

Stable Hydrogen Isotope Fractionations during Autotrophic and Mixotrophic Growth of Microalgae

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ABSTRACT

Isotope effects, studied with precision isotope ratio mass spectrometry, have been used to locate critical steps in the H metabolism of plants. By manipulating the growth conditions of versatile microalgae, the discrimination of H isotopes between water in the growth medium and the organically bonded H in carbohydrates from these microalgae was -100 to -120‰ and was regulated by both the light and the dark reactions of photosynthesis. Photosynthetic electron transport discriminated against the heavy isotope of H and formed a pool of reductant available for biosynthesis that was enriched in the light isotope. Growth in red or white light activated phosphoglyceric acid reduction and H isotope discrimination, when H was fixed into organic matter. An additional fractionation of -30 to -60‰ occurred during the biosynthesis of proteins and lipids and was associated with glycolysis. This fractionation paralleled the isotope effect seen in carbohydrate metabolism, indicating that H metabolism in photosynthesis was coupled with that in dark biosynthetic reactions via the pool of reductant, probably NADPH.

Plants grown under controlled conditions show a reproducible and measurable discrimination (fractionation) of as much as 20‰ between the isotopes of hydrogen, deuterium (D), and protium (H) (1, 11). Cultures of microalgae, natural populations of phytoplankton, macroalgae, mosses, and higher terrestrial plants all have lower concentrations of the heavy isotope of hydrogen (deuterium) than the environmental water. Because there are distinctive isotope fractionations in biochemical processes and because differences of less than 1 µg/g deuterium in the hydrogen isotopic content of organic matter can be measured by precision isotope ratio mass spectrometry, it is possible to conduct tracer experiments with hydrogen at or near the natural abundance levels.

There is a class of biosynthetic problems that is uniquely accessible to measurement of isotope fractionation. The hydrogen in the surrounding H₂O is the ultimate source of all organically bonded hydrogen in autotrophically grown plant cells. The two are connected by a complex network of chemical reactions. Although a difference in reaction rates between H- and D-containing compounds is possible in any of the individual steps, observable fractionation between major reactants and products will occur only when there is a distinctive isotope discrimination in a rate-determining step of the sequence or at a branching point in the flow of metabolites through the network of chemical reactions. Measurements of isotope fractionation can help locate such critical steps or biosynthetic branching points.

Photosynthesis is the major process fractionating hydrogen isotopes in plants (1). Additional fractionation of hydrogen isotopes occurs during biosynthesis. Fatty acids, hydrocarbons, ster-

ols, and other lipids have much lower deuterium concentrations than the organically bonded hydrogen of the total organism (3, 7). This second fractionation step is believed to occur before or during the formation of acetyl-CoA (1).

To understand further the biochemical reactions fractionating hydrogen isotopes in plants and to determine their inter-relationships, with the goal of detecting steps that regulate hydrogen metabolism, the growth conditions of the versatile alga, *Chlorella*, were first manipulated in the following ways: (a) by changing the wavelength of the light source for photosynthesis; (b) by transferring the algae from autotrophic to dark heterotrophic conditions, (c) by growing the algae photoheterotrophically, and (d) by adding the inhibitory compound DCMU. Second, the hydrogen isotopic contents of different classes of compounds, carbohydrates, proteins, and lipids were determined. Third, the uptake of hydrogen from H₂O into cellular organically bound hydrogen was studied by resuspending and growing algae in growth media H₂O with a very slightly enriched deuterium content.

MATERIALS AND METHODS

Algal Culture. Axenic cultures of microalgae were obtained from Dr. Chase Van Baalen, Port Aransas Marine Laboratory, University of Texas. Algae were grown either in test-tube batch cultures in a thermostatically controlled water bath or in a glass, water-jacketed growth chamber. The media used for growth and the exact culture conditions are given in detail by Estep and Hoering (1).

The wavelength of light illuminating *Chlorella* in three experiments (Fig. 1) was controlled by placing sheets of Plexiglas (6 mm) on the sides of the thermostatically controlled water bath. The Plexiglas filters used in the experiments indicated in this paper were blue filter 2424, red filter 2423, and green filter 2092 (some red light near 675 nm is transmitted). The remainder of the bath was shielded with opaque coverings to keep out white light from the room. The cell yield of *Chlorella* grown in green light with glucose was the same as the cell yield with glucose in the dark. The Plexiglas filters transmit broad spectra of light, and these experiments are intended only to demonstrate semiquantitative effects.

The algae were harvested by centrifugation, washed once with distilled H₂O, and dried. Previous work in this laboratory showed the procedure did not change the isotopic composition of the organically bonded hydrogen from the algae (1). The hydrogen bonded into macromolecules of living cells exchanges much more slowly than when bonded into free, simple organic molecules. It is only after the cellular contents have been denatured that hydrogen atoms can exchange freely with H₂O (1). The relative deuterium concentration of the H₂O in the growth medium measured before and after growth was found to be the same.

Separation of Proteins and Lipids. The coccoid, blue-green alga *Agmenellum quadruplicatum* strain PR-6, was grown in the glass water-jacketed growth chamber and harvested daily. The cells

were washed once with distilled H₂O. A solution of 5% (w/v) trichloroacetic acid at 0 to 4 C was added and stirred, and the algal suspension was incubated at 0 to 4 C for 30 min (6). The trichloroacetic acid solution was decanted after centrifugation. The pellet then was extracted with 75% (v/v) ethanol at 50 C for 30 min. The ethanol solution, which extracted cellular lipids, was decanted after centrifugation. A second solution of 5% trichloroacetic acid was added to the algal residue and was incubated at 100 C for 30 min. The solution was removed by centrifugation. The pellet was washed with 75% ethanol, and the sides of the centrifuge tube were wiped with a tissue to remove residual trichloroacetic acid. The pellet (a bright blue color) contained the bulk of the algal protein. Analyses of the amino acid content showed that this fraction was 90 to 99% protein. The trichloroacetic acid solutions, which contained the carbohydrate and nucleic acid fractions, were discarded. The trichloroacetic acid (7.5 g in 150 ml) was difficult to remove from the algal fractions (10 to 20 mg) without possibly fractionating the hydrogen isotopes. This procedure was also carried out with solutions spiked with deuterium. The added deuterium in the extracting solutions did not exchange with the cellular extracts (Fig. 3, day 3).

Hydrogen Isotope Measurements. Six to 10 mg of the dried sample was combusted in a platinum boat at 750 C in an atmosphere of O₂. The H₂O from combustion was trapped with liquid N₂, converted to H₂ over uranium metal at 800 C, and analyzed by an isotope ratio mass spectrometer (Nuclide Corporation RMS-3-60) (1).

In isotope ratio MS, it is more precise to measure relative deuterium concentrations than the absolute abundance. The results of the isotopic analyses are presented in terms of D-H ratios relative to a standard reference material;

$$\delta D = \left[\frac{(D/H)_x - (D/H)_s}{(D/H)_s} \right] \times 10^3$$

where the subscript *x* indicates the unknown sample and *s* is the reference material, which is the Standard Mean Ocean Water (SMOW), from the International Atomic Energy Agency of Vienna, Austria. The absolute atom ratio of D-H in SMOW is 155.76×10^{-6} (2). The isotope fractionation (ΔD) between two substances is defined as follows:

$$\Delta D = \delta D_2 - \delta D_1.$$

This system for combusting organic matter, converting H₂O to H₂

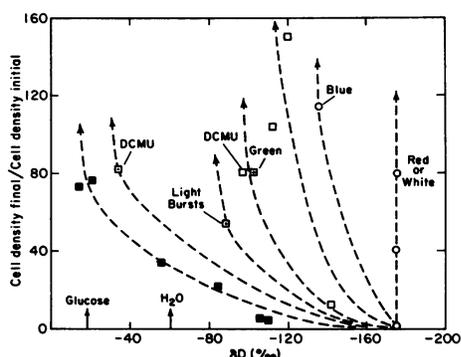


FIG. 1. Hydrogen isotope fractionations during autotrophic and mixotrophic growth of *Chlorella sorokiniana*. The inocula for these experiments were cells grown in white light. The ordinate axis indicates the relative amount of growth that had occurred when the cells were harvested and measured. Experimental points on this graph represent an average determined by analyzing six individual tubes of algae. (○), autotrophic growth in light; (□), photoheterotrophic growth in continuous white light with glucose (3 g/l); (◻), photoheterotrophic growth in limited light with glucose (3 g/l); (■), heterotrophic growth in the dark with glucose (3 g/l).

gas, and measuring D-H ratios is used routinely on a variety of organic matter and typically yields results with a standard deviation of $\pm 3\%$. The data points in Figures 2 to 4 represent an average of three or four analyses.

RESULTS AND DISCUSSION

Isotope Fractionations by *Chlorella*. The hydrogen isotope content of algae grown either photosynthetically or photoheterotrophically indicates that photosynthetically active wavelengths of light stimulate isotope fractionation and that hydrogen can enter the cell from H₂O by several pathways. Growth with red or white light caused a fractionation of the hydrogen isotopes by approximately -115% in *Chlorella*, whereas that caused by growth in blue light was only -75% (Fig. 1). Blue light inhibits the enzyme 3-P-glyceraldehyde dehydrogenase, which reduces P-glyceric acid, the first product of photosynthesis (5). Presumably some of the P-glyceric acid is transferred directly into the pathway of glycolysis when *Chlorella* is illuminated with blue light.

When algae grown photosynthetically were transferred from mineral salts medium to the mineral salts medium plus glucose or acetate, the hydrogen isotope content of the cells was altered dramatically (Fig. 1). The stable isotopic label of glucose was incorporated rapidly (1–6 h) by *Chlorella* in both the light and the dark. Algae grown in full white light with glucose or acetate had a hydrogen isotopic content equal to a mixture of isotopically lighter photosynthetic hydrogen plus the hydrogen from the organic substrate. The organically bonded hydrogen in cells transferred into total darkness reflected the hydrogen isotopic content of the organic substrate rather than the H₂O. Thus, several pathways for incorporating hydrogen from the water and organic substrates in the medium are distinguishable on the basis of isotope discrimination.

Small amounts of light stimulated hydrogen isotope fractionation in photoheterotrophic cells. *Chlorella* grown on glucose in darkness with 0.5 h light/day showed a considerably more negative isotope fractionation than cells grown similarly in complete darkness. Moreover, cells grown with glucose in green light, which is absorbed by Cyt, showed a negative isotope fractionation similar to algae given bursts of light. The additional fractionation cannot be explained by photosynthetic CO₂ fixation because green light and small bursts of light are insufficient to activate the total photosynthetic reactions. It is postulated that light may activate a

Table I. Exchange of Hydrogen Isotopes from Water to Organically Bound Hydrogen in Algae

Species	δD
<i>A. quadruplicatum</i>	
Initial water	-60
Heavy water	+94
Initial cells	-205
Light incubation	
15 min	-205
1 h	-203
3 h	-211
5 h	-203
7 h	-200
Dark incubation, 7 h	-214
<i>C. sorokiniana</i>	
Initial water	-66
Heavy water	+115
Initial cells	-178
Light incubation	
15 min	-156
3 h	-152
5.6 h	-143
Dark incubation, 5.6 h	-158

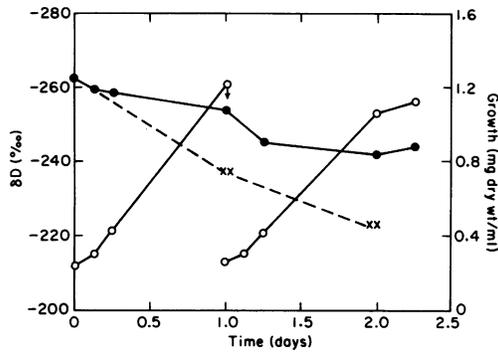


FIG. 2. Hydrogen isotope incorporation from H_2O into organically bonded hydrogen in *Anacystis nidulans*. (●), δD values with time. (XX--X), theoretical δD values for algae in steady-state with the isotopically heavier H_2O ; (○), growth of the algae as measured by mg dry weight/ml culture medium. The culture was diluted with fresh medium after 24 h growth. The δD of the H_2O before the experiment was -52‰ . At 0 h, the δD of H_2O was -21‰ ; at 24 h, the δD of H_2O was -2‰ .

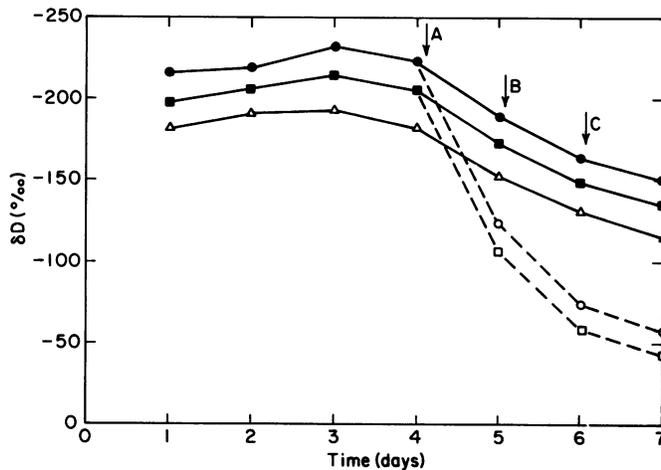


FIG. 3. Hydrogen isotope fractionation in whole algal cells and protein isolated from *A. quadruplicatum*. (●), the δD of protein; (■), the δD of whole algal cells; (---), theoretical δD values for algae in steady state with the isotopically heavier H_2O ; (Δ), the calculated δD values of algal cells minus protein. The δD of the H_2O in the culture medium was -60‰ during days 1 to 4. During days 5 to 7, the arrows indicate the δD of the H_2O in the culture medium was changed to $+65\text{‰}$ (A), $+103\text{‰}$ (B), and $+109\text{‰}$ (C).

Cyt involved in electron transport in either photosynthesis or respiration, or both (4, 11). Electron transport stimulates the formation of proton gradients, which may cause hydrogen isotope fractionation by building pools of hydrogen, which are depleted in deuterium and used in biosynthesis or respiration. An alternative explanation for this light effect may be the light activation of key photosynthetic enzyme(s) involved in isotope fractionation.

DCMU inhibits photosynthesis by totally stopping O_2 evolution and by blocking photophosphorylation, hence inhibiting CO_2 fixation. Photosynthetic electron transport was active, but crippled, however, in photoheterotrophic algae inhibited with DCMU (10). *Chlorella* was grown photoheterotrophically on glucose with $10 \mu M$ DCMU. The δD of these cells was strikingly similar to the δD of the cells grown photoheterotrophically without DCMU. Photosynthetic electron transport may still have been operating and providing reducing power for biosynthesis or the activation of electron transport during respiration (9). Continuous light was needed to produce this hydrogen isotope fractionation, as 0.5 h light plus DCMU did not stimulate any additional fractionation

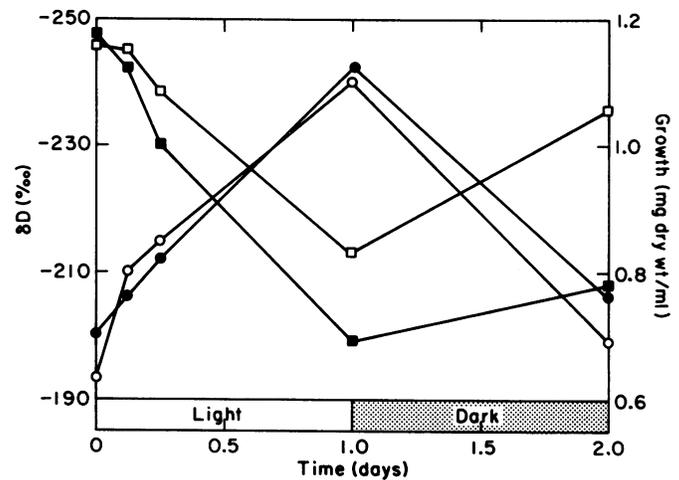


FIG. 4. Fixation of hydrogen into carbohydrates by *A. nidulans* suspended in a bicarbonate-carbonate buffer. Dry weight: (○), $\delta D H_2O = -66$; (●), $\delta D H_2O = +9$. δD values of the total algae through time: (□), $\delta D H_2O = -66$; (■), $\delta D H_2O = +9$.

above that seen in the dark. The primary isotope fractionation may occur during the reduction of NADP during photosynthesis because, in the dark, reducing power is generated from different pathways, glucose 6-P dehydrogenase and the tricarboxylic acid cycle, without fractionating hydrogen isotopes.

Uptake of Water into Cellular Hydrogen. When the algae, *Agmenellum*, *Anacystis nidulans* strain TX20, and *Chlorella*, grown photosynthetically, were suspended in mineral salts media with a slightly increased deuterium content, the new isotopic composition of the H_2O was not displayed immediately (1-6 h) by the cellular organic hydrogen (Table I; Figs. 2 and 3). Even after 2 to 3 days growth, the cellular organic hydrogen was not in isotopic steady-state with the new H_2O in the growth media (Figs. 2 and 3). The dashed lines in Figures 2 and 3 indicate theoretical δD values, calculated by a simple material-balance equation, for algae in isotopic equilibrium with the H_2O in the growth medium. The failure to incorporate hydrogen rapidly from H_2O into cellular-bound hydrogen indicates that hydrogen is metabolized into the cell via a pool of a bound complex, perhaps NADPH or NADH, rather than as H_2O . In addition, the presence of luxury bound hydrogen, reducing power that is present in excess of the cell's present needs, in actively photosynthesizing cells is indicated. Only 16 to 33% of the hydrogen incorporated in algae growing in the deuterium-enriched H_2O was directly attributable to this isotopically heavier H_2O .

Hydrogen Isotope Fractionating in Different Classes of Compounds. The hydrogen isotopic contents of individual classes of compounds are different. Cells of *Anacystis*, washed once with distilled H_2O , were resuspended and incubated in a bicarbonate-carbonate buffer at pH 8.2 at 39 C in the light for 24 h (Fig. 4). The increase in dry weight is attributed to an increase in carbohydrate content, as this phosphate or nitrogen-free buffer inhibits protein metabolism and cell division. By difference calculation, and by forcing algal cells to produce only glucose and carbohydrates, the hydrogen isotope fractionation in carbohydrates is approximately -100‰ . This fractionation is calculated by the simple material balance equation:

$$\begin{aligned} &(\text{initial dry weight algae})(\delta D \text{ initial}) \\ &+ (\text{dry weight carbohydrates})(\delta D \text{ carbohydrates}) \\ &= (\text{total dry weight algae plus carbohydrates})(\delta D \text{ final}). \end{aligned}$$

Subsequently, the algae were transferred into the dark, inhibiting photosynthesis and promoting respiration. After 24 h darkness, the dry weight of the algal suspension had decreased to levels

similar to those at the beginning of the experiment. The algae had metabolized the stored carbohydrates to CO_2 and H_2O to provide cell energy (substrate level phosphorylation). In the case in which the δD of the carbonate buffer was the same as that of the growth medium (CG-10), the δD of the algae after respiration was nearly identical with that of the initial algal cells. The hydrogen incorporated into carbohydrates during the light was eliminated during metabolism without isotope fractionation, indicating that dark respiration does not fractionate hydrogen isotopes. In the experiment in which the deuterium content of the H_2O was increased slightly, some recycling or exchange of hydrogen occurred.

Proteins and lipids isolated from *Agmenellum* have less deuterium than carbohydrates by approximately -30 to -50% ; thus, additional fractionation during the breakdown of glucose to form amino acids and fatty acids is indicated (Fig. 3). Smith and Jacobson also noted a similar fractionation between starch and protein isolated from potato tubers (8). The hydrogen involved in the additional fractionation is related to the pool of bound hydrogen metabolized by the algal cells. Isolated proteins and lipids extracted from *Agmenellum* grown in H_2O spiked with deuterium had hydrogen isotope fractionations that are parallel to the whole organism fractionation. Hydrogen was incorporated into both the protein and lipid fraction and the carbohydrate fraction at a similar rate. A parallel relationship between fractionation and the lack of display of the different δD of the H_2O in all chemical fractions are used to suggest strongly that a common pool of H exists.

CONCLUSIONS

A pool of intracellular, organically bound hydrogen is responsible for providing the hydrogen available for cellular biosynthesis. This pool of hydrogen is generated in the light during photosynthesis and may reflect NADPH concentrations. It does not undergo rapid isotope exchange with the H_2O in the growth media. Stable hydrogen isotope fractionation in plants is governed by this form of bound hydrogen that is generated during photosynthesis. For example, red light activates hydrogen isotope fractionation and P-glyceric acid reduction. In this particular reducing reaction, hydrogen from photosynthetically produced NADPH is fixed into organic matter.

Hydrogen metabolism in biosynthesis and dark reactions is linked to that in the light via the pools of the reductants, NADPH

or NADH, or both. Consequently, additional fractionation occurs during the biosynthesis of proteins and lipids. It was discovered that, whereas the hydrogen from organic substances was incorporated immediately into the algal cells, the hydrogen in the H_2O of the growth medium was not. This lag in the incorporation of hydrogen from H_2O indicates that, in actively photosynthesizing cells, a pool of luxury organically bound reductant is available for cellular hydrogen metabolism. Moreover, increased hydrogen isotope fractionation during photoheterotrophic growth of *Chlorella* in limited light regimes reflects this ability of a plant to produce an abundance of reductant.

Precision stable isotope ratio MS has been used to identify some of the key reactions in plant metabolism that fractionate the hydrogen isotopes. Isotope fractionation is indicative of rate-limiting reactions or branching points. Hence, the enzymes at these critical points that control isotope fractionation also serve to regulate hydrogen metabolism in the plant cell. The complex network of reactions between H_2O and organically bonded hydrogen is regulated principally by photosynthesis, whereas secondary regulation occurs during glycolysis.

LITERATURE CITED

1. ESTEP MF, TC HOERING 1980 Biogeochemistry of the stable hydrogen isotopes. *Geochim Cosmochim Acta* 44: 1197-1206
2. HAGEMANN R, G NIEF, E ROTH 1970 Absolute isotopic scale for deuterium analysis of natural waters. Absolute D/H ratio for SMOW. *Tellus* 22: 712-715
3. HOERING TC 1977 Stable isotopes of hydrogen in Precambrian organic matter. In C Ponamperuma, ed, *Chemical Evolution of the Early Precambrian*. Academic Press, New York, pp 81-85
4. JONES LW, J MYERS 1963 A common link between photosynthesis and respiration in a blue-green alga. *Nature* 199: 670-672
5. MIYACHI S, S MIYACHI, A KAMIYA 1978 Wavelength effects of photosynthetic carbon metabolism in *Chlorella*. *Plant Cell Physiol* 19: 277-288
6. ROBERTS RB, PH ABELSON, DB COWIE, ET BOLTON, RJ BRITTEN 1955 *In Studies of Biosynthesis of Escherichia coli*, Chap 2. Carnegie Institution of Washington, Washington, DC, pp 13-20
7. SMITH BN, S EPSTEIN 1971 Biogeochemistry of the stable isotopes of hydrogen and carbon in salt marsh biota. *Plant Physiol* 46: 129-139
8. SMITH BN, BS JACOBSON 1976 $^2\text{H}/^1\text{H}$ and $^{13}\text{C}/^{12}\text{C}$ ratios for classes of compounds isolated from potato tuber. *Plant Cell Physiol* 17: 1089-1092
9. VAN BAALLEN C, W PULICH 1973 Heterotrophic growth of the microalgae. *Crit Rev Microbiol* 2: 229-255
10. WIESSNER W, DC FORK 1970 The development of system 2 activity in *Chlamydomonas stellata* during transition from photoheterotrophic to autotrophic nutrition. *Carnegie Inst Wash Year Book* 69: 695-699
11. ZIEGLER H, CB OSMOND, W STICHLER, P TRIMBORN 1976 Hydrogen isotope discrimination in higher plants: correlations with photosynthetic pathway and environment. *Planta* 128: 85-92