



# Combined effect of bicarbonate and water in photosynthetic oxygen evolution and carbon neutrality

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**Abstract** Carbon neutrality is widely concerned and highly valued by many countries. Biosphere has always maintained the balance between oxidized organic substances and assimilated organic matter, resulting in net-zero carbon dioxide (CO<sub>2</sub>) emissions and maintaining its own carbon neutrality. Nature has set a good example for human beings to coordinate oxygen (O<sub>2</sub>) balance and CO<sub>2</sub> balance, and achieve carbon neutrality. How does photosynthetic oxygen evolution initiate carbon and water neutrality? My synthesis shows that photosystem II functions as carbonic anhydrase to catalyze the reaction of CO<sub>2</sub> hydration under physiological conditions, and CO<sub>2</sub> hydration coupled with chemical equilibrium,  $H^+ + HCO_3^- \rightarrow 1/2 O_2 + 2e^- + 2H^+ + CO_2$ , occurs in a photosystem II core-complex. Meanwhile, I focused on the revisiting of four classical heavy oxygen (O<sup>18</sup>) labeling experiments and found that bicarbonate can promote photosynthetic oxygen evolution, and that photosynthetic oxygen evolution can alternately come from bicarbonate and water, not only water. Bicarbonate photolysis and water photolysis account for half of the photosynthetic oxygen evolution respectively, which can well explain the bicarbonate effect, Dole effect and plants' environmental adaptability. Photosynthetic oxygen evolution initiated the journey of water metabolism and carbon metabolism in nature, which led to the coupling as 1:1 (mol/mol) stoichiometric relationship between the reduction of CO<sub>2</sub> and oxidation of organic

carbon, coordinated the evolution of the atmosphere, hydrosphere, lithosphere and biosphere, and realized “carbon neutrality” in the whole Earth system.

**Keywords** Bicarbonate photolysis · Bicarbonate effect · Carbonic anhydrase · Dole effect · Water photolysis · Stoichiometric relationship · Carbon metabolism

## 1 Introduction

Carbon neutrality is a widely discussed topic worldwide. Photosynthesis, which converts light energy into chemical energy and inorganic carbon into organic carbon to releases oxygen, is the most important biochemical reaction on Earth and the core link of carbon neutrality. The evolution of photosynthetic oxygen plays a crucial role in photosynthesis. Understanding the photosynthetic oxygen evolution can provide a solution to carbon neutrality in humans.

In 1937, Hill confirmed that in vitro chloroplasts could reduce trivalent iron compounds to divalent iron compounds and release oxygen under light (Hill 1937). Although there have been some disputes, many heavy oxygen (O<sup>18</sup>) isotope labeling experiments have led to a general consensus that the released oxygen comes only from water, which seems to be “conclusive” isotopes evidence (Ruben et al. 1941; Warburg and Krippahl 1958; Stemler and Radmer 1975; Radmer and Ollinger 1980; Stemler 1980, 2002; Hillier et al. 2006). However, can these experiments really prove that the released oxygen during photosynthesis comes solely from water, is excluded from bicarbonate? Let us revisit these heavy oxygen isotope labeling experiments.

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## 2 Reappearance of scenes

In 1941, Ruben et al. reported the results of a heavy oxygen ( $O^{18}$ ) labeling experiment of photosynthesis. In this study, *Chlorella* cells produced heavy oxygen under light conditions when the algae were suspended in heavy oxygen water (0.85%  $O^{18}$ ) containing ordinary potassium bicarbonate and carbonate. However, *Chlorella* cells were suspended in ordinary water and heavy oxygen potassium bicarbonate and carbonate, and the algae evolved unlabeled oxygen in the presence of light. Therefore, Ruben et al. concluded that oxygen originated solely from water according to isotope ratios identical to those of water (Ruben et al. 1941). Many other scientists have reached a similar conclusion (Govindjee et al. 2006).

This conclusion was questioned shortly afterwards. In 1958, Warburg and Krippahl first discovered that bicarbonate can stimulate *Chlorella* to release oxygen under the action of artificial reductants, which was later called the bicarbonate effect, and bicarbonate or  $CO_2$  was necessary for the Hill reaction. They believed that the molecular oxygen of the Hill reactions is evolved not from the splitting of water, but from the splitting of the hydrocarbon (Warburg and Krippahl 1958).

The bicarbonate effect in the Hill reaction has been widely confirmed. However, scientists have proven that oxygen still comes only from water photolysis in the Hill reaction under the stimulation of bicarbonate using  $HC^{18}O_3^-$  as a tracer. In 1975, Stemler and Radmer conducted an  $HC^{18}O_3^-$  labeling experiment using disrupted chloroplasts depleted of bicarbonate/ $CO_2$  and lacking the enzyme carbonic anhydrase (CA) to determine whether bicarbonate acts as the immediate source of  $O_2$ . They found that  $HCO_3^-$ -depleted broken chloroplasts evolved only small amounts of ordinary  $O_2$  without the addition of  $HC^{18}O_3^-$  under illumination. However, the  $HCO_3^-$ -depleted broken chloroplasts with the addition of  $HC^{18}O_3^-$  under illumination released oxygen that was almost entirely unlabeled and less than 0.1% was doubly labeled. Therefore, they concluded that  $HCO_3^-$  was not the immediate source of photosynthetically evolved oxygen, as the administration of bicarbonate produced a fivefold stimulation of oxygen evolution in the chloroplast Hill reaction (Stemler and Radmer 1975).

Single short saturating light flash experiment demonstrated that the evolution of oxygen was accompanied by flash-induced period-four oscillations. The dark-adapted chloroplasts were subjected to the first and second flashes, and little or no  $O_2$  was produced. After the third flash,  $O_2$  was released at most. After the fourth flash, the evolution of  $O_2$  was the second highest, followed by a gradual decrease to a constant value (Allen and Franck 1955; Kok et al. 1970; Joliot 2003). The above flash-induced photosynthetic  $O_2$  has also been experimentally proven that it came only from

water photolysis. In 1980, Radmer and Ollinger determined the evolution of  $O_2$  elicited by short saturating light flashes in  $HCO_3^-$ -depleted chloroplasts in the dark reactivated with  $HC^{18}O_3^-$ . They found that “all” the  $O_2$  evolved was unlabeled. Therefore, they supported that the  $O_2$  evolved from neither bicarbonate nor  $CO_2$  (Radmer and Ollinger 1980).

By the 21st century, many scientists still insisted that water was the sole substrate for  $O_2$  production by photosystem II in contemporary oxygenic organisms. For example, in 2006, Hillier et al. measured the  $^{18}O$  enrichment of the  $O_2$  produced in 50 mM bicarbonate enriched to 96%  $^{18}O$  and 50 mM unlabeled bicarbonate both with 10  $\mu M$  ethoxymolamide (EZ) (a CA inhibitor) for spinach PS II membrane fragments illuminated by 16 saturating light flashes. They found that there was a small ( $\sim 5\%$  relative) increase in the  $^{18}O$  enrichment of the  $O_2$  evolved in the presence of labeled bicarbonate compared with that measured in 50 mM unlabeled bicarbonate. Almost all photosynthetic oxygen that evolves in the presence of labeled or unlabeled bicarbonate is unlabeled oxygen. Accordingly, they concluded that bicarbonate is not a physiologically significant substrate, and the bulk of  $O_2$  produced comes from water; this is because exceedingly low levels of bicarbonate oxidation may occur in contemporary photosystem II (Hillier et al. 2006). The above-mentioned heavy oxygen labeling assessments give the impression that  $O_2$  evolving only from water photolysis is irrefutable (Fig. 1).

## 3 Challenge

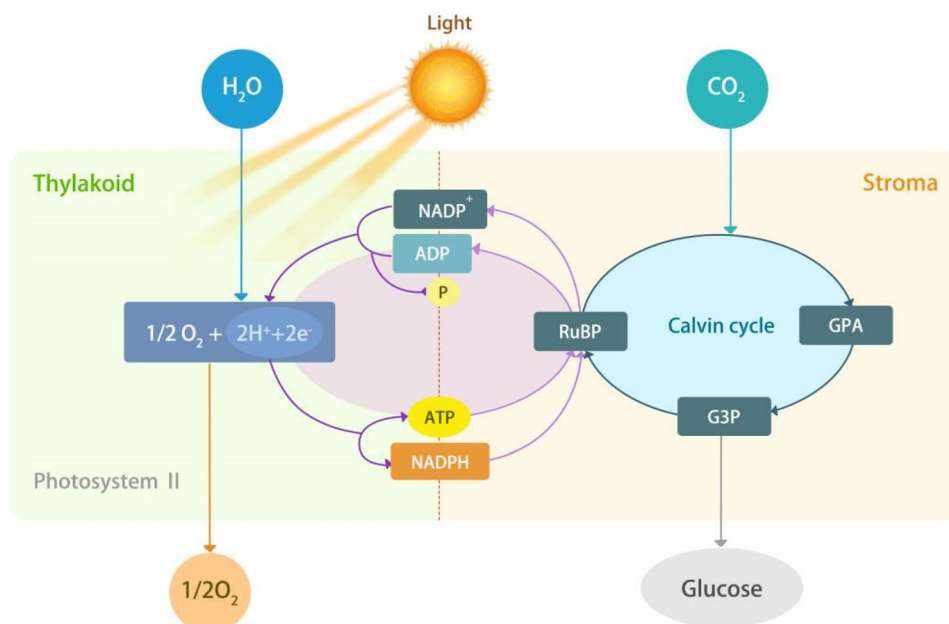
### 3.1 Challenge 1 bicarbonate significantly influences photosynthetic oxygen evolution

Bicarbonate effect in the Hill reaction is the most effective argument against the fact that  $O_2$  evolved only from water photolysis. Oxygen evolution can increase several times or more than ten times under the stimulation of bicarbonate; however, oxygen evolution of *Chlorella* illuminated completely cannot be begin without inorganic carbon (Warburg and Krippahl 1958; Warburg et al. 1965), which demonstrates that the premise of oxygen evolution should be in the presence of inorganic carbon. The role of bicarbonate played in photosynthetic oxygen evolution is significantly greater than that of water.

### 3.2 Challenge 2 existence of Dole effect is strongly conflict with that the oxygen comes only from water photolysis

If the oxygen released by plants during photosynthesis only comes from water, the oxygen isotope composition of oxygen in the atmosphere should be very close to that of seawater. However, atmospheric  $O_2$  is nearly 24‰ more

**Fig. 1** Schematic diagram of photosynthetic oxygen evolution that is generally accepted and written into textbooks. The oxygen evolved comes only from water photolysis; that is, 2 moles of water yield 1 mol of oxygen. Photosynthetic oxygen evolution is not associated with  $\text{CO}_2$  assimilation



enriched in  $^{18}\text{O}$  than seawater, and this enrichment is known as the Dole effect (Metzner 1975). Even if scientists take all factors (including physical, chemical and biological factors) into account, the global  $^{18}\text{O}$  enrichment in atmospheric  $\text{O}_2$  with respect to ocean water (Dole effect) would still be 21‰–24‰ (Bender et al. 1994; Hoffmann et al. 2004; Mader et al. 2017). This indicates that the sources of oxygen in the atmosphere is not only water, but also another source almost equal to water source.

### 3.3 Challenge 3 thermodynamically, bicarbonate photolysis is more feasible than water photolysis in photosystem II

The standard free energy variance in the chemical equilibria ( $\text{H}^+ + \text{HCO}_3^- \rightarrow \frac{1}{2}\text{O}_2 + 2\text{e}^- + 2\text{H}^+ + \text{CO}_2$ ) was 24.8 kcal/mol, and that in the chemical equilibria ( $\text{H}_2\text{O} \rightarrow \frac{1}{2}\text{O}_2 + 2\text{e}^- + 2\text{H}^+$ ) was 37.3 kcal/mol (Disimukes et al. 2001). The free energy required for  $\text{O}_2$  evolution using bicarbonate as the substrate is significantly lower than that required using water as the substrate during photosynthesis of organisms. This result demonstrates that bicarbonate photolysis is more likely to occur during photosynthetic  $\text{O}_2$  evolution than water photolysis.

### 3.4 Challenge 4 thylakoid carbonic anhydrase (CA) and photosystem II with CA activity

#### 3.4.1 Many types of carbonic anhydrases in chloroplasts

Carbonic anhydrase (CA, EC 4.2.1.1) has many physiological functions and is ubiquitously found in most cells

from all kingdoms of life. CA catalyzes the reversible conversion of bicarbonate to  $\text{CO}_2$ , which is  $10^7$  times the rate of non-enzymatic reaction and one of the fastest enzymatic reactions. Many types of CA have been found in chloroplasts. CA has also been found tightly bound to thylakoid membranes, and is called thylakoid CA (tCA) (Pronina et al. 2002; Moskvina et al. 2004; Rudenko et al. 2006, 2007).

Thylakoid CA (tCA) shows an association between enzymatic activity and photosystem II (Khristin et al. 2004; Ignatova et al. 2011; Rudenko et al. 2015). On the one hand, the CA inhibitors, acetazolamide and trifluoromethanesulfonamide, suppress not only  $\text{CO}_2$  hydration but also electron transfer and oxygen evolution in photosystem II. In addition, this inhibition was completely reversed by the addition of bicarbonate (Shitov et al. 2011, 2018). Meanwhile, bicarbonate functions not only as a mobile proton acceptor, but also as a light-driven producer of both  $\text{O}_2$  and  $\text{CO}_2$  (with a constant stoichiometric relationship) (Koroidov et al. 2014). This indicates that bicarbonate is involved in electron transfer as proton acceptor, electron donor, oxygen and  $\text{CO}_2$  evolution in the photosystem II. On the other hand, tCA activity is dependent upon the surrounding redox potential and is similar to that of photosystem II (Moubarak-Milad and Stemler 1994). Similarly, photosystem II inhibitors, hydroxylamine and 3-(3,4-dichlorophenyl)-1,1'-dimethylurea (DCMU), inhibit the activity of tCA (Rudenko et al. 2015). This indicates that tCA catalyzes a redox reaction similar to the photosystem II.

### 3.4.2 Photosystem II with CA function differently from that of typical CA

To date, the CA located in the photosystem II core-complex cannot be completely isolated, thus, it is called intrinsic CA. The intrinsic CA in the photosystem II core-complex undergoes only CO<sub>2</sub> hydration under physiological conditions (Lu and Stemler 2007). Some inhibitors exhibit good inhibitory effects on the activity of CA, but have little effect on oxygen evolution in the photosystem II (Rodionova et al. 2017). This may indicate that CA is not a separable part of the photosystem II core-complex. Photosystem II likely functions both as oxygen evolution and CA, however, it is not as a typical CA.

### 3.4.3 The activity of oxygen evolution in the tCA/ photosystem II is dependent on Mn

The Mn clusters (Mn<sub>4</sub>CaO<sub>5</sub>) of photosystem II, as oxo bridges linking the Mn atoms, may be catalyzed for dioxygen formation to yield oxygen (Umena et al. 2011). The oxygen evolution activity of photosystem II core-complex depends on the presence of Mn. The activity of oxygen evolution was inhibited, but the activity of CO<sub>2</sub> hydration of photosystem II core-complex was not suppressed after removing Mn clusters (Dai et al. 2001). Moreover, CA Mn-substituted the active-site zinc (CA[Mn]) functions as peroxidase and produces O<sub>2</sub> in the presence of bicarbonate (Okrasav and Kazlauskas 2006). This implied that, like CA[Mn], photosystem II core-complex causes bicarbonate oxidation to produce oxygen catalyzed by Mn. The above interaction between the photosystem II and bicarbonate suggests that CO<sub>2</sub> hydration is coupled with the chemical equilibria,  $H^+ + HCO_3^- \rightarrow 1/2 O_2 + 2e^- + 2 H^+ + CO_2$ , and occurs in a photosystem II core-complex.

## 3.5 Challenge 5 sulphid photolysis in anaerobic photosynthetic bacteria during photosynthesis

Some anaerobic photosynthetic bacteria, such as *Oscillatoria limnetica*, split hydrogen sulfide rather than water, and release sulfur rather than oxygen. Thus indicates that photosynthetic organisms can split substances other than water during photosynthesis under light (Cohen et al. 1975). Thus, water is not the sole substrate used for photolysis during photosynthesis.

## 4 Revisiting some heavy oxygen labeling experiments

### 4.1 Revisiting the heavy oxygen (O<sup>18</sup>) labeling experiment from Ruben et al. (1941)

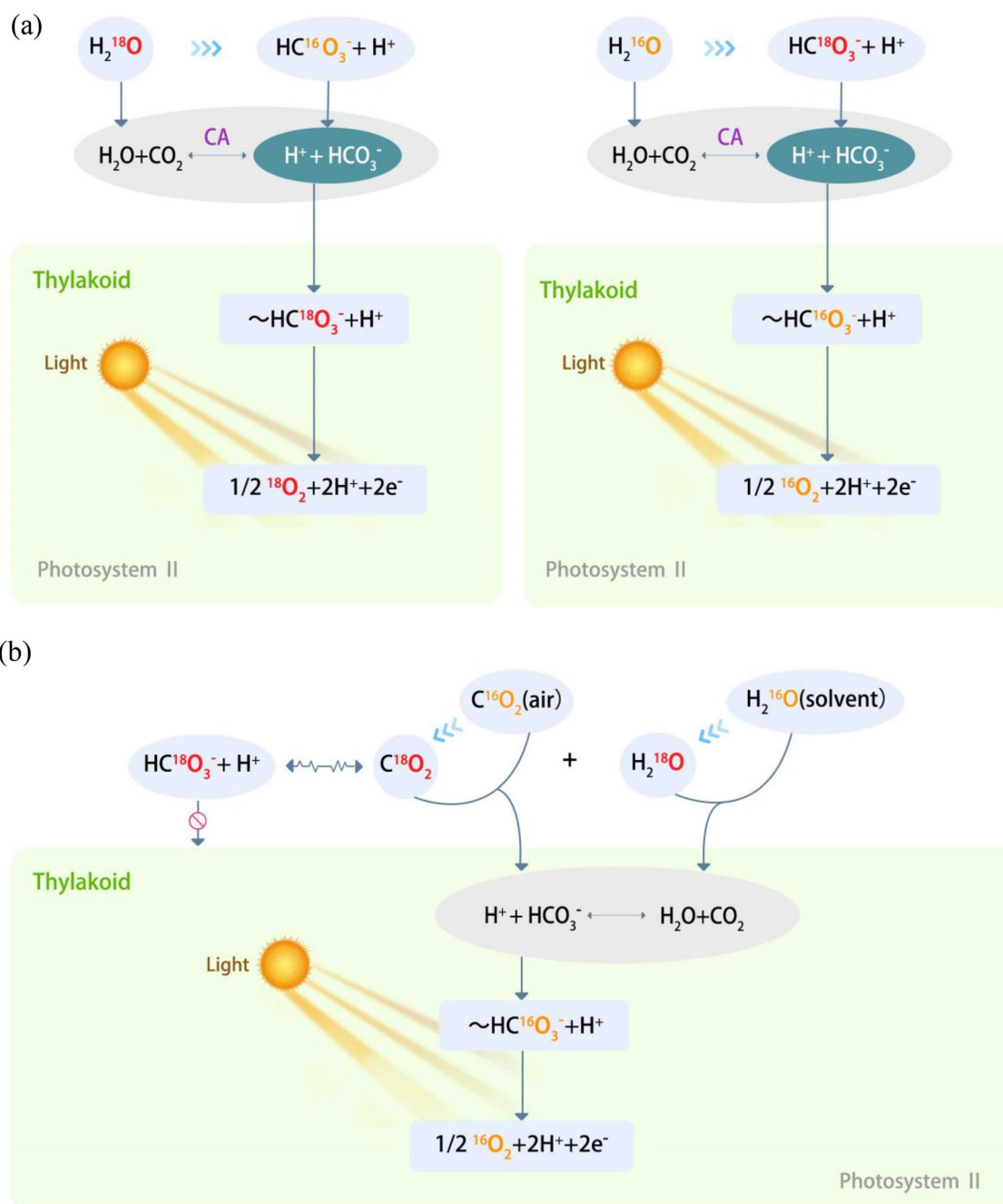
This experiment was performed using active *Chlorella* cells. Algae were suspended in ordinary or heavy oxygen water and heavy oxygen or ordinary potassium bicarbonate and carbonate. They found that the O<sup>18</sup>/O<sup>16</sup> ratio of oxygen evolved during photosynthesis by *Chlorella* is identical to that of water. Therefore, they concluded that the oxygen evolved by *Chlorella* originated only from water. The premise of this conclusion drawn is that the oxygen in group such as OH, C=O, COOH exchanges very slowly with water under the experimental conditions (Ruben et al. 1941).

However, the premise does not occur in the experiment. *Chlorella* has many types of CAs (Hogetsu and Miyachi 1979), and photosystem II in algae inevitably function as CAs. CA rapidly catalyzes the chemical equilibria  $H^+ + HCO_3^- \leftrightarrow H_2O + CO_2$ , thereby speeding up the exchange of heavy oxygen in HC<sup>18</sup>O<sub>3</sub><sup>-</sup> with ordinary oxygen in water, or ordinary oxygen in bicarbonate with heavy oxygen in H<sub>2</sub><sup>18</sup>O. The maximal concentration of bicarbonate used in the experiment was lower than 0.2 M, which was less than 0.4% of the water concentration (55.6 M) in the system. The oxygen in bicarbonate would exchange with almost all oxygen in water. Finally, irrespective of whether water photolysis or bicarbonate photolysis occurs in the experimental system, the O<sup>18</sup>/O<sup>16</sup> ratio of oxygen evolved in photosynthesis by *Chlorella* would always be found identical to that of the water (Fig. 2a).

### 4.2 Revisiting the heavy oxygen (O<sup>18</sup>) labeling experiment from Stemler and Radmer (1975)

This experiment was performed using HCO<sub>3</sub><sup>-</sup>-depleted maize chloroplast fragments. Stemler and Radmer found that HCO<sub>3</sub><sup>-</sup>-depleted broken chloroplasts released oxygen stimulated by HC<sup>18</sup>O<sub>3</sub><sup>-</sup> under illumination, which was almost entirely unlabeled and less than 0.1% doubly labeled. Similarly, they concluded that the photosynthetically evolved oxygen stimulated by bicarbonate also comes from water rather than inorganic carbon. This conclusion is reached on the premise of excluding the influence of exogenous inorganic carbon and effectively controlling the isotope exchange between H<sub>2</sub>O and CO<sub>2</sub> (Stemler and Radmer 1975).

However, although researchers removed nearly all CAs and HCO<sub>3</sub><sup>-</sup> from broken chloroplast fragments, this experiment did not isolate CO<sub>2</sub> from the air, or effectively



**Fig. 2** Bicarbonate photolysis was performed to explain two classic heavy oxygen labeling experiments under continuous light. **(a)** In the experiment from Ruben et al. (Ruben et al. 1941), carbonic anhydrases (CAs) in *Chlorella* rapidly catalyzes  $\text{CO}_2$  hydration and bicarbonate dehydration, thereby speeding up the exchange of heavy oxygen in  $\text{HC}^{18}\text{O}_3^-$  with ordinary oxygen in water, and also the exchange of ordinary oxygen in bicarbonate with heavy oxygen in  $\text{H}_2^{18}\text{O}$ . The concentration of water is much greater than that of inorganic carbon, and the oxygen in bicarbonate exchanges with almost all the oxygen in water. **(b)** In the experiment from Stemler and Radmer (Stemler and Radmer 1975), the action of photosystem II (like CA) causes the exchange of the oxygen in OH, C=O and COOH. Since the amount of unlabeled  $\text{CO}_2$  in the air and the amount of water as a solvent are respectively much greater than the amount of labeled  $\text{CO}_2$  and labeled  $\text{H}_2\text{O}$  converted from labeled bicarbonate, almost all bicarbonates used for photolysis are unlabeled

control the group exchange of oxygen among OH, C=O and COOH in a bicarbonate-stimulated Hill reaction system. When  $\text{NaHC}^{18}\text{O}_3$  was added into the Hill reaction system of  $\text{HCO}_3^-$ -depleted broken chloroplasts during the

dark period, they obtained the maximum signal of  $\text{C}^{18}\text{O}_2$  and recorded a certain amount of ordinary signal of  $\text{CO}_2$  within a few seconds. This indicates that the conversion of bicarbonate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  and the exchange of labeled



$\text{CO}_2$  and unlabeled  $\text{CO}_2$  were performed in the Hill reaction system. Because  $\text{CO}_2$  is a liposoluble substance, it can easily enter the thylakoid membrane. However, the water-soluble bicarbonate remaining in the Hill reaction system could not pass through the thylakoid membrane. After the onset of illumination, the photosystem II in the thylakoid membrane undergoes extremely rapid  $\text{CO}_2$  hydration to form bicarbonate, which then quickly transformed into  $\text{H}_2\text{O}$  and  $\text{CO}_2$  under physiological conditions (Lu and Stemler 2007). The action of photosystem II (like CA) lasts for a long time (more than seconds) (Delosme and Joliot 2002), which almost leads to a full exchange of the oxygen in  $\text{CO}_2$  with the oxygen in  $\text{H}_2\text{O}$ , resulting in minimal heavy oxygen ( $\text{O}^{18}$ ) labeling  $\text{HC}^{18}\text{O}_3$ . Therefore, if the photolysis of bicarbonate occurs in  $\text{HCO}_3^-$ -depleted maize chloroplast fragments, the bicarbonate as the substrate is barely labeled, and the oxygen evolved would also be almost entirely unlabeled (Fig. 2b).

#### 4.3 Revisiting the heavy oxygen ( $\text{O}^{18}$ ) labeling experiment from Radmer and Ollinger (1980)

The experiment was performed using  $\text{HCO}_3^-$ -depleted chloroplasts under the action of short saturating light flashes. Radmer and Ollinger found that oxygen evolved by the  $\text{HCO}_3^-$ -depleted chloroplasts exhibited a 7- to 8-fold decrease in the rate of the Hill reaction via the untreated chloroplasts under continuous light. Meanwhile, the species and magnitude of  $\text{O}_2$  evolved by  $\text{HCO}_3^-$ -depleted chloroplasts were similar to those evolved by depleted chloroplasts reactivated with unlabeled or labeled  $\text{HCO}_3^-$  under the action of short saturating light flashes. In fact, the experiment indicated that the oxygen evolved by  $\text{HCO}_3^-$ -depleted chloroplasts under short saturating light flashes was one order of magnitude less than that under continuous illumination, while being independent of exogenous  $\text{HCO}_3^-$  used for activation (Radmer and Ollinger 1980). However, the existence of bicarbonate photolysis by photosystem II was not ruled out.

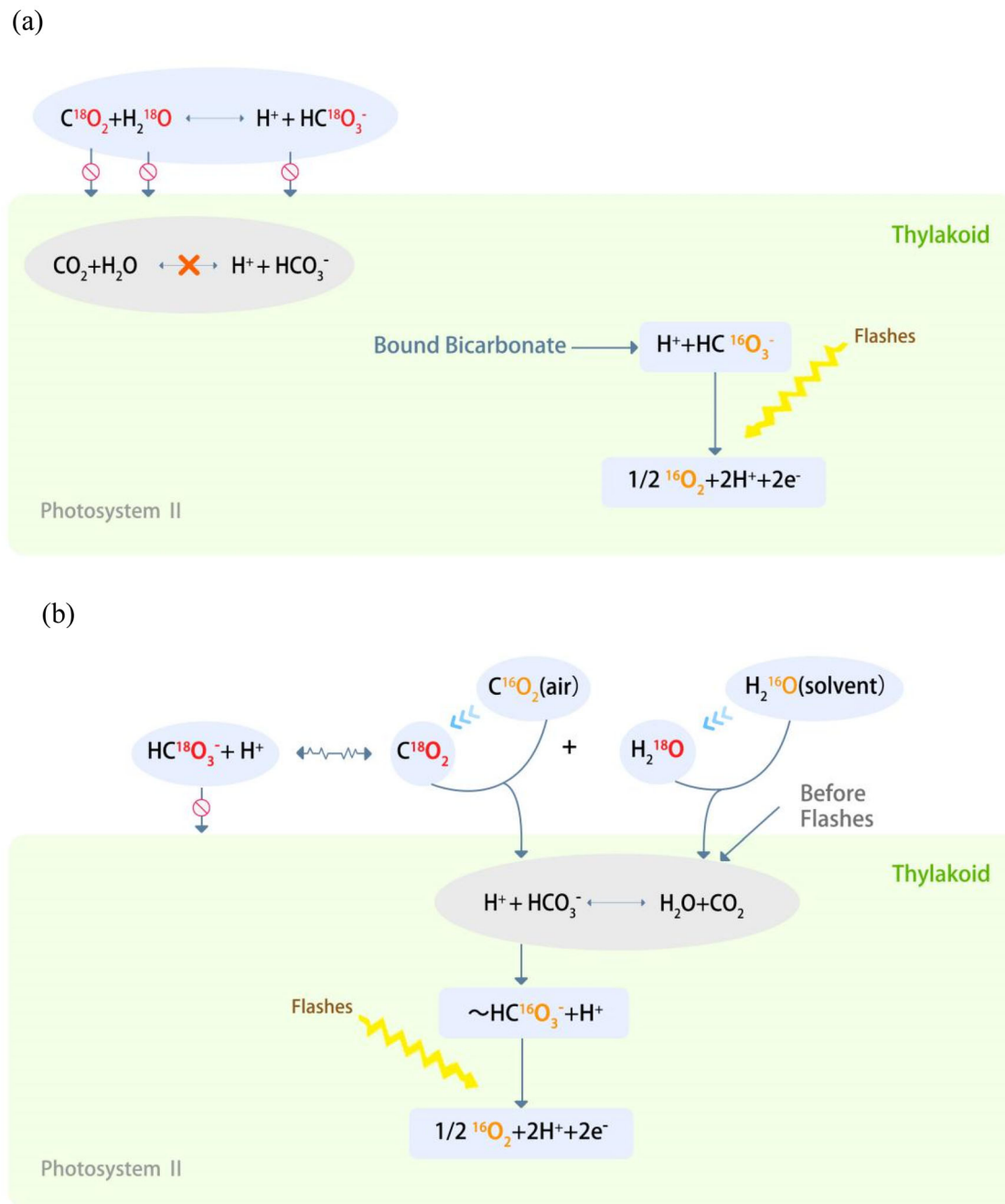
Many studies have shown that a considerable amount of bicarbonate is bound to the vicinity of the oxygen-evolving center of the photosystem II, either (both) in the acceptor or (and) in the donor side of photosystem II, and it seems to flow freely (Van Rensen and Xu 1999; Stemler 2002; Shevela et al. 2012; Koroidov et al. 2014; Tikhonov et al. 2018). However, bound bicarbonate in the photosystem II is not necessary for photosynthetic  $\text{O}_2$  evolution (Van Rensen and Vermaas 1981), and many binding sites for bicarbonate do not always exist, depending on the pH value (Bowden et al. 1991). The architecture of the photosynthetic oxygen-evolving center does or does not display bi(tri)dentate ligand-bound bicarbonate in different observations (Ferreira 2004; Umena et al. 2011). These results

indicate that bicarbonate bound in photosystem II can be transformed into free bicarbonate. Exogenous bicarbonate is a polar substance that cannot easily enter the oxygen-evolving center of the photosystem II under short saturating light flashes. Therefore, assuming that bicarbonate photolysis does occur in the photosystem II, this photosynthetic  $\text{O}_2$  evolution should be elicited by bound bicarbonate rather than by exogenous bicarbonate (Fig. 3a). The evolved  $\text{O}_2$  elicited by bound bicarbonate is of course ordinary oxygen, and the amount of oxygen evolution is also one order of magnitude less than that elicited by exogenous bicarbonate.

The experiment also showed that the labeled  $\text{O}_2$  signal increased 8-fold compared to the unlabeled signal when 100  $\mu\text{l}$  heavy oxygen or 3  $\mu\text{l}$  ordinary water was respectively added in total darkness to dark-adapted normal chloroplasts under short saturating light flashes. This does not bring sole explanation that the oxygen flash yielded comes only from the water. Water does not easily enter the cell membrane, and the most common way for water to pass through chloroplast membrane is simple and slow diffusion. However, there are many CAs in the chloroplast, even in the thylakoids. The soluble CA in the stroma works in coordination with the intrinsic and extrinsic CAs in the thylakoids to form an inorganic carbon pool in the thylakoids, where the rapid group exchange of oxygen in  $\text{H}_2\text{O}$  and inorganic carbon occur. The rate of diffusion on the chloroplast membrane of 100  $\mu\text{l}$  heavy oxygen water was one order of magnitude greater than that of 3  $\mu\text{l}$  ordinary water, and the amount of bicarbonate transformed by heavy oxygen water and  $\text{CO}_2$  was also one order of magnitude greater than that transformed by ordinary water and  $\text{CO}_2$  in the thylakoids. Thus, the labeled  $\text{O}_2$  signal elicited by bicarbonate transformed from 0.1 ml  $\text{H}_2^{18}\text{O}$  is one order of magnitude greater than the unlabeled  $\text{O}_2$  signal elicited by bicarbonate transformed from 3  $\mu\text{l}$  of ordinary water in the photosystem II after bicarbonate photolysis under the action of short saturating light flashes.

#### 4.4 Revisiting the heavy oxygen ( $\text{O}^{18}$ ) labeling experiment from Hillier et al. (2006)

The experiment was performed using spinach photosystem II membrane fragments illuminated by 16 saturating light flashes (5 Hz repetition rate) in the presence of 10  $\mu\text{M}$  ethoxzalamide. Hillier et al. found that almost all the evolved oxygen was ordinary oxygen in the presence of labeled or unlabeled bicarbonate (Hillier et al. 2006). However, the above result cannot draw the conclusion on that bicarbonate is not a physiologically significant substrate. The isotopic exchange of oxygen among bicarbonate,  $\text{CO}_2$  and the solvent water, and the conversion of bicarbonate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$  were significantly inhibited

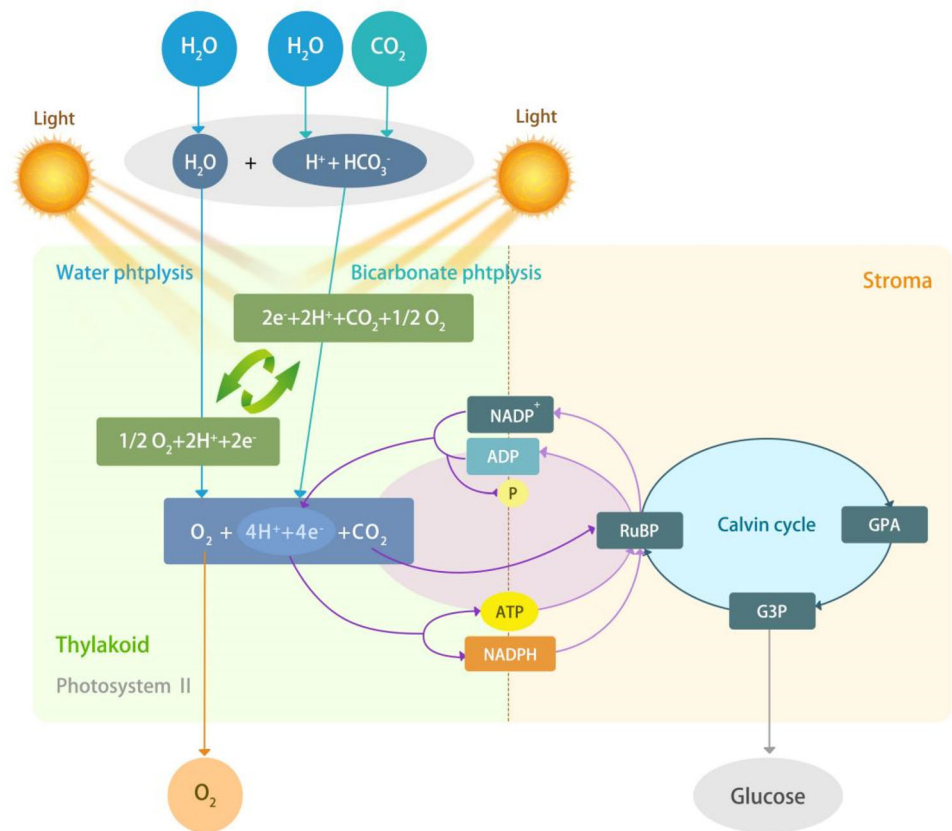


**Fig. 3** Bicarbonate photolysis was performed to explain two classic heavy oxygen labeling experiments under flashes. **(a)** In the experiment from Radmer and Ollinger (Radmer and Ollinger 1980), the oxygen evolved by  $\text{HCO}_3^-$ -depleted chloroplasts under short saturating light flashes comes from bound bicarbonate, and it is independent of exogenous  $\text{HCO}_3^-$  used for activation. **(b)** In the experiment from Hillier et al. (Hillier et al. 2006), most bicarbonates in photosystem II that were produced via the conversion of atmospheric  $\text{CO}_2$  and water as solvent are unlabeled. Moreover, the majority of the bicarbonates used for photolysis are not labeled

when ethoxymethylamine is used to inhibit the activity of intrinsic CAs in photosystem II. However, atmospheric  $\text{CO}_2$  is not removed from the reaction system. Before the 16 flashes, labeled or unlabeled bicarbonate was injected into the reaction system. Obviously, less  $\text{CO}_2$  in the reaction system comes from the added bicarbonate as ethoxymethylamine inhibits the activity of the intrinsic CAs in

photosystem II. In addition, water-soluble bicarbonate cannot easily enter, however, liposoluble  $\text{CO}_2$  can easily enter the photosystem II membrane, where extremely rapid  $\text{CO}_2$  hydration can take place to form bicarbonate (Lu and Stemler 2007), with most bicarbonate in photosystem II being produced via the conversion of water as solvent and atmospheric  $\text{CO}_2$ . Therefore, if bicarbonate photolysis in

**Fig. 4** The combined effort of bicarbonate and water in photosynthetic oxygen evolution. The premise of water photolysis is bicarbonate photolysis. Bicarbonate photolysis and water photolysis work together in a 1:1 (mol/mol) stoichiometric ratio, releasing oxygen and carbon dioxide in a 1:1 (mol/mol) stoichiometry



photosystem II produces oxygen, the oxygen evolved is mostly ordinary oxygen (Fig. 3b).

## 5 Bicarbonate photolysis and water photolysis as well as their roles in photosynthesis

If photosynthetic organisms only perform photosynthetic oxygen evolution through bicarbonate photolysis, the following reaction occurs:  $\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{H}^+ + \text{HCO}_3^- \rightarrow \frac{1}{2} \text{O}_2 + 2\text{e}^- + 2\text{H}^+ + \text{CO}_2$  (Wu 2021a). It can be seen from the reaction equation that 2 moles of bicarbonate provide 1 mol of oxygen and 2 moles of  $\text{CO}_2$ . This would mean assimilation is twice that of dissimilation to maintain the metabolism balance in nature, which seems unreasonable. If photosynthetic oxygen evolution produced by photosynthetic organisms occurs only through water photolysis, the following reaction occurs in photosynthetic organisms:  $\text{H}_2\text{O} \rightarrow \frac{1}{2} \text{O}_2 + 2\text{e}^- + 2\text{H}^+$ . It can be seen from the reaction equation that 2 moles of water can provide only 1 mol of oxygen and there is no stoichiometric relationship between the water photolysis and the carbon dioxide assimilation (Fig. 1). Therefore, both bicarbonate photolysis and water photolysis account for half of the photosynthetic oxygen evolution respectively, which can not

only explain bicarbonate effect and carbon dioxide concentrating mechanisms (CCMS) in photosystem II, but also elaborate the origin of oxygen evolved in overall chemical reaction of photosynthesis more clearly than the traditional explanation (Fig. 4). The synthetic formula of photosynthetic oxygen evolution can be written as follows:  $2\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{H}_2\text{O} + \text{H}^+ + \text{HCO}_3^- \rightarrow \text{O}_2 + 4\text{e}^- + 4\text{H}^+ + \text{CO}_2$ . It can be seen from the synthetic formula that photosynthetic oxygen evolution occurs in the presence of inorganic carbon. In other words, bicarbonate photolysis governs photosynthetic oxygen evolution (Fig. 4).

Bicarbonate photolysis is the premise of water photolysis, which is accompanied by bicarbonate photolysis during the photosynthetic oxygen evolution; this may explain why neither water photolysis nor bicarbonate photolysis is performed by anaerobic photosynthetic bacteria such as sulfur bacteria, in the presence of both inorganic carbon and water. These bacteria live in an acidic environment where inorganic carbon exists in the form of carbon dioxide rather than bicarbonate, and thus they do not undergo bicarbonate photolysis; accordingly, no water photolysis under light (Cohen et al. 1975). These bacteria only have acidic substances such as hydrogen sulfide photolysis, which leads to their photosynthesis without oxygen evolution.



It can be seen from the reaction equation of bicarbonate photolysis that  $\text{CO}_2$  obviously acts as a concurrent post of “catalyst” to accelerate the photosynthetic oxygen evolution in photosynthetic cells (bicarbonate effect), which was called “activated  $\text{CO}_2$ ” by Warburg and Krippahl (Warburg and Krippahl 1958). The bicarbonate-mediated  $\text{CO}_2$  formation closely correlating with  $\text{O}_2$  evolution were found on both the donor and acceptor side of PSII, which confirm the role of  $\text{CO}_2$  as a concurrent “catalyst” and authenticity of the reaction equation of bicarbonate photolysis (Shevela et al. 2020).

The existence of Dole effect is in serious conflict with the view that the oxygen in atmosphere only comes from water photolysis by photosynthetic organism. However, we calculated the  $^{18}\text{O}$  enrichment in atmospheric  $\text{O}_2$  according to that bicarbonate photolysis and water photolysis account for half the photosynthetic oxygen evolution respectively, and found that  $^{18}\text{O}$  enrichment in atmospheric  $\text{O}_2$  with respect to water and bicarbonate (0.2043) is infinite approach to actual  $^{18}\text{O}$  content in the atmosphere (0.2041) (using (Metzner 1975) data to calculate). It demonstrated that the oxygen in the atmosphere sourced undoubtedly from water and bicarbonate evenly, and the Dole effect does not exist when  $^{18}\text{O}$  enrichment in atmospheric  $\text{O}_2$  with respect to water and bicarbonate was considered.

Meanwhile, bicarbonate photolysis can not only release oxygen but also provide  $\text{CO}_2$  for ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), elevate and concentrate the  $\text{CO}_2$  concentration in Rubisco, accelerate the absorption and reduction of inorganic carbon, and suppress the oxidation of organic matter. Carbon dioxide concentrating mechanisms (CCMs) in photosystem II, which are responsible for  $\text{CO}_2$  production via bicarbonate photolysis, tend to be, major, primitive, indispensable and crucial for plant survival and growth. CCMs in the plasma membrane function as subordinate (Wu 2021b).

Last but not least, from the entire process of photosynthetic oxygen evolution, it can be seen that the supply of inorganic carbon determines the water demand of plants, which coordinates the balance of water metabolism and carbon metabolism, and ensures that plants to adapt to the environment. For example, when  $\text{C}_3$  plants were subjected to drought stress, their stomata closed. Accordingly, their carbon dioxide supply has stopped, which terminates water photolysis and bicarbonate photolysis, and eliminates the demand for water simultaneously. When the stomata opening increases with a rise in carbon dioxide supply, water photolysis and bicarbonate photolysis also increase. In addition, when plants are in water deficit, they can boost the utilization of root-derived bicarbonate, which can greatly compensate for the shortage of water supply from the environment (Rao and Wu 2017).

## 6 Synergistic evolution of Earth spheres and carbon neutrality in nature

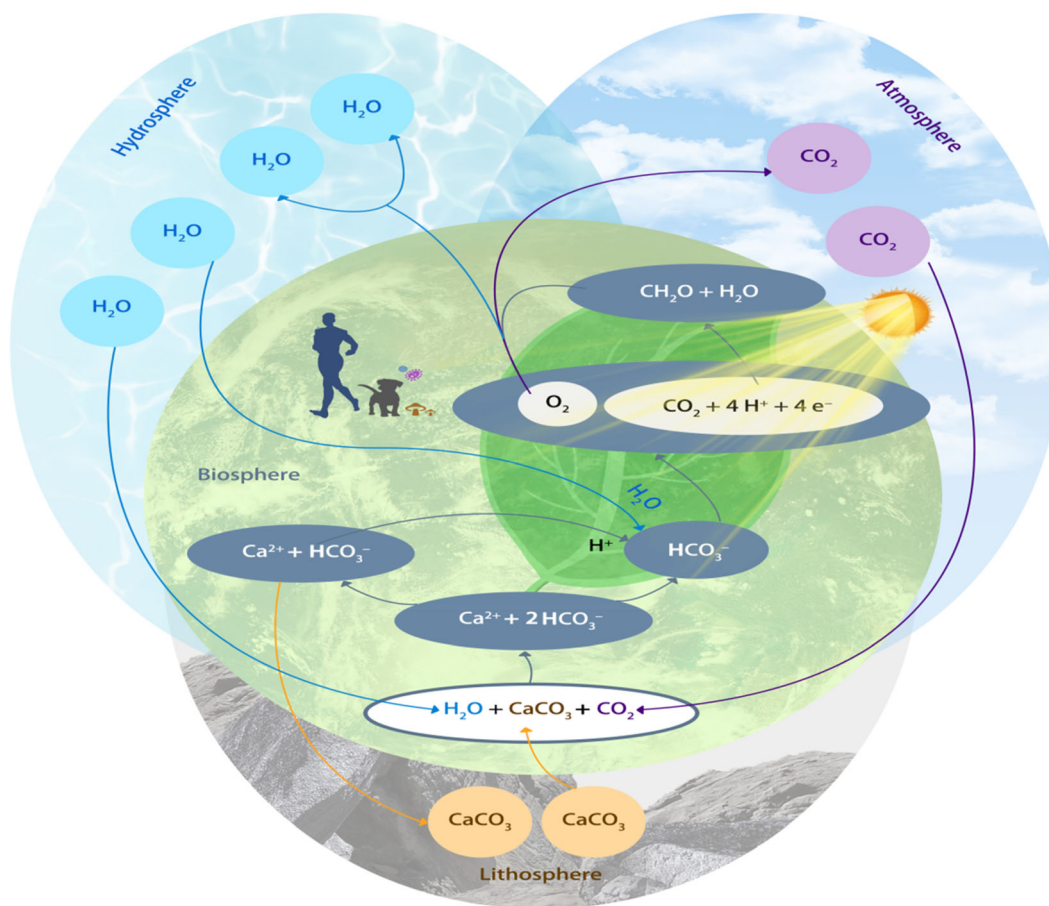
The Earth’s ecosystem can manage its own activities and maintain its own substance balance. Carbon and water balance play key roles in the Earth’s ecosystem. If oxygen evolved only from water photolysis in photosynthetic organisms on Earth in the early stage, it would be difficult for photosynthetic organisms to couple with the hydrosphere and the atmosphere, and it would be even more difficult to couple with the lithosphere (Fig. 1). However, the photosynthetic oxygen evolved from bicarbonate photolysis and water photolysis can stoichiometrically couple with the hydrosphere and atmosphere of the early Earth and then couple with the lithosphere, thereby realizing carbon and water balance (Wu 2022) (Fig. 5).

From the synergistic effects (changes) of  $\text{CO}_2$  and  $\text{H}_2\text{O}$  in the atmosphere, bicarbonate,  $\text{CO}_2$  partial pressure and pH in the hydrosphere, and carbonate rocks in the lithosphere on Earth in the early stage, photosynthetic oxygen evolution is actually stoichiometrically coupled with the atmosphere, hydrosphere and lithosphere (Hart 1978; Kasting 1993; Des Marais 2000; Dismukes et al. 2001; Halevy and Bachan 2017; Wu 2022) (Fig. 5).

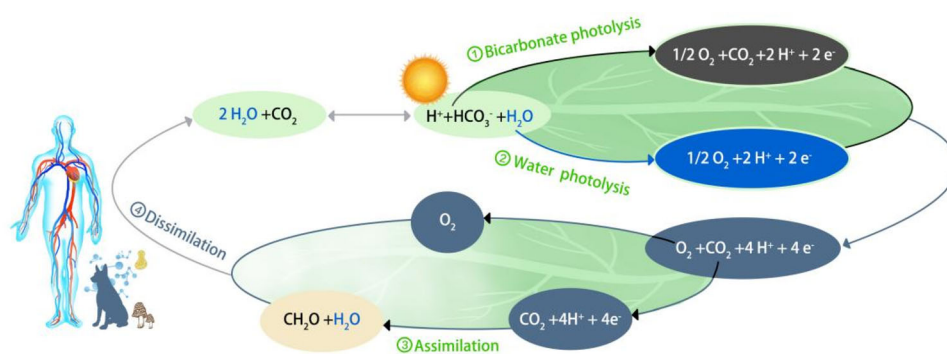
Furthermore, the photosynthetic oxygen evolved from bicarbonate photolysis and water photolysis represents the coupling as 1:1 (mol/mol) stoichiometric relationship between the reduction of  $\text{CO}_2$  and oxidation of organic carbon, as well as between the substance cycle and energy cycle in the biosphere, which contributes to “carbon neutrality” in the whole Earth system (Fig. 6). Introducing a more accurate process of photosynthetic oxygen evolution into textbooks can assist future generations in learning from nature in managing carbon balance and water balance.

## 7 Conclusion

Many types of CA have been found in chloroplasts. Meanwhile, photosystem II functions as CA to catalyze the reaction of  $\text{CO}_2$  hydration under physiological conditions, which leads to almost complete isotope exchange between oxygen in water and oxygen in inorganic carbon in the Hill reaction system. This recent knowledge and research progress enable us to draw new conclusions based on bicarbonate effect, Dole effect, the characteristics of photosystem II, and several classic heavy oxygen labeling experiments. Photosynthetic oxygen evolution should source from both bicarbonate and water as 1:1 (mol/mol) stoichiometric relationship, which initiates the journey of water metabolism and carbon metabolism in nature,



**Fig. 5** The coupling of photosynthetic oxygen evolution and Earth spheres. Carbonate rocks (lithosphere) are dissolved under the action of water (hydrosphere) and carbon dioxide (atmosphere) to form  $\text{Ca}^{2+}$  and bicarbonate (biosphere).  $\text{Ca}^{2+}$  combines with bicarbonate to form the precipitation of calcium carbonate ( $\text{CaCO}_3$ ) into the lithosphere. Plants split bicarbonate and water to release oxygen and carbon dioxide (biosphere). Plants then assimilate carbon dioxide to form carbohydrates ( $\text{CH}_2\text{O}$ ) (biosphere). Finally, organisms utilize oxygen to decompose carbohydrates into carbon dioxide to the atmosphere and water to the hydrosphere



**Fig. 6** Carbon neutrality and water neutrality in nature driven by photosynthetic oxygen evolution. The photosynthetic oxygen evolution, which converts light energy into chemical energy, is performed by the synergistic action of bicarbonate photolysis (①) and water photolysis (②) with a stoichiometric relationship of 1:1 (mol/mol). It produces oxygen and  $\text{CO}_2$  in a stoichiometric ratio of 1:1, which determines that inorganic carbon assimilation (③) is equal to organic carbon dissimilation (④). Finally, from the starting point (photosynthetic oxygen evolution) to the endpoint (carbon dissimilation) of the carbon neutrality strategy, the net flux of both  $\text{CO}_2$  and water in nature is 0

solidifies the relationship of assimilation equal to dissimilation, and provides a demonstration of how to coordinate water and carbon balances to maintain water neutrality and carbon neutrality (Fig. 6). In the future, more direct evidence about that the O<sub>2</sub> evolution in photosynthesis evenly comes from water and bicarbonate will need to provide.

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## Declarations

**Conflict of interest** The author declares no conflict of interest.

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