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Characteristics of Growth and Deuterium Incorporation in *Chlorella ellipsoidea* Grown in Deuterium Oxide

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The effect of deuterium oxide (D_2O) was examined on the cell growth of photoautotrophically growing *Chlorella ellipsoidea*. The growth rate was inversely proportional to the concentration of D_2O in the medium up to 60 mol%. The growth in D_2O medium at concentrations higher than 60 mol% was highly inhibited. However, the cells pre-cultured in 60 mol% D_2O medium became able to grow even in practically 100 mol% D_2O medium. Deuterium (D) was incorporated into cells proportionally to the concentration of D_2O in the medium. The D/H ratio in the cells was lower than that of the cultivation medium. *Chlorella* cells cultured in practically 100 mol% D_2O medium contained *ca.* 70 mol% D. Of the D incorporated into cells, about 20% was easily exchangeable with H, and the remaining 80% was unexchangeable.

Keywords——deuterium oxide; *Chlorella ellipsoidea*; isotope effect; deuterium incorporation; growth inhibition; hydrogen/deuterium exchange; isotope fractionation; cell adaptation

The high mass ratio of deuterium(D)/hydrogen(H) has stimulated interest in the isotope effect of D in biological systems. Actually, since the discovery of D by Urey *et al.*[1] in 1932, a number of studies using D_2O have been performed to reveal the biological isotope effect of D.[2–4] However, the effect has not yet been fully elucidated, possibly because of the complexity arising from differential effects between the solvent effect of D_2O and the isotope effect of D incorporated into biomolecules. The latter effect can be further classified into the isotope effect of easily exchangeable D ("exchangeable" D) in labile regions of molecules, and the other is that of hardly exchangeable D ("unexchangeable" D) in stable regions such as C-D bonds.

To examine these isotope effects, we utilized a photosynthetic unicellar organism, *Chlorella ellipsoidea*, in the present study. The cells were grown in D_2O medium and the D/H ratios (exchangeable and unexchangeable) were determined. Here, we describe the relationship between growth and deuteration of the cells, and the phenomenon of cell adaptation to D_2O.

Materials and Methods

Cell Strain and Culture Conditions——*Chlorella ellipsoidea* Gerneck (C-27) was furnished by Dr. Sansawa of Yakult Central Institute for Microbiological Research, Tokyo. *Chlorella* cells were cultured in Myers 4N (M-4N) medium containing various concentrations of D_2O at 25 °C under 18 klx light and continuous bubbling with a dry gas mixture composed of 5% CO_2 and 95% air (Fig. 1). The M-4N medium consisted of 5 g of KNO_3, 1.25 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 3 mg of $FeSO_4 \cdot 7H_2O$ and 1 ml of Arnon's A-5 solution[5] per liter of H_2O or D_2O, pH 6.8. D_2O (99.8 mol%) was purchased from MSD Isotopes, Division of Merck Frosst Canada, Inc., Montreal, Canada.

Cell Culture in D_2O Medium——The exponentially growing cells, pre-cultured in H_2O medium or 60 mol% D_2O medium, were resuspended on various concentrations of D_2O medium. The cell number was counted with a hemocytometer and the cell density was measured at 660 nm in a spectrophotometer (Spectronic 20-A, Shimadzu Bausch & Lomb).

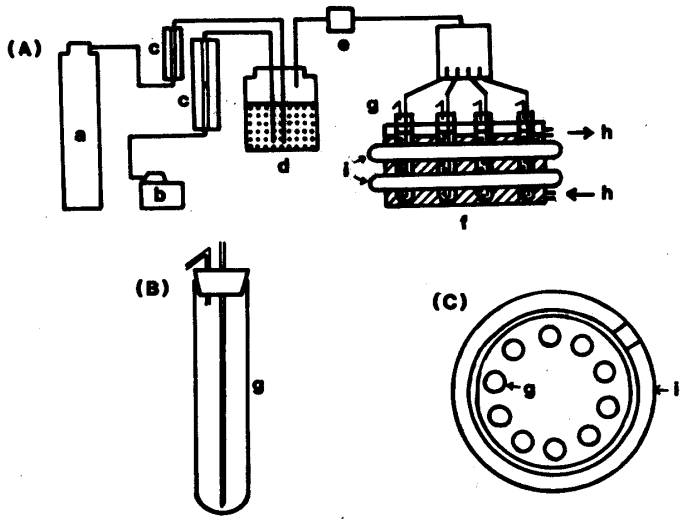

Fig. 1. Equipment for Autotrophic Cultivation of *Chlorella*

(A) Front view, (B) magnified g, (C) top view. a, CO_2 bomb; b, air compressor; c, flow meter; d, silica gel; e, filter; f, water tank; g, test tube (10 ml) containing M-4N medium inoculated with *Chlorella ellipsoidea*; h, isothermal installation (25 °C); i, plant light (32W × 2).

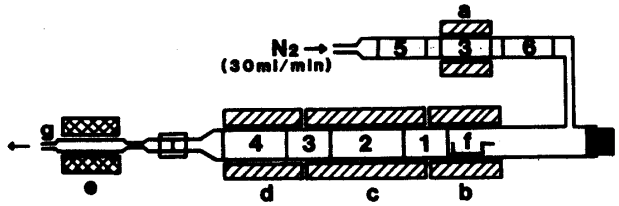

Fig. 2. Combustion Furnace for *Chlorella*

a—d, Furnace (a, 450 °C; b, c, 750 °C; d, 550 °C); e, dry ice; f, Pt-boat for sample; g, water trap.
1, Ni–Cr wire; 2, Co_3O_4; 3, $AgMnO_4$; 4, Cu; 5, silica gel; 6, NaOH, $Mg(ClO_4)_2$.

Deuterium Analysis——*Chlorella* cells harvested by centrifugation were vacuum-dried in a desiccator at 50 °C. An aliquot (2—10 mg) of the dried cells was combusted at 750 °C and then at 550 °C in a microfurnace (Fig. 2). The water obtained by combustion was trapped in a glass tube using dry-ice. The recovery of D in this procedure was confirmed to be theoretical by determining D of a standard sample, acetanilide-d_8. The water obtained (1—5 μl) was diluted with H_2O and analyzed for D in a TCD gas chromatograph equipped with a hydrophobic catalyst (heavy water analyzer HK-102, Shoko Co. Ltd, Tokyo.) The D/H ratio and D_2O concentration were expressed as mol%.

Results

Effect on Cell Growth

Chlorella cells (1.7×10^7 cells/5 ml) were cultured in various concentrations of D_2O medium for 5 d. The growth curves in D_2O medium are shown in Fig. 3 (a) (cell number) and 3 (b) (absorbance at 660 nm). The cell growth was inhibited proportionally to the increase of D_2O in the medium; it was inhibited to about a half of the control in 60 mol% D_2O, and almost completely in 90 mol% D_2O. As can be seen in Fig. 3, the growth curve determined from the absorbance at 660 nm was nearly parallel with that based on the cell number. Therefore, in the following experiments, we determined the cell growth by measuring the absorbance at 660 nm.

Although *Chlorella* cells pre-cultured in H_2O medium ("H-*Chlorella*") could not grow in a high concentration of D_2O in medium, the cells pre-cultured in 60 mol% D_2O medium ("60% D-*Chlorella*") became able to grow even in practically 100 mol% D_2O (Fig. 4), indicating that *Chlorella* cells could "adapt" to D_2O. The growth rate of adapted cells was inversely proportional to the concentration of D_2O. On the other hand, *Chlorella* cultured in practically 100 mol% D_2O medium ("100% D-*Chlorella*") could grow in H_2O medium as easily as H-*Chlorella* after a short lag time (Fig. 5).

H/D Exchange in Cells

Of the D biosynthetically incorporated into various cellular structural components, we distinguished exchangeable D from unexchangeable D. First, the time required for H/D

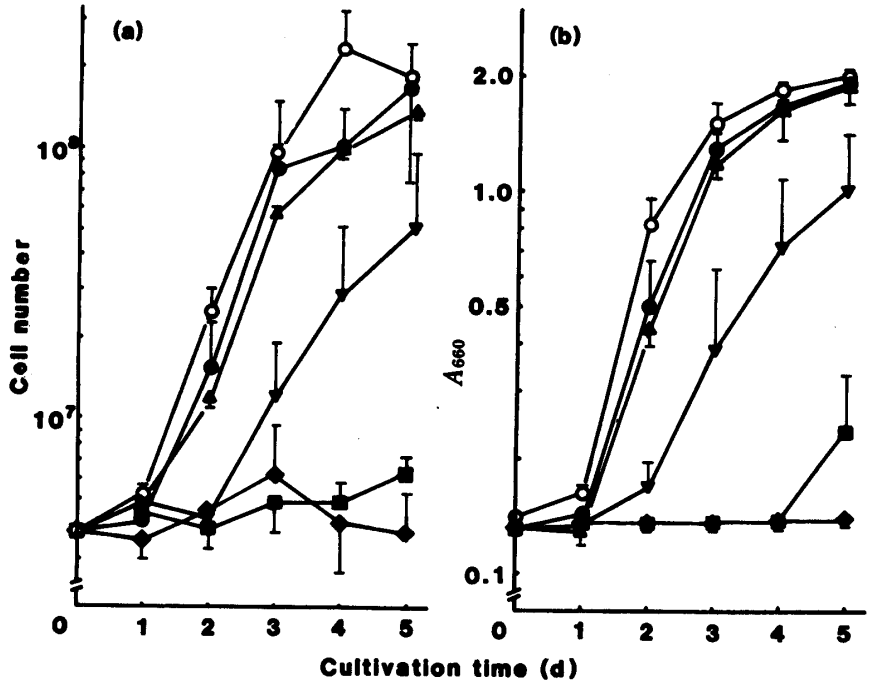

Fig. 3. Growth of *Chlorella ellipsoidea* in D_2O Medium

(a) Cell number, (b) absorbance at 660 nm.

Chlorella cells (1.7×10^7 cells/5 ml) were cultured in various concentrations of D_2O medium for 5 d.

○—○, 0 mol% D_2O (control); ●—●, 20 mol% D_2O; ▲—▲, 40 mol% D_2O; ▼—▼, 60 mol% D_2O; ■—■, 75 mol% D_2O; ◆—◆, 90 mol% D_2O. Each point and bar represent the mean ± S.E. ($n=3$).

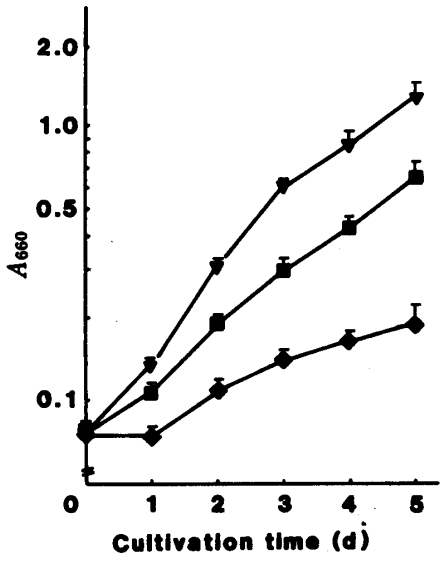

Fig. 4. Growth of 60% D-*Chlorella* in Higher Mol% D_2O Medium

Chlorella cells pre-cultured in 60 mol% D_2O medium for several days were cultured in D_2O medium of higher mol%.

▼—▼, 60 mol% D_2O; ■—■, 80 mol% D_2O; ◆—◆, 100 mol% D_2O. Each point and bar represent the mean ± S.E. ($n=3$).

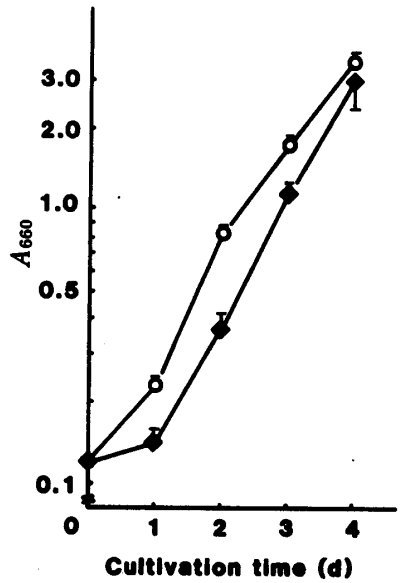

Fig. 5. Growth of 100% D-*Chlorella* in H_2O Medium

H-*Chlorella* (○) or 100% D-*Chlorella* (◆), prepared by culturing in 100 mol% D_2O for more than 10 d, was resuspended in H_2O medium, and cultured for 4 d. Each point and bar represent the mean ± S.E. ($n=3$).

exchange in cells was determined. The cells (5×10^8) of 60% D-*Chlorella* were washed twice with 1 ml of ice-cold H_2O medium, then vacuum-dried and analyzed for the D/H ratio. The result showed that H/D exchange in *Chlorella* proceeded within 10 min and reached an equilibrium (Fig. 6). Therefore, in the following experiments, D-*Chlorella* was washed twice with H_2O medium for 30 min and the residual D was termed unexchangeable D. The portion of exchangeable D was calculated from the difference between the unexchangeable D and total D; the latter was determined on D-*Chlorella* dried without washing.

Incorporation of D in D_2O-Adapted and Nonadapted Chlorella

Chlorella cells were cultured in 20—60 mol% D_2O medium for 5 d and their incorporation of D was determined (Fig. 7). The D/H ratio in cells ($(D/H)_c$) increased linearly in proportion to the D_2O concentration of the medium, *i.e.* the D/H ratio in the medium ($(D/H)_m$). The incorporation ratio of D(IRD) was defined as follows:

$$(IRD)=[(D/H)_c/(D/H)_m]\times 100\ (\%)$$

The IRD in *Chlorella* in any mol% D_2O medium was about 70%. The unexchangeable D was

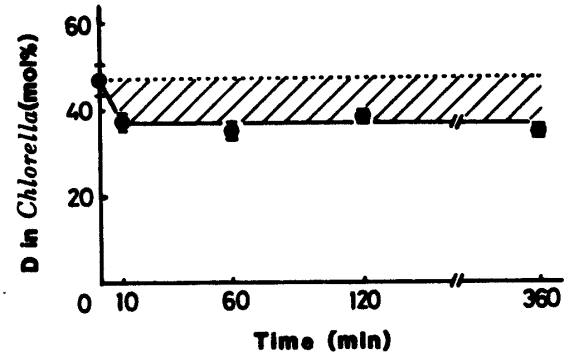

Fig. 6. H/D Exchange in 60% D-*Chlorella*

60% D-*Chlorella* (5×10^8 cells) was washed twice with 1 ml of ice-cold H_2O medium, then the cells were vacuum-dried and analyzed for D/H ratio. The D removed by washing with H_2O was "exchangeable D" ▨, and the remaining D was "unexchangeable D" □. The abscissa indicates the total time of washing. Each point and bar represent the mean ± S.E. ($n=3$).

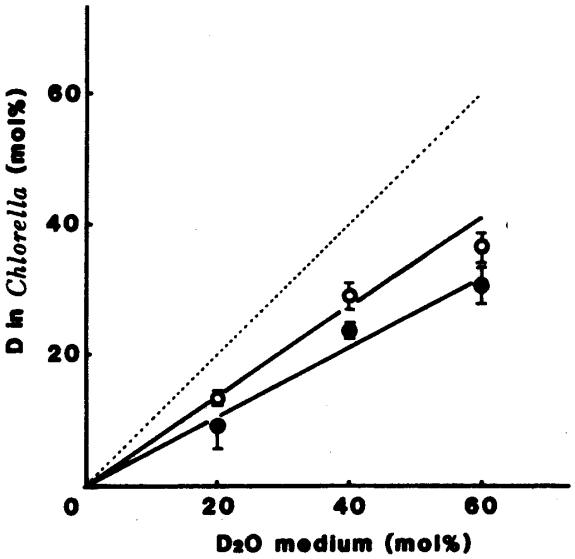

Fig. 7. Incorporation of Deuterium in Non-adapted *Chlorella*

H-*Chlorella* cells were cultured in 20—60 mol% D_2O medium for 5 d. These cells were vacuum-dried and analyzed for D/H ratio. The total D (○) is D in nonwashed *Chlorella*. The unexchangeable D (●) is D in *Chlorella* after washing twice with ice-cold H_2O for 30 min. The dotted line shows the concentration of D_2O in culture medium.

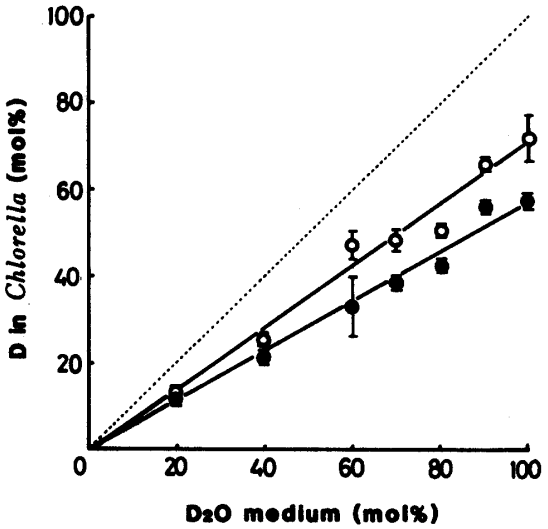

Fig. 8. Incorporation of Deuterium in Adapted *Chlorella*

D_2O-adapted *Chlorella* cells were obtained by culturing the cells twice in D_2O medium for 5 d. The initial *Chlorella* cells used were H-*Chlorella* for adaptation to 20—60 mol% D_2O and 60% D-*Chlorella* for 70—100 mol% D_2O. The D/H ratios in unwashed *Chlorella* (○), and *Chlorella* washed with H_2O medium (●) were determined in the same manner as in Fig. 7.

about 80% of the total D in each *Chlorella*.

Next, the D incorporation in *Chlorella* pre-cultured twice in D_2O medium for 5 d and adapted to D_2O was measured. The D/H ratio also linearly increased proportionally to the concentration of D_2O in medium (Fig. 8). In the cells grown in 100 mol% D_2O medium, the IRD was about 70%, indicating that the D_2O-adapted *Chlorella* took up D in the same manner as the nonadapted organism. In both the nonadapted and adapted cells, cellular isotope fractionation occurred between H and D. The fractionation ratio between H and D in cells (FRD) was defined as follows:

$$(\mathrm{FRD}) = [(\mathrm{D/H})_m - (\mathrm{D/H})_c]/(\mathrm{D/H})_m \times 100 = 100 - (\mathrm{IRD})\ (\%)$$

The FRD was approximately 30%. Of the incorporated D, about 80% was found to be unexchangeable.

Discussion

In agreement with early studies on algae,[6] the growth of *Chlorella* was inhibited by D_2O at high concentrations. Some gigantic "monster" cells were observed in D-*Chlorella*, probably resulting from the inhibition of cell division in D_2O medium.[4,6b] It has been suggested that this inhibition was caused by the effect of D on the functions of tubulin.[7] Cell cycle-regulating proteins[8] might also be affected by D.

A phenomenon of D_2O adaptation was observed in *Chlorella*. In disagreement with previous reports[6b,d] indicating that D_2O-adapted *Chlorella* incorporated D more easily than H, we found that both the adapted and nonadapted *Chlorella* cells took up H more easily than D at any concentration of D_2O in the medium. Further, there was no significant difference of the incorporation ratio of D (IRD) in cells between adapted and nonadapted *Chlorella* cells, at least below 60 mol% D_2O in the medium (Figs. 7 and 8). It therefore seems likely that the cellular availability of D does not alter during adaptation. The adaptation from D to H (Fig. 5) was easier than from H to D (Fig. 4), suggesting the adaptation was caused not by genetic but by physical alteration. However, because a certain lag time prior to growth was required when 100% D-*Chlorella* was transferred into H_2O medium, the deuteration of the unexchangeable H region might have a role in the adaptation from D to H.

In D-*Chlorella* grown in D_2O medium of various mol% of D_2O, the IRD was always about 70%. This suggests that the fractionation ratio between H and D(FRD) was as much as 30% in *Chlorella* cells. Studies on hydrogen isotope fractionation in plant cells have indicated that the FRD was about 20% in natural circumstances where the D concentration was very low.[9] Under the present conditions of very high concentration of D_2O, the FRD may be a little different from that in the case of the very low natural level of D_2O.

In D-*Chlorella* the unexchangeable D, which might reflect the constitutional D,[2d] accounted for about 80% of total D. This constitutional D may have an important isotope effect on biological reactions through alterations in the steric structure of biomolecules and in their reactivity. On the other hand, the exchangeable D may also cause alterations in various reactions. The use of D-*Chlorella* may be useful in elucidating these complex isotope effects and also in elucidating the metabolism of water and hydrogen molecules in cells.

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