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ISOTOPIC COMPOSITION OF THE OXYGEN EVOLVED BY ILLUMINATED SPINACH CHLOROPLASTS AND GRANA WITH $K_2C^{18}O_3$ AS A TRACER

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SUMMARY

The isotopic composition of the O_2 evolved in the Hill reaction by illuminated spinach chloroplasts and grana, using $K_2C^{18}O_3$ as a tracer, was determined. The results indicated that water oxygen was the precursor of the O_2 evolved in the Hill reaction. The O_2 contained a small amount of isotope which appeared to be derived from water oxygen that had exchanged with $K_2C^{18}O_3$. Spinach chloroplasts and grana were found to accelerate the rate of exchange of oxygen between $K_2C^{18}O_3$ and water.

INTRODUCTION

It is generally accepted that the O_2 evolved in photosynthesis is derived from the oxygen of water and is independent of that of CO_2. This evidence is based on the work of a number of investigators who employed either water or CO_2 labeled with ^{18}O as a tracer[1-3]. However, YOSIDA *et al.*[4] reported that two-thirds of the O_2 originates from water and one-third from CO_2. The exchange of oxygen between CO_2 and water[5] sets a practical limit to the usefulness of ^{18}O as a tracer to determine the source of the O_2 evolved in photosynthesis. This exchange is catalyzed by the enzyme, carbonic anhydrase (EC 4.2.1.1), which is present in large quantities in leaves[6]. The data, cited above, on the source of O_2 in photosynthesis, have not included information about the extent of this exchange reaction under the particular experimental conditions. It seemed that a careful investigation of this problem under more rigorous experimental conditions was warranted.

The system chosen for study was one in which spinach chloroplasts photoreduce ferricyanide with the concomitant evolution of a stoichiometric amount of O_2. These chloroplasts are incapable of causing a net reduction of CO_2. However, WARBURG AND KRIPPAHL[7,8] have proved that CO_2 is required for this reaction. For the past few years our laboratory has extensively investigated the role of CO_2 in the Hill reaction[9,10]. Chloroplasts from every species of plants examined show a CO_2 requirement for the Hill reaction. CO_2 may be removed from these chloroplasts resulting in a loss of the Hill reaction. However, if CO_2 is added back, the ability to reduce the oxidant and produce O_2 is restored. If the oxygen atoms of CO_2 contribute to the O_2

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evolved, this should be most easily demonstrated under conditions where added CO_2 is being picked up. The chloroplasts were depleted of endogenous CO_2 in buffer of pH 6.7 and CO_2 was added in the form of K_2CO_3 highly labeled with ^{18}O and then the chloroplasts were illuminated. After illumination the O_2 was collected for isotopic analysis. The exchange of oxygen between CO_2 and water was measured by determining the amount of ^{18}O remaining in the various species of carbonate at the end of the illumination period. The results indicated that water oxygen was the precursor of the O_2 evolved in the Hill reaction. The O_2 contained a small amount of isotope which appeared to be derived from water oxygen that had exchanged with $K_2C^{18}O_3$. In addition, it was found that spinach chloroplasts catalyze an exchange of oxygen between K_2CO_3 and water under experimental conditions where the non-enzymic rate was very low.

MATERIALS AND METHODS

$K_2C^{18}O_3$ was purchased from the Weizman Institute of Science, Rehovoth, (Israel). Spinach chloroplasts were isolated in 0.35 M NaCl and were washed once with the NaCl solution[10]. Grana were prepared by disruption of the chloroplasts in a large volume of distilled water. The chloroplasts or grana were depleted of CO_2 in double-arm Warburg vessels featuring replacement of the usual center well by a trough fused to the wall of the main compartment of the vessel and connected with one side arm[11]. Conventional Warburg vessels of 5 ml volume were employed in the isotope experiments. These vessels were attached to manometers which were modified slightly for gas collection. The volume of gas in the closed system was determined by calibration with mercury. The experiments were carried out in a refrigerated bath maintained at 8.5°. Red or white light was provided from below at sufficient intensity to saturate the reaction. At the end of the illumination, the gas was collected for isotopic analysis.

The amount of ferricyanide reduced was determined on trichloroacetic acid filtrates of the supernatant from the reaction mixtures[10]. The amount of O_2 produced was calculated as ¼ the amount of ferricyanide reduced based on the stoichiometry of the reaction previously observed at pH 6.8 (ref. 10). The relationship between ferricyanide reduction and O_2 evolution was examined at pH 8.6 and the results were the same as at pH 6.8. Since the reactions were carried out in air, the observed value for atom per cent excess ^{18}O was multiplied by a dilution factor. The value for the dilution factor was based on the ratio of O_2 present in the vessel and manometer to the O_2 evolved in the Hill reaction.

The ratio, R, of O_2 with mass 32 to O_2 with a mass of 34 was determined with a Consolidated-Nier isotope-ratio mass spectrometer unless otherwise indicated. The ratio of mass 32 to mass 34 was read with a precision of 1 to 2 parts per 4000. This ratio was determined for a standard sample of air in every experiment. The determination was repeated alternately many times for the standard and experimental sample. Atom per cent ^{18}O was calculated from the following equation:

$$\text{Atom per cent } ^{18}O = \frac{100}{2R + 1}$$

Atom per cent excess ^{18}O was obtained by subtracting normal ^{18}O abundance.

The isotope abundance of the various species of carbonate was determined at the end of the experiment. The vessel contents were frozen and a spatula of dry

citric acid was added. When O_2 was collected, the samples were kept frozen during this period, and the citric acid added later. The vessel was attached to the gas collection apparatus, evacuated and the contents were thawed in cold water. The CO_2 evolved in the first 30 sec was collected for analysis. The isotopic analyses of the CO_2 samples were carried out with a Consolidated Engineering Company mass spectrometer, model 21620.

The ratio of CO_2 with mass 44 to mass 46 and ratio of CO_2 with mass 46 to mass 48 was determined. The following equations were used to calculate the ^{18}O abundance when the isotope concentrations were of the order of 10 atom per cent ^{18}O or higher:

$$\text{Atom per cent } {}^{16}O = \frac{\frac{46}{48} \cdot 100}{2 + \frac{46}{48}}$$

$$\text{Atom per cent } {}^{18}O = 100 - \text{atom per cent } {}^{16}O$$

The contribution of the mass-45 and mass-47 peaks were neglected. The ^{18}O content of the $K_2C^{18}O_3$ was 74 atom per cent ^{18}O, in agreement with the supplier's analysis. Below 10 atom per cent ^{18}O the contribution of the mass-48 peak is negligible. Thus, in this range the ^{18}O abundance of the CO_2 was calculated by using the following equation:

$$\text{Atom per cent } {}^{18}O = \frac{100}{2 \cdot \frac{44}{46} + 1}$$

Atom per cent excess ^{18}O was obtained by subtracting normal ^{18}O abundance.

RESULTS

The oxygen exchange between CO_2 and water was investigated under different experimental conditions. The results in Table I indicate that with the proper choice of experimental conditions, the exchange rate is very slow in the absence of chloroplasts or grana (*cf.* MILLS AND UREY[5]). The addition of chloroplasts or grana markedly accelerates the rate. The exchange between $K_2C^{18}O_3$ and water was measured by determining the ^{18}O content of the CO_2 released by acid at the end of the reaction. The $K_2C^{18}O_3$ contained 74 atom per cent ^{18}O. In all these experiments the chloroplasts or grana were aged in buffer for 1 or 2 h before measuring the exchange reaction. During the 30-min incubation period in Expt. 1, the pH was 8.4. Most of the oxygen had exchanged since the isotopic content of the CO_2 was 0.8 atom per cent excess. Expt. 1b was carried out in the dark in the absence of ferricyanide. The CO_2 contained 0.95 atom per cent excess ^{18}O. Thus, the exchange is not dependent on light or oxidant. The conditions for Expt. 2a were the same as in Expt. 1a except that the chloroplasts were omitted. The CO_2 released at the end of the incubation period contained 66.4 atom per cent excess ^{18}O. The loss of isotope in Expt. 1 was not due to labile CO_2 released from the chloroplasts. Expt. 2b was a duplicate of Expt. 2a except that the chloroplasts were added at the end of the incubation period, just prior to freezing. The isotope content of the CO_2 was 63.3 atom per cent excess ^{18}O.

Chloroplasts contain carbonic anhydrase which was measured by the manometric

TABLE I

THE EFFECT OF CHLOROPLASTS AND GRANA ON THE OXYGEN EXCHANGE BETWEEN K_2CO_3 AND H_2O

Expt. 1: (a). The reaction mixture contained 70 μmoles of potassium pyrophosphate buffer (pH 6.7), 200 μmoles of NaCl and chloroplasts equivalent to 0.1 mg of chlorophyll in a final volume of 1.2 ml. The trough of the vessel contained 0.5 ml of 20% KOH. The vessel was incubated for 2 h at 8.5° in the dark. Then a 1-ml aliquot was transferred to the main compartment of a fresh vessel whose side arm contained 10 μmoles of $K_3Fe(CN)_6$ and 60 μmoles of $K_2C^{18}O_3$ in 0.2 ml of H_2O. At the onset of illumination, the contents of the side arm were tipped in. Control experiments indicated that the pH was 8.4. The vessel was illuminated for 30 min at 8.5°. The contents of the vessel were frozen and the ^{18}O content of the CO_2 released by acid was determined as described in MATERIALS AND METHODS (b). The reaction mixture and the experimental conditions were identical with (a) except that the ferricyanide was omitted and the vessel was kept in the dark during the 30-min incubation period. Expt. 2: (a) The reaction mixtures were the same as in Expt. 1a except that no chloroplasts were added The 2-h preincubation period was omitted. The vessels were not illuminated. (b). The conditions were the same except that chloroplasts equivalent to 0.1 mg of chlorophyll were added at the end of the 30-min incubation period. The ^{18}O content of the CO_2 released by citric acid was determined as described in MATERIALS AND METHODS. Expt. 3: The reaction mixtures contained 70 μmoles of tricine(hydroxymethyl)methylglycine (pH 6.7), 70 μmoles of NaCl, and chloroplasts or grana equivalent to 0.1 mg of chlorophyll, in 1.2 ml H_2O. The contents were incubated in Warburg vessels with KOH in the trough, for 1 h at 8.5° in the dark. At the end of the incubation a 1.0-ml aliquot from each vessel was transferred to a fresh vessel. The side arm of each vessel contained 50 μmoles of $K_2C^{18}O_3$ in 0.2 ml of H_2O. The contents of the side arm were tipped in. The pH of the reaction mixture after adding the $K_2C^{18}O_3$ was 8.6. The vessels were incubated for 30 min at 8.5° in the dark. The ^{18}O content of the CO_2 released by citric acid was determined as described in MATERIALS AND METHODS. Expt. 4: Two vessels were set up. One contained 150 μmoles of potassium phosphate buffer (pH 6.6) and grana equivalent to 0.48 mg of chlorophyll in 1.35 ml of H_2O. The other contained the buffer, but no grana. The contents of the vessels were incubated for 1 h at 8.5° in the dark. An 0.9-ml aliquot from each vessel was transferred to a new vessel containing 300 μmoles of NaCl, 15 μmoles of sodium acetate and 0.021 μmoles of 2,6,3′-trichlorophenolindophenol (total volume 1.2 ml). The side arm of each vessel contained 15 μmoles of $K_2C^{18}O_3$ in 0.35 ml H_2O. The contents of the side arm were tipped in and the vessels incubated in the dark for 30 min at 8.5°. The ^{18}O content of the CO_2 released by citric acid was determined as described in MATERIALS AND METHODS.

Expt. No.	*Experimental conditions*	*Atom % excess ^{18}O in CO_2 released at end of the experiment*
1	Potassium pyrophosphate buffer (pH 8.4), 60 μmoles $K_2C^{18}O_3$;	
	(a) chloroplasts, illuminated, 30 min at 8.5°;	0.8
	(b) chloroplasts, dark, 30 min at 8.5°	0.95
2	(a) same as 1b except that the chloroplasts were omitted, dark, 30 min at 8.5°;	66.4
	(b) same as 1b, chloroplasts added at the end of the 30-min incubation period	63.3
3	Tricine buffer (pH 8.6), 50 μmoles of $K_2C^{18}O_3$;	
	(a) chloroplasts, dark, 30 min at 8.5°;	4.2
	(b) grana, dark, 30 min at 8.5°	10.7
4	Potassium phosphate buffer (pH 6.8), 15 μmoles $K_2C^{18}O_3$;	
	(a) grana, dark, 30 min at 8.5°;	1.6
	(b) no grana, dark, 30 min at 8.5°	20.9

technique of KREBS[12]. The exchange reaction is probably catalyzed by the enzyme present in chloroplasts. KONDO, CHIBA AND KAWAI[13] have purified carbonic anhydrase from spinach. They found it to be a homogeneous protein by electrophoretic analysis. The plant enzyme contains no Zn and is stabilized by 0.1 M NaCl. Since the chloroplasts in Expt. 1 were isolated in 0.35 M NaCl, it seemed feasible that disruption of the

chloroplasts in water to produce grana would result in loss of carbonic anhydrase. This proved to be the case. In Expt. 3, chloroplasts and grana from the same spinach preparation were compared for their ability to accelerate the exchange of oxygen between K_2CO_3 and water. The experiments were carried out in tricine(hydroxymethyl) methylglycine buffer[14] because this buffer has a pK of 7.95 and may be adjusted to the desired pH with hydroxide or carbonate ions. The CO_2 remaining at the end of the experiment when chloroplasts were present, contained 4.2 atom per cent excess ^{18}O and when grana were employed, contained 10.7 atom per cent excess ^{18}O. There was considerable variation in the exchange rate with different batches of spinach. With chloroplasts, the isotope content of the CO_2 released by acid, ranged from 0.2 to 4.2 atom per cent excess ^{18}O in five experiments carried out under the conditions of Expt. 1 or 3. However, with one spinach preparation, the exchange rate was very low as the CO_2 contained 38 atom per cent excess ^{18}O at the end of an experiment which was identical with Expt. 1a. Under the same conditions, with grana, the range was from 10.7 to 38 atom per cent excess ^{18}O.

The exchange was measured in an experiment at pH 6.8 with and without grana, and the results of Expt. 4a indicate that the CO_2 remaining contained 1.6 atom per cent excess ^{18}O when grana were present. Under the same experimental conditions, but without grana, the CO_2 remaining contained 20.9 atom per cent excess. As the pH is lowered the non-enzymic exchange reaction increases rapidly[5].

IZAWA[15] reported that his preparations of chloroplasts and grana did not contain much carbonic anhydrase when measured manometrically. The carbonic anhydrase activity of the chloroplasts employed in these experiments, when determined manometrically, was not as large as would be predicted from the isotope-exchange experiments reported here. The reason for this discrepancy is not known. It does suggest that there might be a mechanism responsible for the rapid exchange of oxygen between CO_2 and the water in the chloroplasts other than that catalyzed by carbonic anhydrase. However, a more detailed examination of the exchange reaction would be needed to investigate this possibility.

IZAWA[15] found that the addition of erythrocyte carbonic anhydrase shortened the time required to obtain the CO_2 effect in the Hill reaction with quinone. I also found that the addition of spinach carbonic anhydrase to the chloroplast–buffer mixture facilitated the removal of CO_2 in the ferricyanide Hill reaction.

The isotopic composition of the O_2 evolved by illuminated spinach chloroplasts and grana with ferricyanide as oxidant is given in Table II. For Expt. 1 the grana were depleted of CO_2 by incubating them in tricine(hydroxymethyl)methylglycine[14] (pH 6.7) with KOH in the trough for 1 h at 8.5°. The reaction mixture was transferred to a fresh flask containing ferricyanide and 50 μmoles of $K_2C^{18}O_3$ (74 atom per cent ^{18}O) in the side arm. The contents of the side arm were tipped in immediately before illumination. The addition of the carbonate altered the pH to 8.6. The vessel was illuminated and at the end of the illumination the O_2 was collected for isotopic analysis. The amount of isotope remaining in the CO_2 released by acid was determined.

The water was not analyzed for its isotope content in the experiments reported here. However, the amount of isotope in the water may be calculated from the amount of ^{18}O in the CO_2 released by acid at the end of the experiment (see footnote *** of Table II). The calculated values are given in the last column of Table II. The O_2 evolved in Expt. 1 contained 0.071 atom per cent excess ^{18}O while at the end of

TABLE II

ISOTOPIC COMPOSITION OF THE OXYGEN EVOLVED BY ILLUMINATED SPINACH CHLOROPLASTS AND GRANA

Expt. 1: The vessel contained 70 μmoles of tricine buffer (pH 6.7) and grana equivalent to 0.1 mg of chlorophyll in a final volume of 1.2 ml. The trough of the vessel contained 0.5 ml of 20% KOH. The vessel was incubated for 1 h at 8.5°. A 1-ml aliquot was transferred to a fresh flask whose side arm contained 10 μmoles of $K_3Fe(CN)_6$ and 50 μmoles of $K_2C^{18}O_3$ in 0.2 ml of water. At the onset of illumination the contents of the side arm were tipped in. Control experiments indicated that the pH was 8.6. The vessel was illuminated for 30 min at 8.5°. The contents of the vessel were frozen and the gas collected for analysis. The ^{18}O content of the CO_2 released by acid was determined. Other experimental details are as described in MATERIALS AND METHODS. Expts. 2, 3, 4, and 5: For each experiment a vessel was set up which contained 70 μmoles of potassium pyrophosphate buffer (pH 6.7), 200 μmoles of NaCl, and chloroplasts equivalent to 0.1 mg of chlorophyll in a final volume of 1.2 ml. The trough of the vessel contained KOH. The vessels were incubated for 2 h at 8.5°. Then a 1-ml aliquot was transferred to a fresh vessel whose side arm contained 10 μmoles of $K_3Fe(CN)_6$ and 60 μmoles of $K_2C^{18}O_3$ in 0.2 ml of water. At the onset of illumination the contents of the side arm were tipped in. The pH was now 8.4. The illumination period was 30 min at 8.5°. Other experimental details are as described for Expt. 1. Expt. 6: The reaction mixture contained 0.027 μmoles of 2,6,3′-trichlorophenolindophenol, 140 μmoles of potassium phosphate buffer (pH 6.6), 350 μmoles of NaCl, 15 μmoles of sodium acetate and grana equivalent to 0.2 mg of chlorophyll in a final volume of 1.5 ml. The trough of the vessel contained KOH. The vessel was incubated for 1 h at 20°. A 1.2-ml aliquot was transferred to another vessel whose side arm contained 15 μmoles of $K_3Fe(CN)_6$ and 15 μmoles of $K_2C^{18}O_3$ in 0.3 ml of water. At the onset of illumination the contents of the side arm were tipped in. The pH was 6.8. The vessel was illuminated for 30 minutes at 8.5°. Other details are as described in Expt. 1. Expt. 7: The experimental procedure was the same as for Expt. 6 except that the reaction mixture employed during illumination contained grana equivalent to 0.3 mg of chlorophyll in a final volume of 1.6 ml.

Expt. No.	*Conditions*	*Dilution factor*	*Atom % excess ^{18}O observed*	*Atom % excess ^{18}O in evolved O_2***	*Atom % excess ^{18}O in CO_2 released at the end of the experiment*	*Atom % excess ^{18}O in water (calculated)****
1	Tricine buffer (pH 8.6), grana, 50 μmoles of $KC_2^{18}O_3$*	23.6	0.003	0.071	21.0	0.119
2	Potassium pyrophosphate buffer (pH 8.4), chloroplasts, 60 μmoles of $K_2C^{18}O_3$	23.2	0.007	0.162	0.25	0.200
3	Same as 2	18.2	0.009	0.164	0.8	0.200
4	Same as 2	24.0	0.007	0.168§	—	0.200
5	Same as 2	20.5	0.005	0.103§	—	0.200
6	Potassium phosphate buffer (pH 6.8), grana, 15 μmoles of $K_2C^{18}O_3$	15.3	0.002	0.031	1.87	0.039
7	Same as 6	10.7	0.003	0.032	0.53	0.037

* The $K_2C^{18}O_3$ contained 74 atom per cent ^{18}O.

** Calculation: (μl of O_2 present)/(μl of O_2 produced) = dilution factor.

*** Sample calculation Expt. 1: μatoms of oxygen in 1.2 ml of water = 66600; 50 μmoles of $K_2C^{18}O_3$ = 150 μatoms of oxygen. Fraction of original $K_2C^{18}O_3$ that exchanged = (74 − 21)/74 = 0.716; 0.716 × 150 μatoms of carbonate oxygen = 107.5; 107.5/66600 = 0.00161 μatom carbonate oxygen per μatom water oxygen. Isotopic composition of carbonate = 74 atom per cent ^{18}O; μatom carbonate oxygen per μatom water × isotopic composition of carbonate = 0.00161 × 74 = 0.119 atom per cent excess ^{18}O in water.

§ The Consolidated Engineering Company mass spectrometer model 21620 was used for the analyses.

the experiment the CO_2 still retained 21 atom per cent excess ^{18}O. The isotope content of the water at the end of Expt. 1 is 0.119 atom per cent excess ^{18}O. If the evolved O_2 had been derived from oxygen of CO_2, its isotope content would have to be above that of the water. Therefore, the O_2 produced appears to be derived from water oxygen. It

must be noted that the kinetics of both the Hill reaction and the exchange reaction complicate the situation. No attempt was made to follow the variation in the rate of either reaction during the illumination period. It is possible that part of the isotope appearing in the O_2 could be derived directly from $K_2C^{18}O_3$ if the labeled O_2 was produced in the first few minutes of illumination before the water was labeled. Earlier experiments have shown that the rate of O_2 evolution declines with time during a 30-min illumination period[10].

The conditions in Expt. 2 were varied by substituting chloroplasts for grana and employing potassium pyrophosphate buffer. The final pH was 8.4. Expts. 3, 4 and 5 were simply duplicates of Expt. 2. The isotope abundance of the oxygen produced was 0.162, 0.164, 0.168, and 0.103 atom per cent excess ^{18}O in Expts. 2, 3, 4 and 5, respectively. The results are not given, but approximately the same amount of enrichment was obtained when fresh chloroplasts were employed under these conditions. Virtually all of the ^{18}O of the carbonate had exchanged with water oxygen as the CO_2 remaining contained 0.25 atom per cent excess ^{18}O in Expt. 2 and 0.8 atom per cent excess ^{18}O in Expt. 3. The calculated value of the ^{18}O content of the water for complete exchange when 60 μmoles of $K_2C^{18}O_3$ are added is 0.200 atom per cent excess ^{18}O.

Expts. 6 and 7 were carried out at a more physiological pH. Grana were freed of CO_2 in potassium phosphate buffer (pH 6.6) and 15 μmoles of $K_2C^{18}O_3$ were added to bring the pH to 6.8. GOOD[16] has shown that ferricyanide reduction may be made completely dependent on CO_2 if certain anions are present. In Expts. 6 and 7, 300 μmoles of NaCl and 15 μmoles of sodium acetate were added. The Hill reaction employed in Expts. 6 and 7 differed from the other experiments in Table II in that catalytic quantities of 2,6,3'-trichlorophenolindophenol were added with substrate amounts of ferricyanide[10]. In Expt. 6, the isotope content of the evolved oxygen was 0.031 atom per cent excess ^{18}O and the calculated isotope content of the water was 0.039 atom per cent excess ^{18}O. The CO_2 released at the end of the experiment contained 1.87 atom per cent excess ^{18}O. Similiar results were obtained in Expt. 7.

DISCUSSION

The data presented here indicate that water oxygen is the precursor of the O_2 evolved in the Hill reaction. However, there are several important reasons for calling attention to the possibility that the oxygen of CO_2 may contribute to the O_2 evolved in the Hill reaction and photosynthesis.

Our knowledge of the relationship between photosynthesis and the ^{18}O content of atmospheric O_2 is inadequate. CO_2 has a greater abundance of ^{18}O than the water with which it is in equilibrium[17]. Atmospheric O_2 contains more ^{18}O than water, but less than CO_2 (ref. 18).

Although there is no direct experimental proof, it is believed that the O_2 of the atmosphere has been produced by photosynthesis. DOLE[19] and GREENE AND VOSKUYL[20] found that the oxygen in the air is about $7.5 \cdot 10^{-5}$ atomic weight units heavier than the oxygen in the water of the oceans. These investigators postulated that the isotopic composition of O_2 evolved in the photosynthetic reaction is an average of that in atmospheric CO_2 and water. This theory could explain quantitatively the observed enrichment of ^{18}O in atmospheric O_2. On the other hand, it has been suggested that the discrepancy in the oxygen isotope content of air and water

is due to isotope exchange reactions between molecular oxygen and water or other oxygen-containing compounds[17].

The present investigation was stimulated by the recent demonstration of the CO_2 requirement for the photoevolution of O_2 by WARBURG AND KRIPPAHL[7,8] and the establishment of conditions where the CO_2 effect is freely reversible[9,10]. WARBURG[21] has suggested that CO_2 is converted into a compound which gives rise to O_2 because of experiments with Chlorella, in which the O_2 precursor can be accumulated in the dark, provided that O_2, P_i, CO_2, and glutamate are present. Since the O_2 precursor is formed from CO_2 and readily decomposes to give CO_2 in the dark, it is regarded as a CO_2 derivative. The fundamental photochemistry is very likely the same in leaves as in Chlorella.

RUBEN, RANDALL, KAMEN AND HYDE[1], using either carbonate or water labeled with ^{18}O, found that the isotopic composition of the O_2 produced by Chlorella was identical with that of the water. KAMEN AND BARKER[18] later pointed out that the rate of equilibration in these experiments was assumed to be that prevailing in the alkaline buffer media, neglecting the possibility of a much faster equilibration inside the cells, where the reaction may be neutral or even slightly acid. HOLT AND FRENCH[3] measured the isotopic composition of the O_2 evolved in the Hill reaction with spinach chloroplasts, using enriched water. They found that the isotopic composition of the O_2 evolved was identical with that of the water. The experiments of DOLE AND JENKS[2] and YOSIDA *et al.*[4] were carried out with normal isotopically equilibrated water and CO_2 and depended on the small difference in the ^{18}O content of these two compounds in isotopic equilibrium[17]. They used the density method of isotopic analysis. YOSIDA *et al.*[4], taking advantage of the difference in the density of fresh water and water made from the O_2 of carbonate compounds, which contains a higher concentration of ^{18}O, found that water prepared from photosynthetic O_2 was about 3.5 parts per million heavier than fresh water. These investigators interpreted their results as indicating about one-third of the photosynthetically produced O_2 must have originated from CO_2. Contradictory results were obtained by DOLE AND JENKS[2] who reported little experimental detail. They found that the O_2 produced by various plants gave water which was only 0.6 to 1.8 parts per million heavier than the water from which it was liberated by the plants. DOLE AND JENKS interpreted the enrichment that they obtained to be the result of the isotope exchange equilibrium at 25° between liquid water and O_2 gas[17].

The results reported here indicate that the O_2 evolved in the Hill reaction is derived from water oxygen. The data on the exchange of oxygen between CO_2 and water catalyzed by chloroplasts and grana, stress the importance of taking into account the intracellular environment of the plant being examined. If the oxygen of CO_2 is a precursor of photosynthetic O_2, it may not be feasible to demonstrate this with present day methods. One can envision individual active centers which function independently. CO_2 may be removed from these centers, but once it goes back in, it stays and cycles during the Hill reaction. Under these circumstances, any labeled O_2 would be produced in the first few cycles. The net isotope content of the evolved O_2 would be so low that it would be extremely difficult to pick up analytically. It would be interesting to examine the isotope content of the O_2 evolved during photosynthesis (net CO_2 consumption using $C^{18}O_2$) under conditions where the intracellular exchange reaction between CO_2 and H_2O was measured.

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