitrate ion as

the source of nitrogen were centrifuged and washed with a nutrient solution containing only ammonium ion (pH 4.5) as a nitrogen source. The cells were resuspended in this nutrient and their ability to store 'CO_2-reducing power' was measured as described above. The experiment was performed in a different manner in that the algae were preilluminated 30 minutes until a maximum was reached. The lights were turned off and the decay rate checked. The 'growth' rate following this decay was very similar to that shown in Figure 6 for normal $[NO_3^-]$ -grown Chlorella.

As a check on the validity of this conclusion, a Chlorella culture was reinoculated three times in NH_4^+ medium and shown to give a 'growth' curve identical with that for normal Chlorella and for cells merely suspended in (NH_4^+) medium.

Photosynthetic $C^{14}O_2$ Fixation by Algae. - The illumination arrangement described for preilluminating algal suspensions was adapted to illuminate a 130 ml. circular vessel with flat sides 20 mm. apart. One cc. of packed cells suspended in 100 ml. of nutrient solution was allowed to photosynthesize 4% CO_2 in air in the vessel for one hour. The gas was then changed to air for 5 minutes, after which the inlet tube was rapidly removed from the vessel and 500 λ (0.10 mc.) of $Na_2C^{14}O_3$ stock solution was rapidly injected into the solution. The vessel was stoppered and shaken vigorously in the light beams for the required time. Simultaneously with cessation of illumination, 20 ml. of acetic acid-hydrochloric acid solution is rapidly injected into the body of the solution. After standing 10 minutes in the dark, the products were isolated as described for dark $C^{14}O_2$ fixations. In an experiment with similar cells killed by rapidly pouring the solution into boiling ethanol, the activity fixed was the same.

Isolation of Radioactive Products from Algae. - The isolation process described below was developed to separate CO_2-fixation products of dark fixations and short photosynthetic fixations into groups of chemically similar compounds which could be further separated by more specialized procedures. Any fractions discarded or not mentioned were not found to possess significant radioactivity.

The algae suspension, having been rapidly killed by an addition of a fifth its volume of acetic acid-hydrochloric acid solution, was allowed to stand for 10 minutes during which unused $C^{14}O_2$ was recivered by aspiration through sodium hydroxide. The suspension was filtered with Celite into a graduated cylinder. In the case of dark fixations and short photosynthetic fixations, no radioactive products remain insoluble. After determining the activity of a suitably sized aliquot (10 to 500 μl.), the solution was subjected to a rapid continuous ether extraction for 15 hours. The ether extract was evaporated to dryness at reduced pressure and taken up in glacial acetic acid for determination of radioactivity. The aqueous phase was evaporated to dryness in vacuo below 20°C, redissolved in 20 ml. H_2O for determination of activity, and successively passed through 25 cc. columns of Duolite C-3 cation exchange resin and Duolite A-3 anion resin. The effluent sugar solution was rendered acid to phenolphthalein, if necessary, by addition of a drop of dilute hydrochloric acid. The resins were washed with 250 ml. of water, and the total effluate was evaporated at reduced pressure to a convenient small volume for determination of radioactivity (Fraction IV).

Fraction II was obtained upon elution of the cation exchange resin with 100 ml. of 7% hydrochloric acid during one hour. Separation of alanine

from this fraction is described below. Further identification of the amino acids synthesized will be described in future publications (9).

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(9). Stepka, W., Benson, A. A. and Calvin, M. Science.

Elution of the anion exchange resin was first done with 100 ml. of 1.5 N ammonium hydroxide during one hour or more after which the column was washed with 250 ml. of water. Evaporation at reduced pressure to a convenient volume gave Fraction III-A. Further elution with ammonia removed no more radioactivity. The resin was then eluted with 30 ml. of 1.5 N sodium hydroxide during one hour. The eluate and the succeeding 250 ml. of water wash was directly passed through a 50 cc. cation resin column (Duolite C-3) which removed the sodium hydroxide. The effluate (Fraction III-B) was evaporated to a convenient volume for determination of radioactivity.

Photosynthesis by Barley Seedlings. - Gaseous $C^{14}O_2$ was fed to the leaves in the apparatus described previously (10). The leaves were stored in liquid nitrogen after the experiments until extraction. In experiment III, the

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(10). Aronoff, S., Benson, A. A., Hassid, W. Z., and Calvin, M., Science 105, 664 (1947)

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products were separated by ether and 80% ethanol extractions of lyophilized leaves. In experiments IV and V, the leaves were subjected to repeated extractions with aqueous acetic acid solution. The combined extracts were evaporated to dryness or to a convenient volume and subjected to continuous ether extraction. Succeeding fractionations were similar to those used

with algal extracts. The results of these experiments are tabulated in Table III.

Identification of Radioactive Products

Fractionation of Ether-Soluble Carboxylic Acids (I). - The continuous ether extract was evaporated to dryness in vacuo and taken up in 0.2 ml. of n-butanol. The solution was diluted with nine volumes of chloroform and the seapration carried out according to the method of Isherwood (11). For the separation of malic and succinic acid, 5 mg. amounts of each were added as

(11). Isherwood, F. A., Biochem. J., 40, 688 (1946)

carriers to produce the detectable indicator color as each emerged from the column. The activities so separated were diluted with added carrier for degradation experiments (12).

(12). Benson, A., and Bassham, J. A., J. Am. Chem. Soc., in press.

Isolation of Radioactive Succinic Acid from Chlorella. - Chlorella cultures 10-14 days old were harvested in a refrigerated Sharples super-centrifuge and resuspended in distilled water. To a CO_2-free suspension of 469 g. of packed cells in one liter of water was added 100 μc. of $C^{14}O_2$, the mixture shaken vigorously for 30 minutes in the dark after which 100 ml. of acetic-hydrochloric acid solution was added to rapidly kill the cells. The radioactive products were separated from the cells by filtration and subjected

to an ether extraction at pH 6.0 which removed very little radioactivity. Continuous ether extraction at pH 1.0 removed 80% of the activity (400,000 c.p.m.). Evaporation of the ether extract gave 50 mg. of crystalline succinic acid containing a small amount of radioactive fumaric acid. The radioactive product was identified as succinic acid by determination of equivalent weight, melting point, distribution coefficient between water and ether at pH 1.0 and between water and ethyl acetate, dependence of distribution coefficient on pH of aqueous phase, titration curve, molecular weight, elementary analysis and by x-ray powder diffraction pattern.

Alanine Determination. - To a suitable aliquot of the cation eluate was added 100 mg. of dl-alanine and excess 3 M $KHCO_3$ solution. N-Benzoyl alanine was prepared in the usual manner by stirring with excess benzoyl chloride. After removing the benzoic acid with ligroin the product was extracted with chloroform and crystallized from acetic acid-petroleum ether solution (yield, 100-200 mg.). The specific activity of the product was usually constant after one crystallization. The alanine activity of the original aliquot is then calculated from the theoretical yield and the specific activity.

Qualitative Identification of Radioactive Amino Acids. - The identification of amino acid constituents of plants as well as identification of radioactive amino acids has been performed using filter paper chromatography radioautograph tachnique (13). The results of these experiments will be reported in future publications (14).

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(13). Fink, Robert and Fink, Kay, Science, 107, 253 (1948)

(14). Stepka, W., Benson, A. A., and Calvin, M., to be published.

Identification of 3-Phosphoglyceric Acid. - To a 200 μl. aliquot of the fraction III-B (30-second photosynthetic Scenedesmus) containing 9250 c.p.m. of C^{14} was added 5.4 mg. of barium 3-phosphoglycerate. The specific activity of the crystalline salt after two recrystallizations from water was found to be 1650 c.p.m./mg. for 0.40 mg. sample. A third recrystallizations gave a specific activity of 1600 c.p.m./mg. for a 0.29 mg. sample, while a fourth recrystallization was 1600 c.p.m./mg. for a 0.49 mg. sample. This assay indicates that 94% of III-A is 3-phosphoglyceric acid but does not represent unequivocal proof since similar compounds may "carry" the radioactivity as well.

Hydrolysis of 3-phosphoglyceric acid was carried out in the following typical manner. To a 141,000 c.p.m. aliquot of III-B (from pre-illuminated Scenedesmus) in 10 ml. of 1.0 N hydrochloric acid was added 3 mg. of barium 3-photphoglycerate and 15 mg. of sucrose. The solution was heated at 100° in an evacuated sealed tube for 8 days. The hydrolysate was evaporated to dryness in vacuo, taken up in water, and was found to have 115,000 c.p.m. It was adsorbed on a 25 cc. Duolite A-3 column and thoroughly washed with water. Upon elution with 200 ml. of 1.5 N ammonium hydroixde, 82,000 c.p.m. was found in the evaporated eluate. In separate experiments it was found that 3-phosphoglyceric acid is not eluted from Duolite A-3 by ammonia while glyceric acid is readily elutable with ammonia.

p-Bromophenacylglycerate was prepared from 112 mg. (0.67 mmols.) of glyceric acid syrup together with the above 82,000 c.p.m. hydrolysate. It was found advisable to use only 100 mg. (0.36 mmols.) p-bromophenacyl bromide in order to obtain a purer product. The specific activity of the crystalline product was found to be 455 $\pm$ 50 c.p.m./mg. which indicates that the hydroly-

sate activity is all glyceric acid.

Solvent distribution constants for the hydrolysate activity were compared with that of authentic glyceric acid and found to be identical within the errors involved. Distribution of p-bromophenacylglycerate (from fraction III-B of 30-second photosynthetic Scenedesmus) between phases in a mixture of 0.50 ml. toluene, 0.20 ml. acetic acid, and 0.07 ml. water was carried out using 5.69 mg. (310 c.p.m./mg.) of the ester. The specific activity of an aliquot of the lower phase was 275 c.p.m./mg. for a 0.030 ml. (0.90 mg.) aliquot. The organic phase had a specific activity of 308 c.p.m./mg. The apparent discrepancy between the radioactive ester and the carrier ester was clarified when it was found that 12% or more of the product from the lower phase was water which caused the low original activity. Evaporation of toluene in preparing a plate of the organic phase had thoroughly dried the sample. Elementary analysis indicated 2-3 moles of water in undried samples of the ester.

Identification Tests on Compounds Appearing in Fraction III-A. - Since carrier triose phosphates are not presently available to us an unequivocal identification of triose phosphate is not possible. The following chemical properties of the radioactive compounds in ammonia-eluates (III-A) have been observed.

The ammonia-eluate was evaporated at reduced pressure and readsorbed on Duolite A-3 resin. A small Fraction (8-15%) of the activity was found in the effluate and may be hexoses, trioses or pyruvaldehyde derived from materials in III-A. Ammonia re-elution removed only 15-20% of the adsorbed radioactivity. The major fraction of the activity was found in the sodium hydroxide eluate (after ammonia elution) and was identified as phosphoglyceric acid.

In a separate 30 second photosynthetic experiment with Scenedesmus, tests were performed to identify triose phosphate. Since Fraction III-A was readily converted to a different substance, phosphoglyceric acid, after the first elution from the resin, it was necessary to use the whole algal extract for identification experiments. To an aliquot was added hydrochloric acid to make the solution 1.0 N HCl. Carrier dihydroxyacetone was added, and the solution was steam distilled. Pyruvaldehyde dinitrophenylosazone was prepared from the distillate. Recrystallization of the product reduced the specific activity considerably to a specific activity corresponding to 10% of that expected if III-A were triose phosphate. This did not change upon successive recrystallization. Acetaldehyde dinitrophenylhydrazone was isolated from the steam distillate using carrier. This may be a break-down product of pyruvaldehyde or phospho-enol pyruvic acid. A small amount of acetic acid was identified in a similar way but no evidence for formic acid or formaldehyde was found.

Identification of Neutral Substances. - Fraction IV, the effluate from the exchange resins, was evaporated to a small volume, and aliquote were co-crystallized with glucose and fructose to constant specific activities. Although glucose and fructose may not have approximately equal activities as indicated by this method, incomplete information obtained using paper chromatography indicates the formation of both hexoses in dark fixation as well as in short photosynthetic experiments. Glucose phenylosazone was prepared using carrier glucose seedlings and fructose from sugars synthesized by photosynthesizing barley seedlings. The total activity in fructose and glucose calculated from the specific activity of the purified osazone was determined

and compared with activities in each of the sugars as determined by co-crystallization. Since the sum of the apparent activities in glucose and fructose was 120% of the activity in glucose phenylosazone, it is obvious that one or both of the radioactive sugars had co-crystallized to a certain extent with the other carrier. For degradation experiments glucose was co-crystallized with suitable amounts of radioactive sugars and gave good yields of radioactive lactic acid with Lactobacillus casei.

Degradation of Products

Succinic Acid. - Samples of labeled succinic acid obtained from separations with the silica gel columns were diluted with carrier and subjected to a Curtius degradation procedure (12). The carboxyl activity is separated as barium carbonate while the methylene carbon atoms are isolated as the hydrochloride of ethylene diamine from the Curtius reaction.

Malic Acid. - Samples of malic acid obtained from the silica gel columns were oxidized with chromic acid (12) to give CO_2 from the carboxyl groups and acetic acid from the α and β carbon atoms. The CO_2 is counted as barium carbonate and the acetic acid is counted as barium acetate.

Alanine. - N-Benzoyl alanine, of known specific activity, was hydrolyzed overnight in refluxing 48% hydrobromic acid. The solution was evaporated to dryness and the residue washed with ether. The alanine was taken up in water and its activity checked.

The decarboxylation of aliquots containing 10-15 mg. of radioactive alanine with ninhydrin was performed using the evacuated U-tube method of Van Slyke. Acetaldehyde activity was determined by dissolving the theoretical amount of 2,4-dinitrophenylhydrazine in glacial acetic acid into which

the products could be distilled. The acetaldehyde, 2,4-dinitrophenylhydrazone was recrystallized to constant specific activity from which total activity in the α and β carbon atoms of alanine may be calculated. In a separate experiment, the acetaldehyde was oxidized to iodoform, the specific activity of which gives the β-carbom activity alone. For determination of carboxyl group activity it was found necessary to decarboxylate an alanine aliquot in an evacuated small flask euqipped with a large bore stopcock, through which the carbon dioxide could later be distilled into dilute carbonate-free sodium hydroxide. The usual procedure which involved heating the alkali in the receiver causes excessive polymerization of acetaldehyde and contaminates the barium carbonate obtained.

Aspartic Acid. - Aspartic acid purified by co-crystallization of carrier with an aliquot of the cation exchange resin eluate is subjected to the same oxidation prodedure as was malic acid.

Glucose. - The degradation of hexose samples was performed using *Lactobacillus casei* according to the method of Aronoff, Barker, and Calvin (15). The validity of this method was confirmed by degradation of synthetic isotopic acetic and lactic acids (16).

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(15). Aronoff, S., Barker, H. A. and Calvin, M., J. Biol. Chem., 169, 459 (1947)

(16). Aronoff, S., Haas, V. A., and Fries, B. A., to be published.

Results

The results obtained by the foregoing methods on several preparations of algae and barley are given in the following tables.

Radioautographs of paper chromatograms of amino acid fraction II and of the total cell extract for photosynthesizing Scenedesmus (30-second) demonstrated the predominance of alanine and aspartic acid among the radioactive amino acids as well as the total absence of any detectable glutamic acid. Similar examination of the amino acid fractions of preilluminated algae gave a similar distribution of radioactivity. With preilluminated Chlorella the predominant amino acid is alanine while aspartic acid is predominant in preilluminated Scenedesmus.

From an examination of Talbe II it would appear thet the most pronounced difference between preilluminated algae and the photosynthesizing algae (30-second) is the large rise in the fraction of radioactivity found in fraction III-A.

Table I

DARK CO_2-FIXATION PRODUCTS OF CHLORELLA[a]

Dark Time--Five Minutes[b]

	PREILLUMINATION[c]	NONE	5 MINUTE	60 MINUTE	120 MINUTE
	Total fixed relative units	1	~ 10	~ 10	~ 10
I	CARBOXYLIC ACIDS in ether extracted[d]	52%	21%	14%	11%
	MALIC ACID[e]	16%	11.5%	7.4%	
	SUCCINIC ACID[e]	5.2%	3.1%	0.5%	
II	AMINO ACIDS[f] adsorbed on cation resin	31%	41%	64%	74%
III	ANIONIC SUBSTANCES[g] adsorbed on anion resin	16%	29%	21%	
IV	SUGARS non-ionized compounds[h]	0.45%	1.0%	0.96%	1.0%

(a) One day old cultures of Chlorella pyrenoidosa. (b) The cells were killed rapidly by adding 20% by volume of galcial acetic acid-hydrochloric (4:1). All radioactive products were in aqueous phase within 5 minutes. Cells removed by filtration. (c) One cc. packed cells per 60 ml. of nutrient solution was illuminated (infra-red removed) using 17,000 lux beams from both sides. A rapid stream of helium passed through the suspensions during the experiments. The maximum fixation was not diminished by illumination periods as long as 17 hours. (d) Rapid continuous 15 hour extractions. (e) Separated by partition chromatography on silica gel column. (f) Eluted from Duolite C-3 resin using 2.5 N hydrochloric acid. (g) Eluted from Duolite A-3 resin using 1.5 N sodium hydroxide. (h) Effluate from both exchange resins.

TABLE II

DISTRIBUTION OF C^{14} IN ALGAE

Alga	Chlorella[a]		Scenedesmus[b]		Chlorella		Scenedesmus	
Pre-Illumination Time	60 Minutes		10 Minutes		Photosynthesizing		Photosynthesizing	
Fixation Time	1 Minute Dark		1 Minute Dark		30 Seconds Light[c]		30 Seconds Light[c]	
Total C^{14} Fixed cpm x 10^{-6}	.	100%	.	100%	3.1	100%	6.2	100%
I Ether extractable acids	.13	13%	.12	12%	.078	2.5%	.64	10%
II Cationic (amino acids)	.51	53%	.38	39%	.44	14%	.68	11%
III-A Anionic. Ammonia elutable[d]	.023	2.4%	.041	4.2%	1.19	38%	2.75	44%
III-B Anionic. Not ammonia elutable[e]	.30	31%	.41	42%	1.13	36%	1.67	27%
IV Neutral Substances (sugars)	.0033	0.3%	.0013	0.1%	.14	4.5%	.29	4.7%

(a) **Chlorella** pyrenoidosa, one day old cultures. (b) Scenedesmus D_3, two day old cultures. (c) Cells rapidly photosynthesizing were given radioactive carbonate and shaken until removed from the light beams and instantly killed. (d) Adsorbed on Duolite A-3 and eluted with 1.5 N ammonium hydroxide. (e) Eluted with sodium hydroxide following the previous ammonia elution.

TABLE III

PHOTOSYNTHETIC PRODUCTS OF BARLEY

	Experiment III	Experiment IV	Experiment V
Age	10 days	7 day + 14 day	11 days
Pretreatment	15 min. dark	15 min. dark	52 min. dark
Time photosynthesizing	75 min.	47 min.	44 min.
Dark respiration time before freezing	5 min.	6 hours	15 min.
Total C^{14} fixed	94×10^6 (100%)	22×10^6 (100%)	98×10^6 (100%)
Insol. in 80% alc.	25%	9%	12%
Ether ext. pH 7			0.2%
I Ether ext. pH 1	1%	3%	3%
II Cation resin eluate	3.3%	4%	3.4%
III Anion resin eluate	2.9%	1.2%	2.5%
IV Neutral substances in resin eluate	58%	88%	52%

TABLE IV

DEGRADATION OF PHOTOSYNTHETIC PRODUCTS

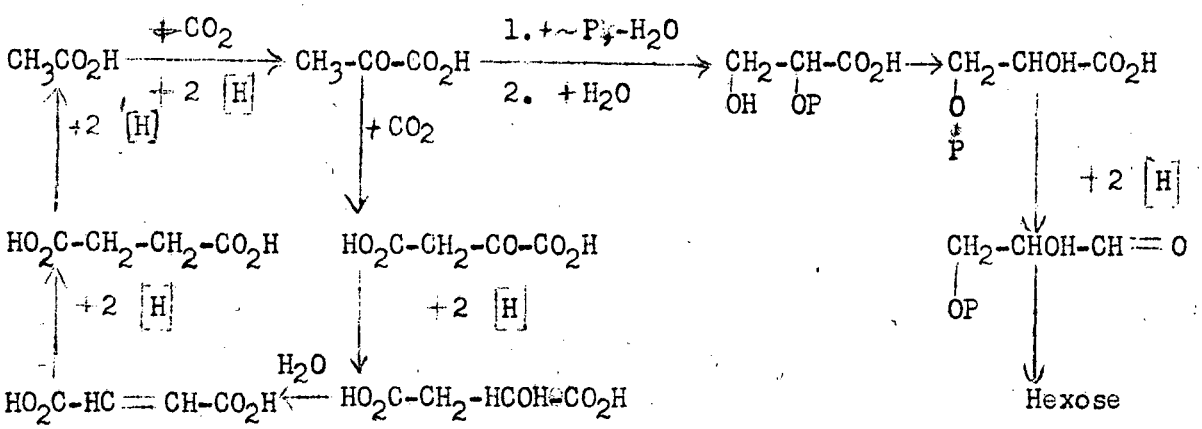
Figures Represent Percentage of C^{14} in the Various Carbon Atoms

Compound Degraded	60' Pre-illum. Chlorella 30' Dark Fix. CO_2	60' Pre-illum. Chlorella 30' Dark Fix. $CH_3\overset{*}{C}O_2H$	60' Pre-illum. Chlorella 5' Dark Fix. $\overset{*}{C}O_2$	30" P.S. Scenedesmus	40 Min. P.S. Barley II	75 Min. P.S. Barley III
Succinic Acid						
$-CH_2-$	2.5					37
$-CO_2H$	97.5					63
Malic Acid						
$-CH_2-CHOH-$			0.86	6.5		
$-CO_2H-$			99	93.5		
Alanine						
$-CO_2H$	89	66	98.1			26[a]
$-CHNH_2-$	10					49
$-CH_3$	0.5	34	2			25
Aspartic Acid				4		
				96		
Glucose						
3,4	76			87	61	36
2,5	17			6.5	24	37
1,6	7			6.5	15	27

(a) The low C^{14} content of the carboxyl group is probably due to the low specific activity of $C^{14}O_2$ photosynthesized by the plants during the last part of the experiment.

DISCUSSION

The deductive arguments leading to the following proposed scheme of the path of carbon in photosynthesis have already been presented (6). Our point of view here will be not to try and demonstrate that it is the only scheme capable of accounting for the results but rather to show that it can account for presently available experimental data.



- cycle A-

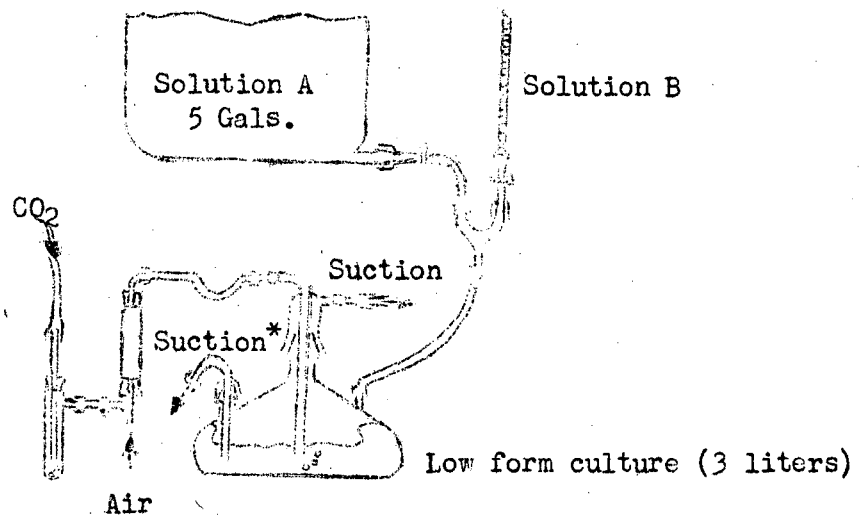
An examination of Cycle A, running in a clockwise direction, shows that its net result is the reduction of two molecules of carbon dioxide to one molecule of acetic acid. The energy required to accomplish this is obtained in the form of reducing equivalents, designated [H], from the photo-chemical apparatus involving chlorophyll and other substances. We do not say that all of these reducing equivalents have their immediate origin in the photochemical apparatus, and we cannot say, as yet, which ones do.

It is clear from an examination of the cycle that if it is running with tracer carbon dioxide, the tracer will appear first in the carboxyl groups of the three- and four-carbon acids, then, in the carboxyl group of the acetic acid and the α-carbon atoms of the three- and four-carbon acids, and, finally, in the methyl group of the acetic acid and of the β-carbon atoms of the pyruvic

acid. If the cycle should be stopped (the plant killed) after a relatively short period of operation, the specific activity of the carbon in each of the above named positions will be found to decrease in the order named. This is in accord with the available data shown in Table III.

There are at least four positions at which the products accumulated in this cycle A may be drained off to produce the normal constituents required by the plant for its life, growth, and storage. Thus, the pyruvic acid may be drained off through glyceric acid and glyceraldehyde to hexose by the direct reversal of the usual glycolytic mechanism. This would produce a hexose in which the specific radioactivity would be highest in the center pair of carbon atoms, decreasing toward each end. The earliest amino acids which are formed, namely alanine (serine) and aspartic acid, probably have their origin in the pyruvic acid and the oxalacetic acid, respectively. Finally, the two-carbon fragment corresponding to acetic acid may undergo β-condensation to aceto-acetic acid and form fats.

It should be pointed out that the compounds as given in the above scheme merely represent the nature of the chemical transformations, and they are not intended to specify the precise chemical form through which the transformations take place. These are all almost certainly enzymatic transformations. Each of the above listed compounds in the cycle may undergo the whole scheme of transformations without ever becoming free molecules unattached to their corresponding enzymes, or they may be passed from enzyme to enzyme in some specially activated form; for example, the acetic acid may be passed around only as an acetyl group attached, perhaps, to phosphate or in some other special form. Our method of isolation would not allow the detection of any labile form of these intermediates, since all such forms would have had ample opportunity to hydrolyze down to the simple compounds as shown.



* For Harvesting

Figure 1 - Apparatus for continuous Culture of Algae

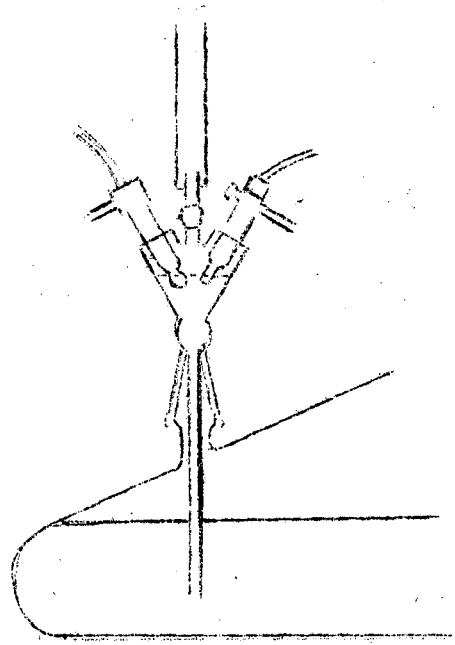


Figure 2 - Micro-pH electrode attachment for Continuous Culture Flask

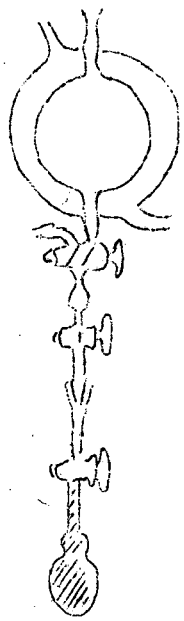


Figure 3 - Vessel for Pre-illumination of Algal Suspensions

Dark fixation is carried out in the small (30 ml.) blackened flask which is detached after receiving an aliquot of algal suspensions.

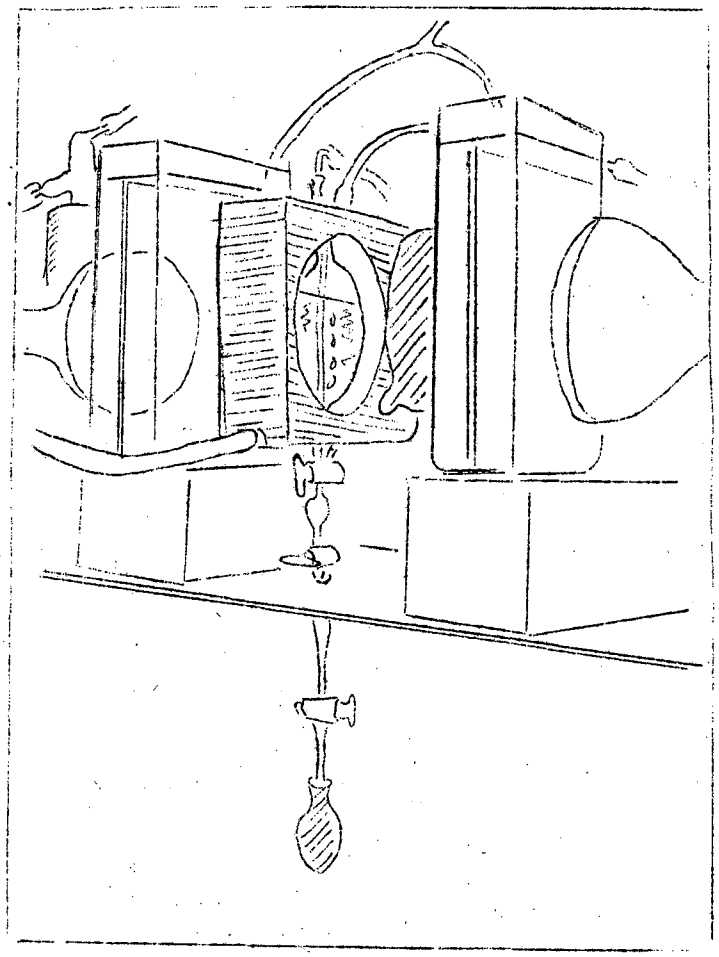
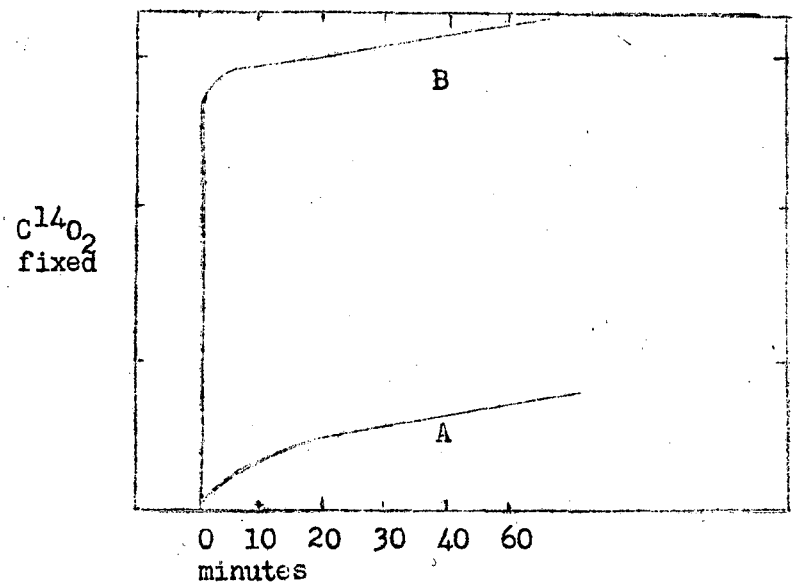


Figure 4 - Apparatus for Pre-illumination of Algal Suspensions

The dark fixation flask is protected from the light beams by being located under a shelf. Helium or 4% CO_2 is admitted to the vessel through the upper tube inserted in the illumination vessel.



RATE OF DARK FIXATION (SCENEDESMUS)

Figure 5 - Curve A represents dark CO_2 fixation by one day old Scenedesmus cultures after being in darkness one hour in the presence of 5% CO_2 in nitrogen. Curve B represents the dark fixation of CO_2 by the same cells immediately after 10 minutes pre-illumination.

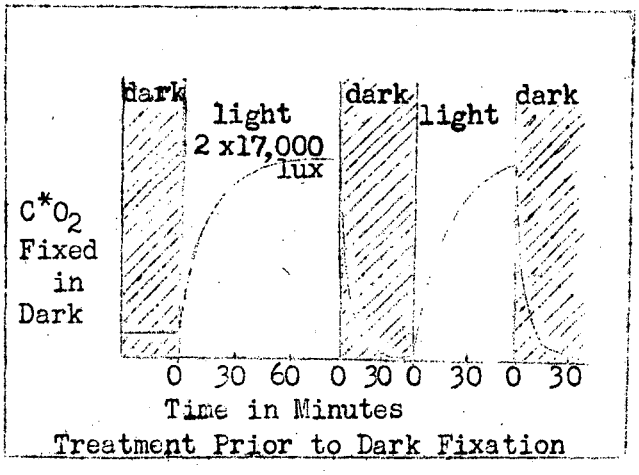
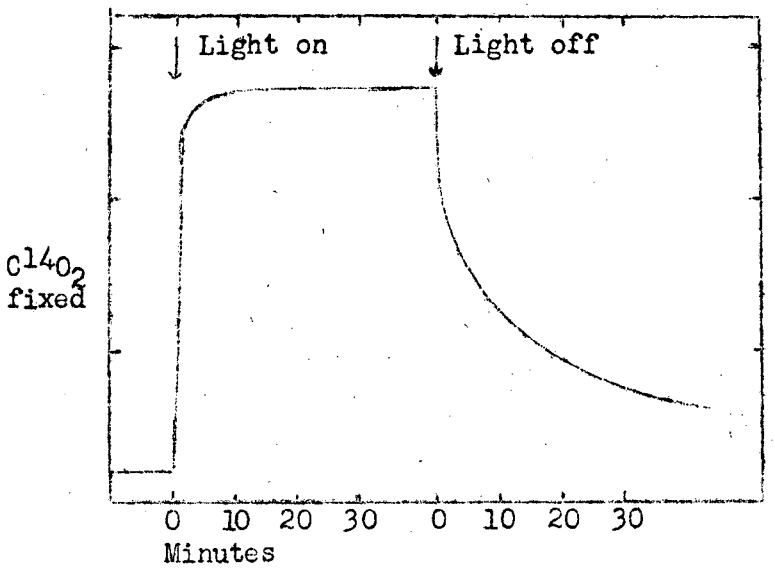


Figure 6 - Effect of Pre-illumination on Dark $C^{14}O_2$ Fixation by Chlorella

The curve represents $\overset{*}{C}O_2$ fixed by equal aliquots of algae during 5 minutes in the dark as a function of the pretreatment described by the abcissa.



Effect of Pre-illumination upon 1 minute Dark Fixation
(Scenedesmus)

Figure 7.