Reports

Source of Photosynthetic Oxygen in Bicarbonate-Stimulated Hill Reaction

Abstract. *The bicarbonate ion can stimulate oxygen evolution fivefold in chloroplasts performing a Hill reaction (oxidation of water to molecular oxygen and reduction of an artificial oxidant). When bicarbonate containing oxygen-18 is administered, however, the evolved oxygen contains only oxygen-16. Bicarbonate, therefore, cannot be the immediate source of photosynthetically evolved oxygen.*

It is commonly accepted that molecular oxygen evolved during photosynthesis arises from the splitting of H_2O molecules and not, as originally postulated by Warburg (*1*), from the splitting of CO_2 molecules. However, the direct evidence to support this belief has been controversial. Experiments using the tracer ^{18}O to reveal the source of O_2 can be ambiguous if the normal isotopic exchange between H_2O and CO_2 is not controlled. Important factors in speeding isotopic exchange are low *p*H (*2*) and the presence of the enzyme carbonic anhydrase. Ruben *et al.* (*3*), using whole algal cells, could not control these critical internal factors (*4*). Thus the tracer method, as previously applied, could not allow definite conclusions [see criticisms by Warburg (*1*)].

Recently, it has become evident (*5–7*) that the bicarbonate ion, HCO_3^-, does in fact play a critical and direct role in photosystem II activity leading to oxygen evolution. This effect, first discovered by Warburg and Krippahl (*8*) and offered as proof of Warburg's theory (*1*), is distinct from the well-known role of CO_2 as a terminal electron acceptor in photosynthesis. Removal of HCO_3^- from broken chloroplast fragments can suppress the Hill reaction more than 90 percent (*6*). To see whether or not HCO_3^- was acting as the immediate source of O_2, as postulated by Warburg and more recently by Metzner (*9*), we again resorted to tracer methods. The material used was isolated broken chloroplast fragments instead of whole cells. This minimized the problems of low intracellular *p*H and removed nearly all carbonic anhydrase (*10*), thus effectively controlling the isotopic exchange reaction between H_2O and CO_2. In addition, by depleting chloroplasts of HCO_3^-, oxygen evolution was made dependent on an exogenous source of this ion. When labeled bicarbonate, $HC^{18}O_3^-$, was resupplied, oxygen evolution commenced at a high rate. The oxygen given off, however, was unlabeled—that is, $^{16}O_2$.

The experiment was performed as follows: HCO_3^--depleted maize chloroplasts (*11*) were suspended in CO_2-free reaction mixture containing a Hill oxidant (ferricyanide) and placed in the reaction cell attached to the mass spectrometer (*12*). The chloroplasts were illuminated for a brief period (1 minute), $HC^{18}O_3^-$ (*13*) was injected during a subsequent dark period, and the chloroplasts were then reilluminated. The molecular masses observed throughout the experiment were 32 ($^{16}O_2$), 34 ($^{16,18}O_2$), 36 ($^{18}O_2$), 44 ($C^{16}O_2$), and 48 ($C^{18}O_2$).

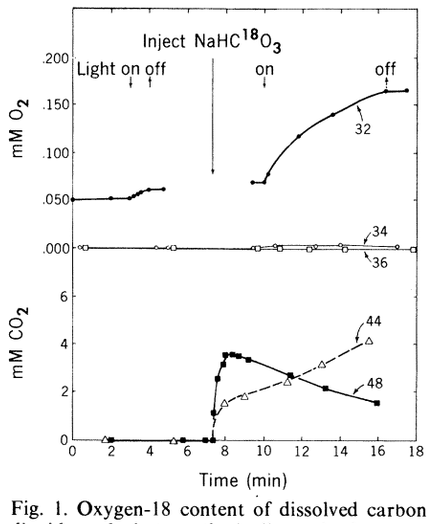

Fig. 1. Oxygen-18 content of dissolved carbon dioxide and photosynthetically evolved oxygen with time. The reaction mixture contained 0.175*M* NaCl, 0.1*M* sodium formate, 0.05*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, *p*H 7.5, 2 m*M* $K_3Fe(CN)_6$, and chlorophyll 100 μg/ml. Light from a projection lamp was filtered through 12 inches (30.5 cm) of water and a 3-110 filter (Corning) and focused onto the reaction vessel. The intensity was about one-half of saturation. Mass numbers represented by each curve are indicated: 32 ($^{16}O_2$), 34 ($^{16,18}O_2$), 36 ($^{18}O_2$), 44 ($C^{16}O_2$), and 48 ($C^{18}O_2$).

The results are presented in Fig. 1. During the first period of illumination, HCO_3^- depleted chloroplasts gave off only small amounts of O_2, seen as an increase in mass number 32. At 7.3 minutes, 20 μl of $NaHC^{18}O_3$ stock solution was injected into the reaction cell. Within a few seconds the mass 48 ($C^{18}O_2$) signal reached a peak and slowly began to decline as the ^{18}O was exchanged for the ^{16}O in the unlabeled H_2O. [It is clear from the figure that some unlabeled CO_2 (mass 44) was also injected, probably due to incomplete isotopic equilibration between labeled H_2O and HCO_3^- in the stock solution.] The amount of unlabeled CO_2 increased with time, as expected. Nevertheless the rate of isotopic exchange was slow, indicating an absence of carbonic anhydrase. In other experiments (data not shown) addition of a small amount of this enzyme produced a very rapid and complete reduction of the mass 48 signal. Since the exchange reaction was slow under our conditions, we can see that at the onset of the second period of illumination (at 10 minutes) there still existed in solution more $C^{18}O_2$ than $C^{16}O_2$. While singly labeled CO_2 ($C^{16,18}O_2$) was not measured (mass number 46) we can infer that a significant amount of this species was also present at the time of the second light period.

With the onset of the second light period at 10 minutes, oxygen evolution again commenced. Due to the presence of HCO_3^-, the initial rate was about five times the rate seen during the first light period; that is, the usual HCO_3^- effect was very much in evidence (*14*). We note, however, that while HCO_3^- was predominantly labeled with ^{18}O at this time, the O_2 that was evolved was almost completely unlabeled and contained less than 0.1 percent doubly labeled O_2. A small amount of singly labeled O_2 ($^{16,18}O_2$) was evolved which was consistent with the amount of ^{18}O in the water (20 μl of 97.2 percent $H_2{}^{18}O$ was also injected as part of the HCO_3^- stock solution, so that the final reaction mixture was about 2 percent $H_2{}^{18}O$).

The conclusions to be drawn from this experiment, unlike those of the past, seem inescapable. Although the addition of $HC^{18}O_3^-$ caused a fivefold stimulation of oxygen evolution in chloroplast Hill reaction, the O_2 evolved was almost exclusively $^{16}O_2$. This indicates that the added HCO_3^- was not the immediate source of photosynthetically evolved O_2. To conclude otherwise we must assume that there is a very rapid exchange of oxygen between water and a small pool of "active" HCO_3^- localized inside the grana membranes and not reflected in the external medium. This

small pool of unlabeled HCO_3^- (which cannot be dilutable with labeled HCO_3^- from the external medium) must be proposed as the source of oxygen. Lacking any supporting evidence, such a scheme must be considered improbable at the present time.

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References and Notes

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11. Maize (*Zea mays*) chloroplasts were isolated by the procedure described in (*6*). The source and manner of isolating chloroplasts, however, is not of great importance here. More critical is the HCO_3^- depletion procedure. To deplete chloroplasts of HCO_3^-, they were suspended at a concentration of 50 μg of chlorophyll per milliliter in a solution containing 0.175*M* NaCl, 0.1*M* sodium formate, and 0.05*M* sodium phosphate buffer, *p*H 5.0 [higher *p*H for chloroplasts from C_3 plants; see (*5*)]. The solution was made CO_2-free by boiling before it was used. The chloroplast suspension was flushed with a continuous stream of pure N_2 or Ar gas for 10 minutes in the dark at room temperature. Portions were then drawn off with a syringe and placed in cooled screw-capped test tubes previously flushed with N_2. After centrifugation, the supernatant was poured off and the tubes were placed in ice. The HCO_3^--depleted chloroplasts were later suspended in CO_2-free reaction mixture and assays were conducted.
12. The mass spectrometer inlet system used in these experiments was similar to one described earlier [G. Hoch and B. Kok, *Arch. Biochem. Biophys.* **101**, 160 (1963)]. It utilized a semipermeable membrane (MEM 213, General Electric) which allowed dissolved gases (but not the liquid phase) to enter the mass spectrometer from the (~1 ml) reaction vessel. The response time of the system, end-to-end, was about 3 seconds. The quadrupole mass spectrometer (Extranuclear Laboratories) was programmed by using a peak selection–stepper system developed by O. Ollinger.
13. A stock solution of $HC^{18}O_3^-$ was made by dissolving ordinary $NaHC^{16}O_3$ in water (obtained from Monsanto Research Corporation) containing 97.2 percent ^{18}O. Several days were allowed for isotopic equilibration.
14. A more rapid rate of photoinactivation, reflected by a decreasing rate of O_2 evolution in the light, is usually observed once chloroplasts are HCO_3^--depleted. The reason for this is not clear [see (*7*)].
15. Partially supported by NSF grants GB36751, GT33537, and NSFC-704 and ERDA contract E (11-1) 3326. Preliminary experiments were done at the University of Illinois, Urbana, with the cooperation of G. Haight and Govindjee. While the methods employed proved limiting, the results appeared similar to some of those presented here. We are grateful for the valuable experience gained through their support. We thank B. Kok for permission to use the facilities at the Martin Marietta Laboratories in Baltimore and for helpful discussion. Carnegie Institution of Washington, Department of Plant Biology, Publication No. 555.
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Vibrational States of the Biopolymer Polyglycine II: Theory and Experiment

Abstract. *The density of vibrational states, and hence the heat capacity, has been calculated for the parallel-chain hexagonal lattice of 3_1 helical polyglycine. The agreement with experimental results in the temperature range from 1 to 20 K, including an anomaly near 8 K, is the best obtained thus far for homopolypeptides.*

Simple polypeptides, homopolypeptides in particular, have long been considered as model systems for the study of proteins (*1*). Solid-state and polymer physics have now advanced to the stage where one can treat the vibrations of polymers by using techniques that have been well established for simple molecules and for oligomers, based on springlike interactions between the atoms of the polymer. However, the computational difficulties become progressively much more severe as one considers polymers more complicated than polyethylene. We have calculated here the density of vibrational states and thence the specific heat for the simplest homopolypeptide, polyglycine II (PG II), and also measured the low-temperature specific heat from 1 to 20 K. The specific heat for PG II measurements are by far the lowest in temperature yet reported.

The specific heat provides a test of how the vibrational modes are distributed over different frequencies (that is, the density of states). The theory and experiment are in fair agreement, particularly with respect to the unexpected specific heat behavior near 8 K; PG II is thus the most complicated polymer for which a first-principles vibrational (lattice dynamical) calculation is in good agreement with experiment. Even though this study bears the same relation to biological systems as many structural studies on proteins (for example, in neither case are the studies carried out on the molecule under true intracellular aqueous conditions), the approach is valuable, particularly for the low-energy skeletal modes.

Polyglycine, or poly(–COCHRNH–) with R = H, is dimorphic in the solid state, existing as a β sheet structure (PG I) or as 3_1 helices (PG II). In these calculations the helices were assumed to be arranged in a parallel array with their axes on a hexagonal lattice. Theoretical (*2*) and experimental (*3*) results indicate that the helices are arranged antiparallel as well as parallel. However, to lessen the computational difficulties the model was restricted to the parallel arrangement only. The main differences between this calculation and earlier normal mode calculations (*4–8*) is that in this model all atoms are considered explicitly and the interchain interactions are included. The valence force field developed (*4*) for the polyamides and PG I was used for the intrachain interactions, and only the hydrogen bonding interactions were included in the interchain force field. The lattice dynamical secular equation was approximated with the use of a perturbation technique in which the secular equation was initially transformed with the eigenvectors of the isolated chain calculation (that is, no interchain interactions) and then truncated to the 21 lowest-frequency modes. This method yielded the approximate frequencies below 350 cm^{-1} and at selected wave vectors was shown to yield frequencies in excellent agreement with those found by diagonalization of the full secular determinant. The smaller secular equation leads to a substantial savings in computational time since a large number of wave vectors must be sampled to adequately describe the density of states. The vibrational frequencies above 350 cm^{-1} were assumed to be dependent only on the wave vector components in the chain axis direction. The resultant density of states is shown in Fig. 1. The higher frequencies (greater than 300 cm^{-1}) have little influence on low-temperature specific heat.

The experimental input to the lattice

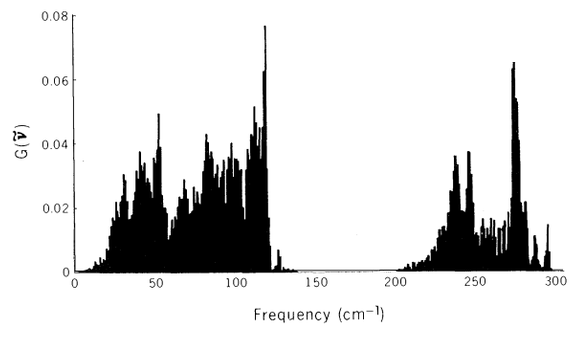

Fig. 1. Density of vibrational states $G(\tilde{\nu})$ versus frequency for PG II. The number of total states is normalized to the number of vibrations in one chemical repeat unit, that is, 21.