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Photosynthesis with Radioactive Carbon. II. Chemical Properties of the Intermediates

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In a previous communication to THIS JOURNAL[1] preliminary observations were reported on the reduction[2] of carbon dioxide by green plants (barley, sunflower, wheat) using as an indicator radioactive carbon (C^{11}). Many of the results were unexpected, and it seemed possible they were due to interchange reactions involving carbon dioxide. Only ~15% of the assimilated carbon dioxide was found in reducing sugars. In addition a dark assimilation of carbon dioxide was observed. We have since extended these experiments in detail to the green alga, *Chlorella pyrenoidosa*, which is more suited for quantitative experimentation of photosynthesis.[3] Moreover, the rate of photosynthesis per unit volume of material is far greater than in the higher plants. For example, 100 cu. mm. of *Chlorella* cells suspended in 10 cc. of water reduces more $C^{11}O_2$ than a quantity of barley sufficient to fill a 10-liter desiccator.

It now seems quite definite that the process being studied is photosynthesis since it has been possible to demonstrate the absence of extraneous interchange reactions involving carbon dioxide. Moreover, the effect of various inhibitors on photosynthesis measured by the radioactive technique is similar to that found by the usual manometric methods. *Chlorella* like the higher plants takes up small quantities of carbon dioxide in the dark. This dark reaction is reversible and is independent of the chlorophyll concentration. The carbon dioxide is incorporated in the dark reaction into a carboxyl group in a polyhydroxy molecule, presumably carbohydrate in nature. Attempts to identify the radioactive material formed with a large number of compounds likely to occur in plants have thus far been unsuccessful. It is of interest to note that no radioactive aldehydes (formaldehyde, etc.) could be detected.

(1) Ruben, Hassid and Kamen, THIS JOURNAL, **61**, 661 (1939).

(2) The terms oxidation and reduction usually imply a loss or gain of electrons. This idea has no theoretical significance when applied to the carbon compounds because the electron pairs are shared. While this limitation is generally recognized these terms are widely used for the sake of convenience and brevity. We shall employ the usual terminology (*cf.* "The Oxidation States of the Elements and their Potentials in Aqueous Solutions," W. M. Latimer, p. 118, Prentice-Hall, New York, N. Y., 1938) which does not consider the energy relationships or bond types involved, but refers to the type of atom to which carbon is bonded.

(3) *Cf.* Emerson, *Ergeb. Enzymforsch.*, V, 305 (1936).

Experimental

Preparation of C^*O_2.—The radioactive carbon was prepared in the Berkeley cyclotron by the bombardment of B_2O_3 with 8 Mev. deuterons. The nuclear reaction is

$$_5B^{10} + {}_1D^2 \longrightarrow {}_6C^{11} + {}_0n^1 \qquad (1)$$

Although elementary boron gives the highest yield of C^{11}, B_2O_3 or H_3BO_3 are more satisfactory targets since the newly formed C^* is expelled under bombardment almost completely as volatile oxides of carbon.[4] Thus the extraction of the radioactivity was achieved with minimum loss of time. This is an important consideration since C^{11} decays to half value in twenty-one minutes. The type of target chamber described by Kurie[5] was used in this work.

The active gas from the target chamber was passed into a heated combustion tube containing cupric oxide and oxidized to carbon dioxide. The carbon dioxide was trapped in a U-tube immersed in liquid air. For most experiments, the presence of a small quantity of carrier gas (carbon monoxide or methane) in the target chamber during bombardment was desirable in order to provide upon oxidation sufficient carbon dioxide for the maintenance of the maximal photosynthesis by the algae. The requisite quantity of carrier was determined by the conditions of the experiment (amount of algae used, time of exposure to C^*O_2, etc.). It was, however, desirable to use a minimum amount of carrier in order to avoid excessive dilution of the $C^{11}O_2$.

Handling of the Algae.—Glass vessels (Warburg type) containing aqueous algal suspensions were connected to a glass line and shaken by a motor-driven stirrer. A glass-bottomed water-bath was employed to maintain the algae at a constant temperature. The U-tube could be connected and the radioactive gas admitted as desired. The light source consisted of a battery of four 500-watt lamps mounted below the water-bath. Photometric measurements indicated the intensity of the light to be uniform within 5 or 10% over the area in which the vessels were suspended and sufficient for light saturation of photosynthesis. Before exposure to C^*O_2 the cells were removed from the nutrient medium by centrifugation, the volume measured and the cells resuspended in distilled water or a phosphate buffer (*p*H 7). Induction periods were avoided by shaking the suspensions for twenty to thirty minutes with an inactive carbon dioxide–air mixture prior to C^*O_2 introduction. In order to obtain reproducible results all conditions relating to the growth and handling of the cells were carefully controlled.[6] To check the activity of the

(4) Yost, Ridenour and Shinohara (*J. Chem. Phys.*, **3**, 133 (1935)) found this to be the case to a lesser extent when bombarding at lower energies and intensity than those employed in our experiments.

(5) F. N. D. Kurie, *Rev. Sci. Instruments*, **10**, 199 (1939).

(6) We are indebted to Dr. W. Arnold, Professor H. A. Barker and Professor R. Emerson for the strain of *Chlorella* used and also for advice and assistance in their culture. In all experiments fresh pure cultures of *Chlorella* were used.

cells, manometric measurements were made frequently by the well-known Warburg method. In most of the experiments the gas above the algal suspension was evacuated just prior to introduction of the C^*O_2–air (1–5% C^*O_2) mixture. Short evacuation periods or the presence of radioactive carbon dioxide had no detectable effect on the rate of *Chlorella* photosynthesis indicating that the *Chlorella* functioned in the normal fashion. An aliquot of the gas was withdrawn and absorbed in sodium hydroxide for the determination of the total activity to which the algae had been exposed.

For experiments on dark uptake of C^*O_2, extensive precautions were taken to eliminate stray light, the room being darkened completely or the vessels wrapped in black cloth. At various intervals the vessels were withdrawn and immersed in boiling water for several minutes to terminate cell activity. *In all experiments before the radioactivity was measured, dissolved carbon dioxide and carbonates were removed by addition of inactive bicarbonate followed by acidification and vigorous boiling. Prepared samples of radioactive carbonate subjected to this treatment quickly lost all activity so that it was certain that any remaining activity was carbon in oxidation states lower than +4.*[2]

Determination of Radioactivity.—The radioactivity of solutions and cell suspensions was measured by pipetting an aliquot on a blotter (5 × 7 cm.). The blotter was dried, covered with cellophane and wrapped around a thin-walled Geiger counter. Precipitates were also covered with cellophane and counted in the same fashion. Errors due to self absorption of the emitted radiation (positrons) were negligible. This method of counting samples could be used for non-volatile radioactive material only. The determination of volatile activity was accomplished by the use of a thin-walled cylindrical vessel made to contain a thin layer of liquid and designed to slip over the counter.

Proof that the Observations are on Photosynthesis.—It is apparent that the sensitivity and the nature of the indicator method is such that the occurrence of exchange reactions will complicate the results obtained. It is therefore essential to determine to what extent, if any, interchange reactions participate. This can be done by comparing the rate of photosynthesis measured by the radioactive technique with that obtained by the manometric method. The radioactive method should yield high results if any carbon dioxide exchange reactions are involved.

The results of such experiments are shown in Table I.

TABLE I

Method	Rate of photosynthesis (cu. mm. CO_2 per cu. mm. per min.)
Manometer (Warburg)	0.22 ± 0.01
Radioactive	.21 ± .01

The rate is the same within the experimental error, and it is reasonable to conclude that, in *Chlorella*, exchange processes can only account for a small fraction of the reduced radio-carbon.

In addition it is known that photosynthesis in *Chlorella* is more sensitive to certain poisons than is respiration. It is thus possible to inhibit photosynthesis completely[7] and leave respiration unaffected. This fact affords additional means of showing the absence of reversible reactions involving carbon dioxide in the respiratory process. The results are shown in Table II.

TABLE II

PHOTOSYNTHESIS (ARBITRARY UNITS)

Method	No inhibitor	10^{-3} *M* HCN	10^{-3} *M* Phenylurethane
Radioactive	100	0.003	5
Manometric	100	0(< 3)	5
Respiration (same units)			
Manometric	5	6	8

It is clear that inhibitors in amounts insufficient to decrease the respiration (oxidation) rate result in the expected decrease of C^*O_2 photosynthesis. This can be considered as conclusive evidence that reversible reactions in respiration can only account for a very small fraction of the assimilated carbon dioxide in *Chlorella*.

Figure 1 shows the amount of C^*O_2 reduced by the algae as a function of the time exposed to light. As expected the rate is constant.

Preparation of Algae for Chemical Analysis.—A few remarks are desirable on the procedure used in the attempts to identify the radio-activity with known compounds. The sensitivity of the method is such that the activity may be contained in as low as 10^{-8} g. of material. It becomes necessary to add various substances (to act as carriers) suspected of being identical, or similar to the radioactive molecules, to facilitate isolation. The possibility exists, of course, that the C* is in a molecule as yet unknown. But it should nevertheless be possible to obtain definite chemical evidence concerning its nature. It is necessary, then, to add a large variety of carrier substances, to work with crystalline precipitates, to wash all precipitates thoroughly, and to reprecipitate many times when possible. In order to simplify the identification of the primary products, short time (one to five minutes) exposures to C^*O_2 were used. After exposure to C^*O_2 the cells were killed and the insoluble cell material was sharply separated from the aqueous extract by centrifugation and filtration through a no. 4 Jena glass filter. Various methods for killing and extraction

(7) Warburg, "Über die katalytischen Wirkungen der lebendigen Substanz," Julius Springer, Berlin, 1928, p. 341.

were used. They involved the addition of a variety of aldehydes, mineral and organic acids, alcohols, ketones and cyanide; also gentle heating or grinding in the cold with abrasives in the presence of the substances mentioned above.

Prior to the extraction and chemical analysis a small quantity of a carrier mixture was added. This carrier solution contained the following substances (including many compounds which have been suggested as possible intermediates by workers in the past): formaldehyde, acetaldehyde, propionaldehyde, glycolic aldehyde, glyceric aldehyde, methanol, ethanol, glycol, glycerol, erythritol, glucose, sucrose, starch, hexose monophosphate, glycine, alanine, arginine, histidine, albumin, acetone and the acids formic, acetic, propionic, butyric, oxalic, succinic, malic, citric, maleic, fumaric, glycolic, pyruvic, glyceric, tartaric, lactic, ascorbic, glucuronic, glutamic, aspartic and glutaric. The methods used in isolating the various constituents of this mixture and testing for radioactivity will be described below.

Volatile Material.—A number of attempts were made to isolate radioactive formaldehyde and certain other volatile compounds since they have played a prominent role in many theories of photosynthesis. The analyses were made on cells which were exposed to C^*O_2 for approximately one minute in the light. Formaldehyde, etc., was added, the suspension boiled, and the vapors were led into a solution of 2,4-dinitrophenylhydrazine. The resulting hydrazones were inactive. In other experiments no measurable loss in activity was found when the water-soluble active material was evaporated to dryness. The possibility exists that labile volatile molecules present at low concentrations had condensed or polymerized in the killing process. A number of different methods of killing (see above) and extraction were used to circumvent this possibility, and the same result was obtained in each case. It would seem from the above experiments that the vapor pressure of the active material at 120° is small, thus eliminating formaldehyde, acetaldehyde, propionaldehyde, formic, acetic, propionic acids, etc.

Solubility.—Moreover, the activity was not extractable from water by ethyl ether, petroleum ether, chloroform or butyl alcohol. It is possible that the "water soluble" fraction is in reality nearly insoluble since the amount observed in these experiments may be much less than the solubility. As pointed out before, the method is easily capable of detecting 10^{-8} gram of radioactive ($C^{11} + C^{12} + C^{13}$) material. However, no activity was removed by shaking the aqueous extract with adsorbents such as talc, charcoal and powdered glass.

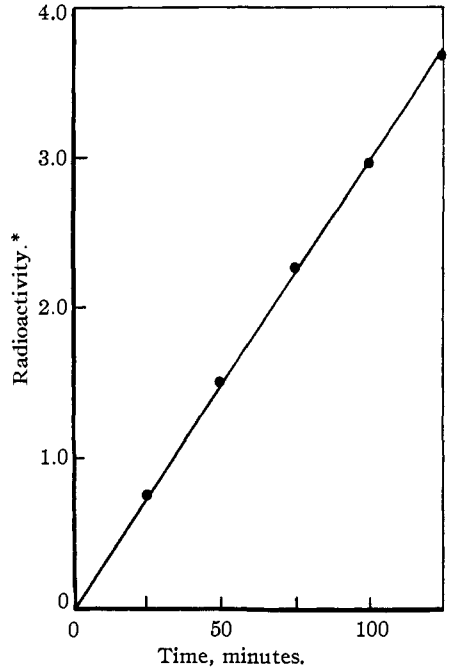

Fig. 1.—Reduction of C^*O_2 in light. The C* content of the algae is expressed in arbitrary units. One radioactive unit corresponds to 6.3 cu. mm. C^*O_2 per cu. mm. of algae.

Proteins.—Heat coagulation of albumin added to the active solution failed to bring down any activity. The precipitate obtained by the addition of trichloroacetic acid was likewise found inactive. This treatment should bring down proteinaceous material.

Amino Acids.—The mono-amino mono-carboxylic acids (tyrosine, alanine, glycine, phenylalanine, proline, etc.) were extracted with butyl alcohol by the procedure of Dakin.[8]

The basic amino acids (arginine, lysine, histidine, etc.) were brought down with phosphotungstic acid. The precipitate was slightly active. However, several reprecipitations removed all of the activity.

Pigments.—Since the water-soluble fraction contains the great bulk of the activity and is colorless, it is unlikely that the active molecules are chlorophyll, carotene, or xanthophyll. A Tswett column packed with talc failed to adsorb

(8) Dakin, *Z. physiol. Chem.*, **130**, 159 (1923).

any significant amount of activity. Furthermore, the activity is not adsorbed on charcoal nor can it be extracted from water by organic solvents.

Soluble Sugars.—Osazones prepared with phenylhydrazine in the usual manner contained approximately 1% of the radioactivity. Reprecipitation always resulted in marked diminution of this small amount of activity. In separate experiments, non-reducing sugars were hydrolyzed by boiling with 1 to 4 *N* hydrochloric acid for periods ranging up to one hour. These osazones were also inactive.

Starch.—Starch was precipitated by the addition of ammonium sulfate. The precipitate was inactive.

Carbohydrates of the Cell-Wall Constituents.—The water-insoluble fraction of the *Chlorella* was hydrolyzed by boiling with 72% sulfuric acid. After cooling, the solution was diluted with water and neutralized with sodium hydroxide. Ninety-five per cent. alcohol was added to crystallize the sodium sulfate. The salt was filtered off and the solution evaporated to a small volume. The osazones prepared from this solution were inactive.

Molecules Containing the Carbonyl Group.—The insoluble hydrazones of 2,4-dinitrophenylhydrazine were slightly active. A single reprecipitation removed all of the activity.

It is possible that an active hydrazone formed in these experiments failed to come down due to the minute amount (*i. e.*, absence of correct carrier) of the material involved. However, this objection does not apply to the distribution of a substance between two immiscible liquids. Hydrazones of 2,4-dinitrophenylhydrazine are soluble in chloroform, and therefore any active hydrazone, regardless of concentration[9] should be found in the chloroform. The chloroform layer in such an experiment was inactive. The hydrazones of large polar molecules may be relatively insoluble in organic solvents, but nevertheless it can be concluded that small molecules containing carbonyl groups are inactive.

Alcoholic Hydroxyl Group.—The Schotten-Baumann reaction with benzoyl chloride was employed in the determination of hydroxyl groups. The esters were extracted from the aqueous solution with chloroform. The chloroform layer contained ~20% of the activity despite repeated washing.

(9) Grahame and Seaborg, THIS JOURNAL, **60**, 2524 (1938), have shown the distribution coefficient of a solute between two immiscible liquids is constant at very low concentrations.

This amount may be considered as a lower limit for the amount of radioactive molecules containing alcoholic hydroxyl groups, since the benzoylation may be incomplete.

Carboxyl Groups.—As found in the previous work on barley, organic salts of Ba^{++}, Ca^{++} or Pb^{++} insoluble in 80% ethanol were very active. Even after repeated reprecipitation, 35 to 40% of the water-soluble radioactivity was contained in these heavy metal salts. This behavior is not unique for carboxyl groups, since other acid groups are known to form insoluble barium salts. Moreover, this test cannot give information concerning the location of the C* in the molecule. It should be possible to obtain more direct evidence by decarboxylation, *i. e.*

$$(RCOO)_2Ba \longrightarrow BaCO_3 + R_2CO \qquad (2)$$

From *Chlorella* exposed to C^*O_2 for twenty minutes in the light, the dry barium salt was prepared and after several reprecipitations heated in a stream of nitrogen for two hours at 250°. No activity could be detected in any of the vapors produced in the reaction. However, the barium carbonate formed contained approximately 5% of the C* originally precipitated with Ba^{++}. This was shown by acidifying the residue and collecting the evolved gas in sodium hydroxide. While this experiment affords evidence for the presence of —C*OOH, the distribution of the C* between carboxyl and other groups in the molecule is still unknown because of the uncertainty of the yield. Experiments to be described later show this distribution varies with the elapsed time of the C^*O_2 photosynthesis.

Specific Organic Acids.—The suggestion that plant acids are intermediates in photosynthesis is prominent in the literature.[10] As pointed out above, formic, acetic and propionic acids are inactive. The isolation of a number of the more common plant acids was carried out, and the results are summarized in Table III.

Phosphorylated Sugars.—Hexosephosphates are very often present and are precipitable by Ba^{++}. The phosphate can be removed by acid hydrolysis. Accordingly a portion of the water-soluble activity was refluxed for one hour with 1 *N* hydrochloric acid and the resulting solution examined for active sugars. The osazones, however, were inactive. Furthermore, no loss in Ba insoluble activity was observed.

(10) For an excellent summary, see H. A. Spoehr, "Photosynthesis," Chem. Cat. Co., New York, N. Y., 1926.

TABLE III

Acid	Method	Remarks
Ascorbic	Catalytic oxidation[11] to dicarbonyl and precipitate of hydrazone with 2,4-dinitrophenylhydrazine	Hydrazone inactive
Citric and malic	Precipitated with bismuth subnitrate in presence of mannitol[12]	Two reprecipitations sufficed to remove all activity
Fumaric	Insoluble mercurous salt in dilute HNO_3[13]	Inactive
Maleic	Converted to fumaric by action of Br_2 + light and precipitated as mercurous fumarate	Inactive
Succinic	Hydrolytic precipitate with $FeCl_3$	Inactive
Oxalic	Calcium oxalate in acetic acid solution	First precipitate active. After 3 reprecipitations no activity could be detected
Tartaric	Insolubility of potassium acid tartrate in 80 per cent. ethanol[14]	First precipitate was active. Two reprecipitations removed all activity

The Dark Uptake at C^*O_2.—The higher plants (barley, sunflower, etc.) were found to assimilate C^*O_2 in the absence of light. Likewise *Chlorella* carry on a measurable C^*O_2 accumulation in the dark. In all these experiments extensive precautions were taken to eliminate stray light during the exposure to C^*O_2 and also during extraction of the cellular material.

The time course of the dark reduction is shown in Fig. 2. The apparent saturation value corresponds to approximately 0.2 mm.^{3} C^*O_2 per mm.^{3} of cells. (The average uptake of C^*O_2 in the light is 0.22 mm.^{3} C^*O_2 per min. per cu. mm. cells.) The saturation value for the dark C^*O_2 uptake has been found to be independent of previous dark periods varying from one minute to two hours.

Since the dark assimilation of carbon dioxide is but a small fraction of the light reduction, it is necessary to determine by additional experiments whether or not this reaction has any relation to the photosynthesis. This was done by comparing the effects of various inhibitors which (as pointed out above) are known to stop photosynthesis. The results are shown in Table IV.

(11) Roe and Hally, *J. Biol. Chem.*, **128**, 329 (1939).
(12) Täufel, *Z. Untersuch. Lebensm.*, **71**, 297 (1936).
(13) Stotz, *J. Biol. Chem.*, **118**, 471 (1937).
(14) Hartmann and Hillig, *J. Assoc. Official Agr. Chem.*, **13**, 103 (1930).

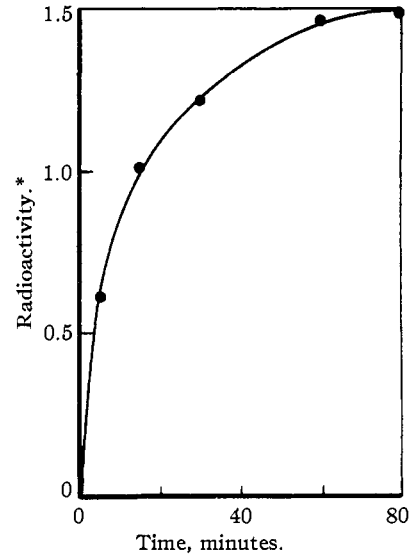

Fig. 2.—C^*O_2 uptake by *Chlorella* in the dark. Measured in arbitrary units (1.5 units corresponds to approximately 0.2 cu. mm. of C^*O_2 per cu. mm. of algae).

TABLE IV

COMPARATIVE EFFECTS OF VARIOUS INHIBITORS

	No inhibitor	10^{-2} *M* KCN	2537 Å. Hg line[b]
Photosynthesis (manometrically)	100[a]	0(< 3)	5
Respiration (manometrically)	5[a]	6	5
Dark uptake of C^*O_2	100[a]	0.003	5

[a] In arbitrary units. [b] We are indebted to Mr. Henry W. Anderson for the use of the Hg arc.

It is readily apparent that controlled quantities of diverse inhibitors all have the same effect on the dark reduction of C^*O_2. Under these conditions respiration is uninhibited; in fact, it is slightly accelerated. In addition to these results, evidence will be presented below which suggests strongly that the dark uptake is the first step in photosynthesis.

The following experiments were made to find out whether the dark uptake is reversible. After the algae were shaken with 3% C^*O_2 in the dark for twenty minutes the C^*O_2 was pumped off and 5% inactive carbon dioxide was introduced for a few minutes and then pumped out. This flushing procedure with inactive 5% carbon dioxide was repeated twice. Aliquots of the algae were taken before and after the flushing with inactive carbon

dioxide and the C* content measured. The decrease in radioactivity brought about by the flushing was 5–10 per cent.

In another experiment the algae were shaken with 3% C^*O_2 for twenty minutes in the dark. The system was then evacuated, the C^*O_2 collected in a liquid air trap, the vessels opened to the atmosphere and an aliquot of the algal suspension removed and measured for reduced C*. Precautions were taken to exclude light while the samples of algae were withdrawn and prepared for the radioactivity measurements. The system was again evacuated, the liquid air trap warmed and the C^*O_2 flushed by air back into the vessels. The procedure was repeated four more times at intervals of twenty minutes (forty minutes between the last two). The results are shown in Fig. 3, Curve B. Curve A is a reproduction of Fig. 2 and shows how without the pumpings the reaction slows up long before reaching the maximum obtainable by the pumping technique.

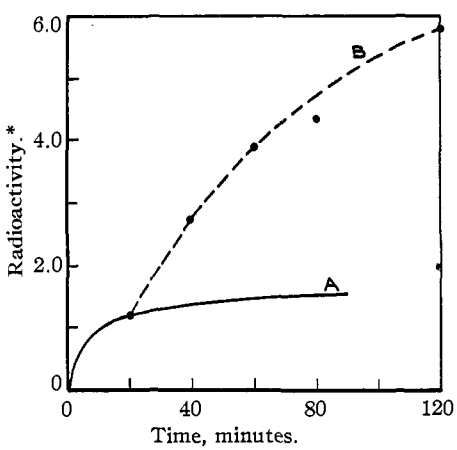

Fig. 3.—Dark uptake of C^*O_2 by *Chlorella*. Curve B was obtained by the removal and reintroduction of C^*O_2 at the specified intervals while curve A represents the course of the reaction when the pumping procedure is omitted.

The amount of C^*O_2 which has been fixed at the last point on Curve B is ~0.8 mm.[3] (or ~3 $\times 10^{-8}$ mole) per cu. mm. of algae If the molecular weight of the product is ~1000 (for which evidence is presented in the following papers) the weight of the product is about 3×10^{-5} g. per cu. mm. of algae or ~3% of the wet weight of the cells.

To explain why it is necessary to alternately raise and lower the C^*O_2 pressure in order to reach the maximum value several suggestions offer themselves. It is possible that the dark reaction does continue at a fairly rapid rate but that after the first twenty minutes or so very little $C^{11}O_2$ is incorporated by the reaction because of carbon dioxide (inactive) production by respiration within the cell coupled with inadequate mixing with the C^*O_2 present outside the cell. However, the cell wall is highly permeable to carbon dioxide (as shown by rapid rate of photosynthesis) and it seems unlikely that slowness of diffusion is responsible for the course of curve A. It may be that curve A is characteristic[14a] of the dark reaction itself.

We may represent the dark reaction by the equation

$$X + CO_2 \rightleftharpoons Y \qquad (3)$$

and may explain the results obtained if we assume (1) it is reversible, (2) the rate (that is, of course, the rate in both directions—not the net rate) is very slow at equilibrium and (3) the forward rate is greater when the system is far from equilibrium. We may picture, then, that the concentrations are such that the quantity of Y present is decreased 10–20% when the carbon dioxide pressure is reduced by the pumping in these experiments and is increased to its original value when the original carbon dioxide pressure is restored. The new radioactive Y is, however, only 10–20% of the total Y when equilibrium (elementic[15]) is reached and the interchange between the C^*O_2 and Y at this equilibrium will now be considerably slower accounting for the flat portion of curve A. If the pressure is again reduced and then increased with C^*O_2 as in obtaining the later points of curve B, 10–20% new radioactive Y is again formed. This can continue until Y has been made sufficiently radioactive so that further pumping actually removes about as much C* as is put in by each restoration.

The average concentration of chlorophyll in *Chlorella* cells is about 10^{-8} mole/cu. mm. of cells.[2] The close correspondence between this figure and the amount of Y calculated from the pumping experiments has been considered very suggestive. Using a rapid spectrographic method McAllister[16] has obtained evidence indicating the formation in wheat during active photosynthesis of a material which combines with or absorbs carbon dioxide. He has suggested this intermediate may be chlorophyll because approximately

(14a) This point is being investigated further.
(15) *Cf.* Mills and Urey, THIS JOURNAL, **62**, 1019 (1940).
(16) McAllister, *J. Gen. Physiol.*, **22**, 613 (1939).

one carbon dioxide molecule is taken up for each chlorophyll molecule present in the leaf. This was a reasonable interpretation of the data, especially since it had been assumed in many theories of photosynthesis that the primary step is a direct combination of carbon dioxide with chlorophyll. Unfortunately, however, our results show that the dark reduction of carbon dioxide is independent of the chlorophyll concentration both in *Chlorella* (*vulgaris* and *pyrenoidosa*) and barley plants. Barley grown in complete darkness and which contained no detectable amount of chlorophyll was exposed to C^*O_2 in the dark for thirty minutes. In the same chamber as a control a similar quantity of barley with the normal concentration of chlorophyll was present. The amounts of C^*O_2 assimilated by the normal and the etiolated plants were identical within 5%. Experiments with *Chlorella vulgaris* were even more convincing. Emerson and Arnold[17] have found that *Chlorella vulgaris* grown in red light contains $\sim$ four times as much chlorophyll as cells grown in blue light. Using high intensity neon and mercury arcs, we have obtained fairly dense cultures in which there was approximately a four-fold variation in chlorophyll concentration per cell. These cells when exposed to C^*O_2 in the dark under identical conditions assimilate the same amount of C^*O_2.

The amount of C^*O_2 reduced in the dark while independent of chlorophyll concentration is, however, related to the capacity for carrying out photosynthesis. This is to be expected if it is the primary step in the process. The two species of *Chlorella* furnish an excellent means for the establishment of this relationship since it is known that *Chlorella pyrenoidosa* can photosynthesize at a greater rate than *vulgaris*. In several experiments it was found that the dark C^*O_2 assimilation by *pyrenoidosa* was 2–3 times greater than in *vulgaris*.

As in the case of the light reduction, the chemical identity of the radioactive compounds formed in the dark is as yet unknown. It has, however, been possible to show they possess similar chemical properties. While the amount of radioactive material formed in the dark is considerably less than that formed in the light, the same chemical tests described above were carried out. The radioactivity can be extracted almost completely from the algae by gentle boiling for $\sim$ one minute. The bulk (at least 70%) of this activity is precipitated by Ba^{++} in 80% ethanol. When the barium salts are dry distilled under the same conditions as the material formed in the light (see above), a much higher fraction as radioactive barium carbonate is formed. Thirty to fifty per cent. of the C* present in the Ba^{++} salt is converted into carbonate, whereas in the light $\sim$5% conversion was obtained. If all the C* was in —C*OOH and the decarboxylation was complete one should find 50% of the C* in barium carbonate. It seems therefore that the greater fraction if not all the C^*O_2 has been reduced to —C*OOH. The low yield of active carbonate in the case of the light reduction material is indication that C* is then distributed in other groups in the molecule.

Benzoylation of the dark radioactive extract resulted in an active ester showing the presence of alcoholic groups. It is not necessary to cite all the experiments performed since they have been described in some detail for the light reduction products.

Briefly then it may be stated the light and dark reduction products have many common properties.

Acknowledgments.—We are indebted to Professors G. N. Lewis, W. M. Latimer, W. F. Libby, W. C. Bray, T. D. Stewart, C. B. Van Niel, D. R. Hoagland, R. Emerson, and Drs. D. C. DeVault, H. Tarver, W. Arnold, and others too numerous to mention for many very valuable discussions and suggestions. We are grateful to Professor E. O. Lawrence and the members of the Radiation Laboratory for their interest and coöperation. Thanks are also due to the Rockefeller Foundation for support to the Radiation Laboratory.

Summary

1. The light and dark assimilation of C^*O_2 reported previously on higher plants has been studied in detail in the unicellular green alga *Chlorella*.

2. The characteristics of the time course of the C^*O_2 uptake are described.

3. Simultaneous measurements on the rate of carbon dioxide reduction by the Warburg manometric method and the radioactive technique give identical rates within the experimental error. It can be concluded therefore that exchange reactions account for little of the C^*O_2 reduced; practically all must be photosynthesis.

4. The effects of various inhibitors (hydrogen

(17) Emerson and Arnold, *J. Gen. Physiol.*, **15**, 391 (1932).

cyanide, phenylurethan and ultraviolet light) administered in controlled dosage so that photosynthesis is either partially or completely stopped while respiration is unaffected have been studied. The light and dark C^*O_2 reductions show the same sensitivity as normal photosynthesis to these widely different modes of injury.

5. The dark assimilation is reversible and apparently independent of the chlorophyll concentration.

6. *Chlorella pyrenoidosa*, which has a higher rate of photosynthesis than *Chlorella vulgaris*, also possesses a greater capacity for assimilating C^*O_2 in the dark. This is in agreement with the suggestion that the dark C^*O_2 reduction may be the primary step in photosynthesis.

7. Chemical tests on the water-soluble material formed in the light and the dark indicate the presence of at least one alcoholic hydroxyl and one carboxyl group in the active molecules. Attempts to identify the active compound as one of a large number of organic substances known to exist in plants (*i. e.*, sugars, aldehydes, ketones, proteins, etc.) were not successful.

8. The major part, if not all, of the C^*O_2 taken up in the dark was found in carboxyl groups. A smaller but appreciable fraction of the C^*O_2 reduced in the light was in COOH groups.

9. No radioactive carbon was found in formaldehyde (and other volatile substances) even after very short (one minute) exposures to C^*O_2.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, THE RADIATION LABORATORY, UNIVERSITY OF CALIFORNIA, AND THE DEPARTMENT OF CHEMISTRY, STANFORD UNIVERSITY]

Photosynthesis with Radioactive Carbon. III. Ultracentrifugation of Intermediate Products

BY S. RUBEN, M. D. KAMEN AND L. H. PERRY

Considerable progress toward a solution of the problem of photosynthesis will be achieved when the identity of the molecules formed in the intermediate stages of carbon dioxide reduction is known. It is the object of this work to isolate and identify the primary products formed by the dark and light reduction of carbon dioxide using the radioactive isotopes of carbon as tracers. Since many specific chemical tests[1] have been unsuccessful, a more general method of attack is necessary. Knowledge of the molecular weight is the first objective.

A survey of the various methods available for molecular weight determinations indicated the most satisfactory are based upon sedimentation and diffusion properties. With the ultracentrifuge molecular weights can be found by (1) the sedimentation velocity or (2) sedimentation equilibrium. For sedimentation equilibrium spinning for long periods (days) is required and therefore is excluded,[2] since the half-life of C^{11} is only twenty-one minutes. The sedimentation velocity method which involves rotation periods of the order of an hour was used. The transparent ultracentrifuge is most widely used, the concentration gradient being measured by optical methods while the top is in rotation. This technique cannot be employed in the C* work since the radioactive molecular species are present only at high dilution. The sedimentation must be followed by radioactivity measurements. The opaque analytical ultracentrifuge[3,4] developed by McBain and his students is particularly suited for this problem because the top can be stopped and a sample from the periphery removed for analysis.

After ordinary centrifugation the supernatant aqueous extract from *Chlorella* exposed to C^*O_2 was filtered through a no. 4 Jena sintered glass disk. This solution was introduced into a 37-mm. rotor according to the method described by McBain and Leyda.[4] The possibility of spurious sedimentation due to adsorption on the metallic immobilizers was eliminated in experiments in which convection currents were set up in the top. No change ($< 0.5\%$) in the specific activity could be detected, indicating no measurable adsorption. The results of five successful runs are summarized in Table I.[5]

(1) *Cf.* preceding paper, THIS JOURNAL, **62**, 3443 (1940).

(2) The production of strong samples of long-lived radio-carbon will make possible these and other long term experiments: *cf.* Ruben and Kamen, *Phys. Rev.*, **57**, 549 (1940).

(3) McBain, *Chem. Rev.*, **24**, 289 (1939).

(4) McBain and Leyda, THIS JOURNAL, **60**, 2998 (1938).

(5) Several of these runs were carried out at the Shell Development Company at Emeryville. We are grateful to Dr. T. F. Ford and Mr. D. C. Waldman for their help and coöperation.