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ISOTOPE RATIOS IN PHOTOSYNTHETIC OXYGEN

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Summary

Axenic suspensions of the fresh water green alga *Ankistrodesmus braunii* were illuminated under aerobic conditions. The released gas mixture was introduced into the ion source of an isotope mass spectrometer, which recorded the $^{18}O/^{16}O$ ratio. The ^{18}O content of the photosynthetic oxygen ($\approx 0.199\%$) exceeded that of the cell water ($\approx 0.197\%$) significantly.

Introduction

If the release of molecular oxygen in photosynthesis should be connected with rearrangements of interatomic bonds (i.e. O-H and O-O bonds), we have to expect kinetic isotope effects [1]. Since the zero point energy of an O-H bond depends both on the masses of the involved oxygen and hydrogen atoms, there should be a significant discrimination both between ^{16}O and ^{18}O and between ^{1}H and ^{2}H [2]. Water decomposition should therefore produce oxygen with an isotope ratio different from that of water. As the ^{18}O-H bond is somewhat more reluctantly decomposed [3,4], the released O_2 should demonstrate an ^{16}O enrichment. This assumption can actually be confirmed both in electrolysis [5] and in the chemical water decomposition [6]. On the other hand, it is well known that atmospheric oxygen (which is thought to be the result of photosynthesis) is significantly enriched in ^{18}O [7—11]. There are two possibilities to explain this shift: within the leaves of terrestrial plants the higher evaporation rate of $H_2{}^{16}O$ molecules may lead to an ^{18}O enrichment in the cell water, so that the claimed substrate of photosynthesis is different from normal fresh water [12—14]. Moreover there is good evidence, that cell respiration prefers the lighter oxygen isotope, leaving behind the ^{18}O [15—17]. A quanti-

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Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

tative comparison makes it, however, doubtful, whether the actually observed oxygen isotope differences between the (ocean and fresh) water and the atmosphere can be exclusively caused by transpiration and respiration [18]. In this situation a new series of experiments was started to look for oxygen isotope discrimination in the photosynthetic process itself.

Material and Methods

The experiments were performed with axenic suspensions of the unicellular green alga *Ankistrodesmus braunii* (strain 202-7c). Before the photosynthesis experiments, they were removed from the culture vessels and transferred to closed glass bottles, which remained up to 4 h under dim light conditions. Aliquots (40 ml each) of the suspension were pipetted into a round-bottomed reaction flask placed inside a temperature-controlled water bath (28°C). The top portion of this glass had two gas outlets, which were connected both to a vacuum pump (Edwards Speedivac II) and a helium cylinder. A preevacuated (50 ml) sampling glass was attached to the neck.

To remove the air, the gas space of the flask was flushed with several liters of helium. Then one of the taps was closed and the inert gas removed by 5 s evacuation of the reaction vessel. This treatment does not degas the aqueous medium. So it was possible to keep the cells under aerobic conditions, avoiding fermentation process. Then the tap at the second outlet was closed and the suspension (in complete darkness) vigorously agitated for 5 min. By this means an equilibration of the dissolved oxygen between the gas space and the suspension medium was obtained.

In some cases the cell suspensions were provided with some μl of a DCMU solution (final concentration 10^{-5} M). After this standardized pretreatment the algae were illuminated for periods between 1 and 40 min. The light intensity within the reaction flask was approx. 1 kW $\cdot$ m^{-2}, the average photosynthesis rate approx. 160 μmol $O_2 \cdot$ mg^{-1} chlorophyll $\cdot$ h^{-1}. Control samples remained for the same time intervals under complete darkness. At the end of the light periods the tap of the sampling glass ($p \approx 1$ μbar) was opened for 3 s. By this means 60% of the free gas out of the reaction flask were collected.

All gas analyses were performed with the collected oxygen itself, i.e. there was no conversion of O_2 into CO_2. This rather unusual procedure [17,19] was chosen to avoid oxygen losses during the otherwise necessary carbon oxidation. Since after short illumination periods only small amounts (μmol) of oxygen can be recovered, it was necessary to mix the O_2 with a suitable carrier gas. For this reason the sampling glasses were — up to a final pressure of 1 bar — filled with helium. They were then attached to the inlet of an isotope mass spectrometer with a triple collector (Varian MAT 250). The acceleration voltage was set to 6 kV. A glass with an air/helium ($\approx 1 : 10$) mixture (connected to the reference line of the instrument) served as laboratory standard.

To remove both CO_2 and water vapour, the sampling glasses were immersed into deeply precooled (—140°C) isopentane [17]. This leaves the remaining gases (oxygen, nitrogen, inert gases) unfrozen, whereas in liquid nitrogen some oxygen is retained on the cold glass walls.

The spectrometer measured alternatively the ion currents caused by the introduced masses 32 ($^{16}O_2$), 33 ($^{17}O^{16}O$) and 34 ($^{18}O^{16}O$). It was connected to a desk computer (Hewlett Packard 9815 A), which printed the ratios 34/32, 33/32 and 34/33. From 6–10 consecutive data sets it calculated the mean δ values:

$$\delta = \left[\frac{^{18(17)}O/^{16}O_{\text{sample}} - {}^{18(17)}O/^{16}O_{\text{standard}}}{^{18(17)}O/^{16}O_{\text{standard}}}\right] \times 1000$$

together with their standard deviations. Due to their higher accuracy only the $^{18}O/^{16}O$ ratios were further evaluated. The ^{18}O content of the atmospheric oxygen may be regarded as constant [20]. So the recorded δ values could easily be related to the common international standard (standard mean ocean water).

To determine their ^{18}O content both the suspension and the cell water were collected from freshly harvested (superficially dried) algal cells, which were subjected to an exhaustive vacuum distillation. All water samples were equilibrated with CO_2, the ^{18}O content of which was determined by the usual procedure.

Results

All buffers and nutrient media were prepared with distilled water, which had a δ value of −14.2‰ related to standard mean ocean water (i.e. −37.9‰ related to the laboratory standard). In the nutrient medium the δ values (presumably due to the preferential loss of $H_2{}^{16}O$ (caused by evaporation)) gradually increased up to −12.8‰. Further control experiments showed, that the ^{18}O content of the dissolved air (with $\delta = +22.2 \pm 0.9‰$) was practically identical to that of atmospheric air ($\delta = +23.7 \pm 0.3‰$). This fact is in agreement with the very small solubility difference between ^{16}O and ^{18}O [21]. The δ values of the gas samples collected from DCMU controls (see below) did not differ from air standards ($\delta = +22.9 \pm 0.8‰$).

Before the photosynthesis experiment the dissolved air was not, or only partly, removed. So the collected gas samples must be a mixture of air and photosynthetic oxygen. After short illumination periods there should accordingly be an extreme ‘contamination’ by residual air. It was therefore assumed, that the ‘real’ isotope ratio of photosynthetic oxygen could only be measured after prolonged light periods. If even extended illumination should not lead to constant values, it seemed promising to obtain the final value by extrapolation. The data need a correction, however: cells respire both in light and darkness. This process may lead to a decisive, perhaps the only measurable [22,23], oxygen isotope discrimination. To determine the quantitative influence of cell respiration, control experiments were necessary. Some samples were kept in darkness. As a light-induced gas exchange independent from photosynthesis would be conceivable, another set of suspensions was illuminated in the presence of a potent photosynthesis inhibitor (10^{-5} M DCMU). It was anticipated, that the difference between the recovered O_2 with and without the addition of DCMU indicates the percentage of photosynthetic oxygen related to the total O_2 obtained.

As expected, there was a definite dependence of the δ values from the illumination time. Fig. 1 gives the observed (averaged) δ values as a function of

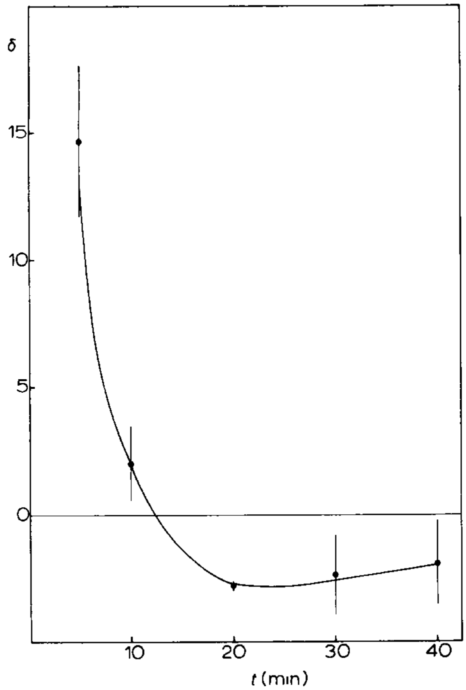

Fig. 1. ^{18}O content (given as δ values, relative to standard mean ocean water) of oxygen samples obtained from illuminated algal suspensions as function of the illumination time. The points represent the averages of (between 10 and 19) measured values, the vertical bars the standard errors of the mean.

photosynthesis time. Due to the high initial air content (with $\delta = +23.7‰$) the curves start with strongly positive ($\delta \approx +15‰$) data. After $\geqslant 20$ min the δ values are between −2.0 and −2.5‰. A more complex situation is given, if we relate the observed δ values to the total amount of O_2 obtained (Fig. 2). To limit the variation of the single values, all data given are the average of ten consecutive results [24]. With 500 μl O_2 a relative minimum is observed; the final values (>1500 μl O_2) lie between −2 and −3‰.

We can now try to correct these curves by a comparison of the apparent δ values with coordinated DCMU controls. This was achieved with the equation

$$V_C \cdot \delta_C + (V_S - V_C) \cdot \delta = V_S \cdot \delta_S$$

with V_S and V_C, δ_S and δ_C as the collected O_2 volumes and δ values for the samples (S) and the controls (C), respectively; δ should be taken as the 'real' value. If the recovered O_2 samples would be mixtures of air and photosynthetic oxygen only, the corrected δ values must be independent from time and volume of released O_2. But surprisingly we obtain even more pronounced minimum curves (Fig. 3), approximating a final δ value between −5 and −6‰.

With the DCMU controls there finally remains another method of evaluation. By quantitative comparisons of the released O_2 volumes with the corresponding values of proper DCMU controls we obtain the percentage of 'contamination' by air. There should be a strictly linear correlation between these data and the observed δ values. Extrapolation to 'zero contamination' (=100% photosyn-

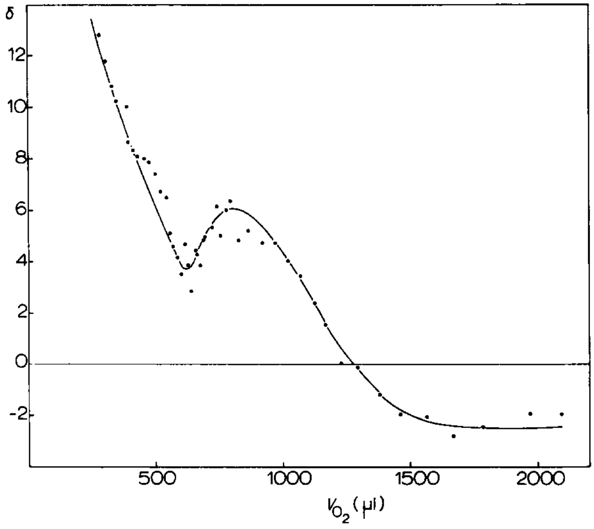

Fig. 2. ^{18}O content (given as δ values, relative to standard mean ocean water) of oxygen samples obtained from illuminated algal suspensions as function of the released O_2 volume. Each point represents the average value of 10 consecutive data (see Ref. 24).

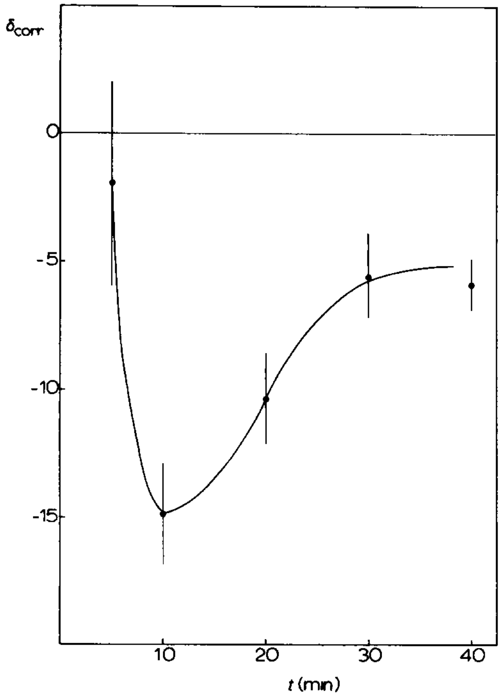

Fig. 3. ^{18}O content (given as 'corrected' δ values, relative to standard mean ocean water) of oxygen samples obtained from illuminated algal suspensions. The directly measured data (see Fig. 1) were compared to corresponding DCMU controls (details see text). The points represent the averages of (between 10 and 19) measured values, the vertical bars the standard errors of the mean.

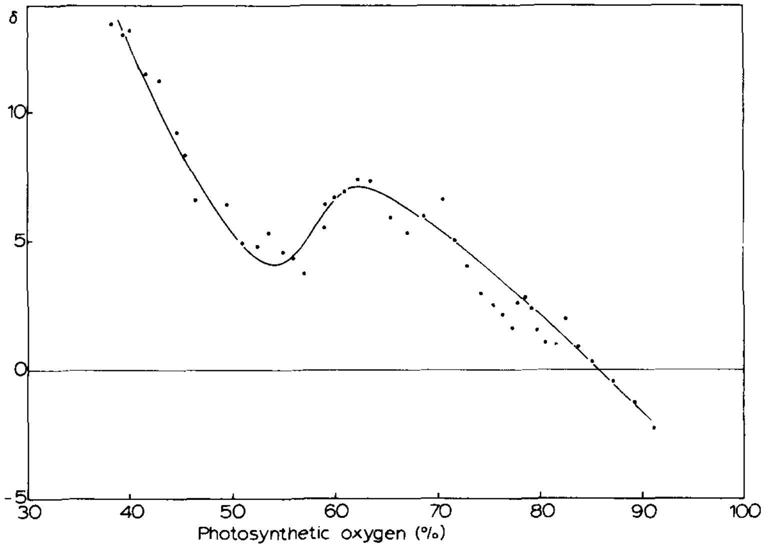

Fig. 4. ^{18}O content (given as δ values, relative to standard mean ocean water) of oxygen samples obtained from illuminated algal suspensions as function of the percentage share of photosynthetic oxygen (details see text). Each point represents the average value of 10 consecutive data (see Ref. 24).

thetic oxygen) should then give the 'real' δ values. Fig. 4 demonstrates, however, that even this curve passes a relative minimum region. Extrapolation leads to a final δ value between −5 and −6‰.

So there remains only the possibility to calculate the 'real' δ values from all curves obtained. Taken all our data together, we may assume that the 'real' δ value of the photosynthetic oxygen produced by the studied fresh-water alga is very near to that standard mean ocean water ($^{18}O = 0.1995\%$). Slightly below the standard mean ocean water value, it corresponds to ≈0.199%.

Respiring cells preferentially consume ^{16}O; dark controls should therefore demonstrate a slight isotope shift to higher $^{18}O/^{18}O$ ratios, i.e. to values >23.7‰ (δ value of the atmospheric oxygen [17]). In fact, however, an average δ value of only +19.2 ± 1.8‰ was obtained. This shows that these values must be falsified by the release of an oxygen 'fraction' of a different $^{18}O/^{16}O$ ratio. The same happens in the presence of a photosynthesis inhibitor (DCMU). The deviation to smaller values demonstrates, that the ^{18}O content of the 'additional' oxygen must be definitely lower than that of air.

Discussion

Photosynthetic oxygen has a significantly higher ^{18}O content than the cell water. The extrapolated δ value corresponds to approx. ~0.1990% with a standard error of the mean of ≤0.0004%, compared to 0.1972 ± 0.0003% for cell water. The surprising discrepancy in the time dependence of the δ values (minimum curves) favours the assumption, that illuminated algae release a mixture of at least two oxygen 'fractions'. The easiest explanation would be the working hypothesis, that the cells 'store' rather considerable amounts of oxygen. These may either be loosely bound or dissolved in the lipid phase. As soon as the cell produces photosynthetic oxygen, this will probably become mixed with the already present oxygen. It is only after rather long photosynthesis periods, that practically all formerly 'stored' O_2 is replaced by freshly produced oxygen. If we assume, that the 'stored' O_2 has a lower ^{18}O content than photosynthetic oxygen (see above), we easily understand the minimum curves obtained: the initially taken samples contain a high proportion of air — with a strongly positive δ value. The first minutes produce a mixture between photosynthetic and stored O_2, both with a much smaller δ value. This drops the three components mixture to lower ^{18}O percentages. The more the air is replaced by photosynthetic oxygen (with progressively smaller shares in stored O_2), the more the δ values must approximate their final values.

If a fresh-water sample with the ^{18}O content of the cell water would be electrolytically decomposed, the ^{18}O percentage of the released oxygen can be predicted. Since the share of the heavier isotope in the resulting O_2 is diminished by a factor of 0.99 [5], we would obtain an ^{18}O content of $0.1972 \times 0.99 = 0.1952\%$. If the photosynthetic oxygen would be the product of water decomposition, it should show a comparable ^{18}O decrease related to the cell water. The fact, that the ^{18}O content of the photosynthetic oxygen exceeds that of the cell water, is unexpected.

There remain two conceivable interpretations: several authors have assumed, that the photosynthetic reactions themselves do not at all exhibit any measurable isotope effect [22,23]. They have postulated, that the observed shifts are the mere consequence of the preferential ^{16}O consumption during the cell respiration [15—17]. The respiration rate of algal cells is by a factor of approx. 5—10 smaller than the photosynthesis rate [16,25]. So it remains doubtful, whether the discrimination rate of this process is high enough (see Ref. 18). At least all published isotope effects [15—17,26] are definitely too small. This discrepancy speaks against a pure respiratory effect (see also Ref. 25).

On the other hand, the photosynthetic oxygen could conceivably be produced from an as yet unknown kind of 'bound' water [27—29], which could be enriched in ^{18}O. This interpretation would be in accordance with the observation, that e.g. water inside hydration shells of inorganic ions is significantly enriched in the heavier oxygen isotope [30].

A quite different suggestion would be the hypothesis, that the electron source in photosynthesis is not the water but a 'photolyte', the oxygen of which has a higher $^{18}O/^{16}O$ ratio than the cell water [31]. We have to realize, that there is an uneven distribution of isotopes in equilibrium systems like an H_2O/CO_2 mixture. The accumulation of the heavier isotope in the heavier species [3,4] leads to a significant ^{18}O enrichment both in the CO_2 and the HCO_3^-. Writing the equation

$$\begin{array}{ccccccc} 2H_2O & + & CO_2 & \rightleftharpoons & HCO_3^- & + & H_3O^+ \\ (1.000) & & (1.041) & & (1.026) & & (1.000) \end{array}$$

we find relative isotope abundances as given in the brackets [32—34]. By this exchange HCO_3^- anions are formed, which possess 2.6% more ^{18}O than water. If the oxidized chlorophyll molecules would regain their missing electrons from bicarbonate (or a form of 'bound bicarbonate') the resulting $HCO_3^\bullet$ radicals should decompose, producing oxygen with an increased ^{18}O content [31]. Should this interpretation be correct, mass spectrometric determinations with ^{18}O-labelled bicarbonate must indicate, that part of the CO_2-oxygen finds its way into the photosynthetic O_2. Literature contains several data, which seem to support this assumption [35,36], but due to possible failures in their interpretation we cannot take them as really conclusive [18]. A valuable complement can be expected from the study of hydrogen isotope effects. Under continuous illumination the photosynthesis rate in H_2O exceeds that in ^2H_2O by a factor of >2 [37,38]. If experimental conditions are chosen, which make the oxygen releasing step the rate-limiting reaction, this discrimination disappears [39] or decreases to a very small factor [40,41]. This result makes the assumption of an O-H bond breakage rather unlikely.

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