RESPONSE OF *CHLORELLA* TO A DEUTERIUM ENVIRONMENT*

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INTRODUCTION

With the development of nuclear magnetic resonance instruments for the detection of protons it became possible to consider the use of these particles as a tracer substance in a low-proton environment. As this element is one of the important participants in the process of photosynthesis, it became desirable to attempt to obtain a photosynthetic organism in which all the cellular hydrogen was replaced by deuterium (which is not detected by proton magnetic resonance) in order that the pathway of hydrogen (protons) could be traced in the metabolic sequences involved in photosynthesis. In an analogous way ^{14}C has been used to trace the path of carbon[1].

A number of other considerations were also involved. An investigation was necessary into the metabolism of deuterated cells in order to find whether derangement of normal metabolic function ensued consequent to deuteration. A principal feature of the structure of such biologically important macromolecules as proteins and nucleic acids is the maintenance of their structure by virtue of the participation of many hydrogen bonds[2,3]. One may expect that the hydrogen bonds formed by deuterium will be different, at least in their energy, from those formed by the proton[4]. The multiplication which would occur in a macromolecule of even a small difference between a proton and a deuteron bond would certainly have some effect upon its structure. The sensitivity of enzyme function to structure, and the presumed sensitivity of nucleic acid function (genetic and mitotic) to its structure would lead one to expect a noticeable effect on the metabolic pattern and reproductive behavior of an organism.

MEYER[5] has reported the ability of *Chlorella* to grow, albeit very slowly, in very high concentrations of heavy water, while other workers have reported inhibition of growth in the presence of this substance with complete cessation above certain concentrations[6]. A study has therefore been made with *Chlorella pyrenoidosa* of the growth in and adaptation to various concentrations of deuterium oxide together with effects on the rates and incorporation patterns of carbon dioxide fixation. An extensive investigation has already been made into the path of carbon in photosynthesis in green algae grown in water of natural isotopic composition[1].

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METHODS

Growth

Algae for inoculation into experimental media were grown in continuous culture conditions described by HOLM-HANSEN, *et al.*[7] These cells also served as a source of unadapted algae. For investigations into the effect of D_2O on growth, 0.5 ml aliquots of cell suspension were inoculated into 55 ml of MYER's medium containing graded concentrations of D_2O, in 250 ml Erlenmeyer flasks. The flasks were shaken at 24° above a light source for a number of days during which a continuous stream of filtered air containing 4% (v/v) CO_2 was passed through the cultures. At the end of the growth period the extent of growth was determined by four procedures: (i) an aliquot of the cell suspension was centrifuged, washed and evaporated to dryness in a tared vessel to give the dry weight; (ii) the optical density at 665 mμ was determined with a Cary spectrophotometer, using the opal-glass techniques described by SHIBATA *et al.*[8]; (iii) a third sample was centrifuged in a graduated tube for 15 min at 1900 *g* in order to determine the volume of the packed cells; (iv) the number of cells in a known volume of suspension was counted microscopically using a haemo-cytometer. From the last two measurements the average cell volume was calculated. Similar measurements were performed on the cell suspension used as the inoculum for the growth experiments.

A similar experiment was performed in which the cells of the inoculum had become adapted (see RESULTS section) to grow in a concentration of 60% (v/v) D_2O.

Photosynthesis

Cells adapted to various known D_2O concentrations were washed and resuspended in distilled water containing graded quantities of D_2O. A 10 ml sample of each cell suspension, containing 0.27 ml of wet packed cells, was pipetted into a flattened test tube.* The tubes were placed in a water bath at 24° and illuminated from both sides with 150 watt RS-P-2 photospot lamps, the suspension being simultaneously flushed with air containing 1% (v/v) CO_2 for about 30 min in order to obtain the cells in a steady metabolic state. At the end of this period the gas inlet was removed from one tube at a time, 150 μl of 0.036 *N* $NaH^{14}CO_3$ (60 μC) was injected, the tube stoppered and shaken by hand in the water bath between the light sources for 3 min. The stopper was then removed and the cell suspension rapidly poured into 4 volumes of boiling ethanol to kill and extract the cells.

After extraction the cell debris was centrifuged and re-extracted with 10 ml of 20% (v/v) boiling ethanol. The extracts were pooled, evaporated to a small volume and chromatographed on washed Whatman No. 4 filter paper, using phenol–water as the solvent in the first dimension and *n*-butanol–propionic acid–water as that in the second dimension. Radioactive materials were detected by radioautography, identified and the radioactivity in each determined with a thin-window Scott tube[9]. Radio assays were made of the total cell suspension after photosynthesis and of the ethanol extracts by drying suitable portions in the presence of a drop of acetic acid with a current of warm air onto rotating aluminum planchettes[10]. The latter were counted with Scott tubes as before.

Preparation of samples for deuterium analysis

MYER's medium (1 l) containing the desired amount of D_2O was inoculated with unadapted cells or cells adapted to grow in the presence of 60% (v/v) D_2O. After a growth period of some days the cells were centrifuged at 1900 *g* for 30 min to produce a stiff paste. A sample of the supernantant medium was retained for D_2O analysis. The cell paste was lyophilized and the condensed water retained as the "intracellular water". The remaining cells were finally dried to constant weight at 105° and were then combusted in a current of O_2. The water from this combustion represented the oxidized form of the bound cellular hydrogen.

Deuterium analysis

Samples were analyzed for protons by nuclear magnetic resonance on a Varian V-4300 B spectrometer[11] against a series of standard H_2O/D_2O mixtures. The experimental values were obtained by interpolation.

RESULTS

Growth of unadapted cells in deuterium oxide

The growth of unadapted cells for 4 days in the presence of increasing quantities of heavy water is shown in Fig. 1. With some fluctuations all the criteria of growth

* The bottom 14 cm of 22.5 cm × 1.8 cm test tubes were flattened to produce parallel-sided vessels 2.4 cm wide and 1 cm thick.

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used showed substantially the same picture. At concentrations below 33% D_2O there was a small inhibition of growth compared with the control in H_2O; above 33% D_2O, however, growth was very markedly reduced, while there was a simultaneous increase in the average cell volume.

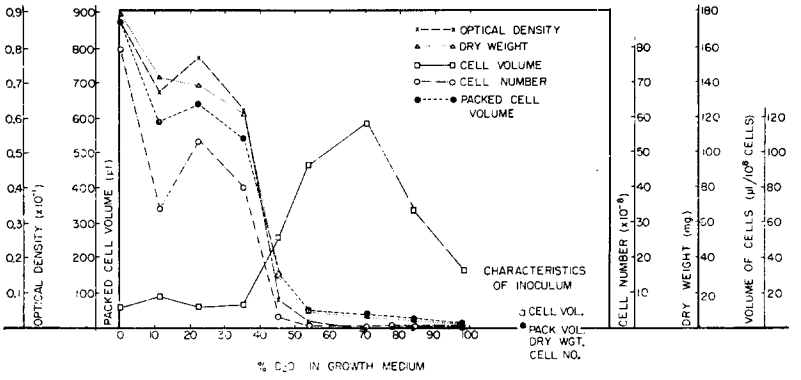

Fig. 1. Growth of unadapted *Chlorella* in the presence of increasing concentrations of D_2O in the medium. The points plotted relate to the values obtained from 55.5 ml of inoculated medium after 4 days' growth. The characteristics of the inoculum indicate the values found for 55.5 ml of medium immediately after inoculation. The cells were grown in the presence of light at 24°, the flasks being shaken and flushed continuously with air containing 4% (v/v) CO_2.

Adaptation to growth in the presence of deuterium oxide

By repeated subculture in gradually increasing concentrations of D_2O it was possible to adapt cells to grow in concentrations as high as about 99%. However, above about 65% the rate of growth could never be raised to the rate of normal growth. At the upper end of the D_2O range, growth was extremely slow, sporadic and unpredictable. A typical sequence of adaptation was the passage of unadapted cells once through 35% D_2O, followed by three successive subcultures into 60% D_2O, one in 87%, two in 90% three in 95% and finally into 99% D_2O. The period between subcultures (during which time there was an approximately tenfold increase in cell material) ranged from 4 to 7 days at 35% and 60% D_2O, to 3 to 4 weeks at 95 to 99% D_2O.

Growth of adapted cells in the presence of deuterium oxide

Cells adapted by successive subculture to grow in the presence of 60% D_2O were grown for 4 days in the presence of increasing concentrations of heavy water in an experiment similar to that performed with unadapted cells. While there was a gradually increasing inhibition of growth between 0% and 60% D_2O, growth was seriously reduced above 70% D_2O, above which concentration the cell volume also showed a marked enlargement (Fig. 2). Adaptation thus enabled the organism to overcome the serious block to growth above 33% D_2O found with unadapted cells, though strong inhibitory effects were still observed at the highest D_2O concentrations.

It is interesting to note that there was no rise in the average cell volume of adapted cells in D_2O concentrations as high as 60 to 70%, indicating that the cell size at which division takes place had become adapted to a D_2O environment, and

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remained the same as it is in ordinary water. The overall metabolic rate, however, as measured by the increases in cellular material, did not adapt to the same extent; thus the rate of growth was progressively inhibited as the concentration of D_2O increased.

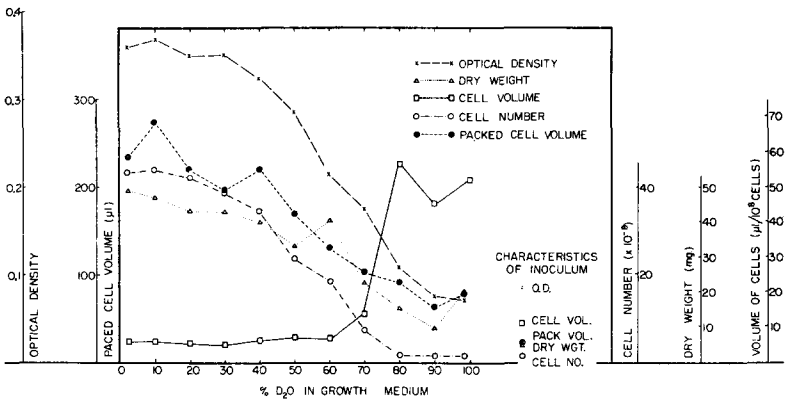

Fig. 2. Growth of adapted *Chlorella* in the presence of increasing concentrations of D_2O in the medium. The points plotted relate to the values obtained from 55.5 ml of inoculated medium after 4 days' growth. The characteristics of the inoculum indicate the values found for 55.5 ml of medium immediately after inoculation. The cells were grown in the presence of light at 24°, the flasks being shaken and flushed continuously with air containing 4% (v/v) CO_2.

Intracellular deuterium concentrations in unadapted and adapted cells grown in the presence of D_2O

(*a*) *Unadapted cells*: 25 ml of cell suspension containing a packed cell volume of 0.125 ml of unadapted cells were inoculated into 975 ml of growth medium, producing a final concentration of D_2O in the medium of about 23.5%. The cells were grown in the presence of air plus 4% (v/v) CO_2 for 8 days. The cells were harvested, the volume of the packed cells after growth being 9 ml; there was thus a 72-fold increase of cell material during growth.

The deuterium content of the water of the medium, of the intracellular water obtained by lyophilization of the packed cells, and of the water produced by combustion of the dried cells was measured. The D_2O concentrations of these three samples were 21%, 24% and 13% respectively, confirming the finding of WEINBERGER AND PORTER[12] that unadapted cells select protons from the growth medium in preference to deuterons.

(*b*) *Adapted cells*: a similar experiment was performed with adapted cells. After algae previously adapted to grow in 60% D_2O had been grown in 1000 ml of medium containing approximately this level of D_2O, the medium contained 63.7% D_2O, the intracellular water 63.4% D_2O, while the water obtained by combustion of the dried cells had a D_2O content of 69.8%. It would appear that adapted cells showed some preferential uptake of D_2O rather than H_2O from the medium in contrast to unadapted cells in which the reverse effect was noted.

Effect of increasing deuterium content on photosynthesis

Unadapted cells were suspended in distilled water containing 0%, 33%, 66% and

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TABLE

Percentages of the total $^{14}CO_2$ found in the ethanol-soluble fraction which are present in a number of known *B*, cells adapted to and grown in 60% D_2O, and suspended in the D_2O concentrations shown. *C*, cells adapted of D_2O for study o

	A			
Cells grown in % D_2O	0	0	0	0
Cells suspended in % D_2O	0	33	66	99.5
Ribulose monophosphate	0.59	0.64	0.83	1.2
Fructose monophosphate	9.2	9.0	9.1	9.3
Glucose monophosphate and Sedoheptulose monophosphate	27.8	20.4	16.4	14.9
Ribulose diphosphate	10.3	16.7	21.0	23.3
Fructose diphosphate	2.3	1.0	0.68	0.76
Glucose diphosphate	0.79	0.66	0.54	0.49
Uridine diphosphoglucose	3.9	3.2	3.6	3.1
Phosphoglyceric acid	9.9	7.9	10.7	11.1
Phosphoglycollic acid	1.1	7.3	3.1	9.5
Phosphoenolpyruvic acid	0.0	0.0	0.0	0.0
Fumarate	0.66	0.82	0.84	0.93
Malate	3.9	5.8	5.6	7.1
Citrate	0.05	0.04	0.25	0.87
Aspartate	2.9	1.6	1.5	1.2
Alanine	5.5	5.5	5.9	4.5
Citrulline	0.0	0.0	0.12	0.73
Glycine	0.78	0.45	0.0	0.0
Serine	3.0	2.1	1.6	1.0
Glutamic acid	0.0	0.08	1.2	1.2
Sucrose	10.4	9.6	11.0	2.9
Unidentified 1	2.0	2.2	2.5	2.0
Unidentified 2	0.39	0.42	0.56	0.45
Unidentified 3	0.25	1.2	0.81	2.0
2-Carboxy-3-keto-ribitol-1,5-diphosphate	0.82	0.67	0.68	0.40
Total recovered	96.53	97.28	98.51	98.93
Total in phosphates	66.70	67.47	66.63	74.05
Total in organic acids	4.61	6.66	6.69	8.90
Total in amino acids	12.18	9.73	10.32	8.63
Total in $^{14}CO_2$ fixed/100 μl of packed cells (c.p.m. $\cdot 10^{-5}$)	54.23	37.74	40.06	27.91
% in EtOH-soluble fraction	83	89	99.8	99.5

99.5% D_2O, giving the same cellular concentration in each suspension. Each suspension was supplied with equal quantities of$NaH^{14}CO_3$ and photosynthesis was allowed to proceed for 3 min. The total quantities of ^{14}C fixed, the quantities present in the hot ethanol extracts, and the percentages of the total ethanol-soluble activities present in a number of intermediary metabolites are given in Table I-A.

A similar experiment was performed with cells adapted to and grown in 60% D_2O. The results are also shown in Table I-B. A final experiment was performed in which cells adapted to grow in 60% D_2O were grown in four D_2O concentrations, 0%, 20%, 40% and 60%, and were resuspended in distilled water containing these concentrations of D_2O respectively. After 3 min periods of photosynthesis the cell extracts were analyzed as above with the activities in various fractions presented in Table I-C.

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I

substances after 3 min of photosynthesis. *A*, unadapted cells suspended in the D_2O concentrations shown. to grow in 60% D_2O, grown in the D_2O concentrations shown, and suspended in the same concentration photosynthesis.

B				*C*			
60	60	60	60	0	20	40	60
0	33	66	99.5	0	20	40	60
0.29	0.17	0.27	0.0	0.22	0.19	0.30	0.0
11.2	5.2	6.7	8.6	9.5	12.3	11.5	13.2
19.7	14.3	12.8	11.4	31.5	28.8	23.0	22.3
1.9	1.8	3.0	3.1	0.74	2.8	2.9	2.0
2.4	0.7	1.0	1.5	2.1	2.0	0.79	2.2
0.44	0.23	0.49	2.4	0.87	0.88	0.97	0.45
3.8	1.4	1.0	0.56	3.2	3.8	3.5	1.0
18.1	12.2	17.4	19.4	26.8	22.5	25.6	11.1
0.64	0.86	0.56	0.92	1.7	1.2	1.4	1.2
1.7	0.57	0.0	0.0	0.30	0.74	0.73	0.23
2.7	2.2	2.1	2.4	0.57	0.78	1.5	3.3
16.5	11.3	14.7	15.6	2.7	4.1	9.7	19.3
1.1	2.6	2.0	1.6	0.0	0.0	0.14	0.97
4.5	10.4	9.2	11.7	2.8	5.1	4.9	5.0
3.1	13.5	13.7	9.7	8.8	5.0	2.7	3.4
2.1	2.7	1.8	1.9	0.0	0.16	0.27	2.4
0.10	0.43	0.20	0.56	0.30	0.24	0.36	0.0
1.9	3.4	2.0	2.3	1.7	2.4	2.5	2.7
1.5	6.1	4.9	3.1	0.0	0.18	0.42	2.0
0.0	0.25	0.18	0.0	2.1	2.6	2.4	0.0
1.6	1.3	1.5	1.2	3.0	2.6	2.6	1.1
0.0	0.07	0.21	0.69	0.19	0.21	0.08	0.90
0.30	1.4	1.3	0.0	0.0	0.0	0.0	0.52
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
95.57	93.08	97.01	98.63	99.09	98.58	98.26	95.27
60.17	37.43	43.22	47.88	76.93	75.21	70.69	53.68
20.30	16.10	18.80	19.60	3.27	4.88	11.34	23.57
13.20	36.53	31.80	29.26	13.60	13.08	11.15	15.50
22.94	10.70	5.15	1.87	3.52	4.52	5.74	5.53
90	93	96	99	80	77	82	89

DISCUSSION

Effect of deuterium on growth, adaptation and deuterium incorporation

Cells having a previous history of growth in the absence of deuterium are markedly inhibited by the presence of heavy water at concentrations above about 30%. By adapting the organism through repeated subculture in media of increasingly higher concentrations of heavy water, growth can be supported in D_2O concentrations approaching 100%. Although inhibition of growth at high concentrations remains a considerable factor even with adapted cells, the block to growth at 33% D_2O exhibited by unadapted cells is overcome to a considerable extent. The increase in the average cell volume becomes very marked with unadapted cells cultured at

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concentrations above 33%; with adapted cells this effect does not become significant until over 70% D_2O is present.

The increase in cell size in the presence of D_2O has been observed by WEINBERGER AND PORTER[12] with *Chlorella* and by KATZ *et al.*[13] with animal cells and appears to signify that while cell enlargement can continue in the presence of D_2O one or more vital stages in cell division is inhibited.

The phenomenon of adaptation also involves a shift from the preferential absorption of H from the medium by unadapted cells to a preferential absorption of D with cells adapted to growing in the presence of D_2O. This may imply a considerable alteration in the proton-deuteron composition of the genetic and enzymic constituents of the adapted cells, a subject worthy of further investigation.

Effect of deuterium on photosynthesis

Unadapted cells showed a fall in the total quantity of $^{14}CO_2$ fixed by photosynthesis with increasing D_2O concentration, together with a fall of the amount incorporated into the ethanol-insoluble fraction (proteins, polysaccharides, nucleic acids, etc.). As the D_2O content of the incubation medium was increased, a greater proportion of the fixed radioactivity was found in many organic and amino acids. Activity was found in citrulline at the highest D_2O concentrations, while there was a fall of activity in sucrose. There was a greater proportional increase of activity in ribulose diphosphate than in phosphoglyceric acid, with fluctuating activities in the other sugar phosphates.

There is thus a tendency for the $^{14}CO_2$ fixation to shift from the characteristic pattern of photosynthesis to that found in the incorporation of $^{14}CO_2$ in the dark. The high levels of activity found in the Krebs cycle and amino acids may be a reflection of the inhibition of protein synthesis. The rise in the ratio of activities of ribulose diphosphate to phosphoglyceric acid might indicate some inhibition of $^{14}CO_2$ incorporation by the carboxydismutase route, leading to an accumulation of the pentose phosphates with fluctuations among the other sugar phosphates of the photosynthetic cycle. In addition the presence of activity in citrulline where none is seen when unadapted cells are photosynthesizing in the absence of deuterium would appear to indicate that the suppression of CO_2 fixation by combination with ribulose diphosphate has resulted in the accumulation of either intracellular CO_2 or of some light-dependent active CO_2 which may then be diverted to the ornithine–citrulline route[14]. As unadapted cells contain no nonexchangeable deuterium in their enzyme molecules, all these effects must be due primarily to the presence of deuterium in the environment and in exchangeable positions in the macromolecules.

The effects on photosynthesis of increasing the deuterium concentration in the environment of cells grown in 60% D_2O shows many aspects similar to the effects on unadapted algae. Thus, with increasing D_2O concentration there was a fall in overall activity fixed, a fall in activity fixed into the ethanol-insoluble fraction, a rise in the ^{14}C present in the amino acids and a low activity in sucrose, together with fluctuations in sugar phosphates. However, although the ratio of activity in ribulose diphosphate to that in phosphoglyceric acid rose as with unadapted cells, the ratio was very low at all concentrations of D_2O. The high phosphoglyceric acid pool may denote an impairment of the photosynthetic reduction of this substance due to the presence of incorporated deuterium in the enzymes. The presence of active citrulline

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at all D_2O concentrations would argue for an effect due to differences between deuterium and hydrogen enzymes, superimposed on the environmental effect of D_2O on citrulline noted with unadapted cells.

A slightly different picture is presented by adapted cells grown in different concentrations of D_2O and suspended in these same concentrations for the photosynthesis experiments. Whilst with increasing amounts of heavy water there was a marked rise in the incorporation of radioactive carbon into the organic acids, there was no increase in the amino acids, possibly indicating a balance between partially suppressed protein synthesis and the pool of free amino acids. The activities in ribulose diphosphate and phosphoglyceric acid showed similar distributions to the previous experiment with adapted cells. Activity was found in citrulline only as the D_2O concentration increased, stressing again that the degree of adaptation is largely responsible for determining the incorporation of ^{14}C into citrulline. The total incorporation of $^{14}CO_2$ in this experiment showed no consistent fall with adaptation to increasing levels of D_2O, though there was some fall in the quantities incorporated into the ethanol-insoluble fraction: the significance of this in view of the drop in the total $^{14}CO_2$ fixed with increasing D_2O concentrations in the two previous experiments reported is not clear.

The effects of deuterium oxide on the metabolism of *Chlorella* can be separated to some extent into those changes due to the presence of D_2O in the environment with little or no incorporation into the larger molecules of the cells, and those associated with adaptation of the cells with the consequent utilization of relatively large amounts of deuterium for general synthetic purposes. The lowered rates of the total incorporation of $^{14}CO_2$ by both adapted and unadapted cells shows evidence of this effect being largely due to a deuterium environment, at least up to the concentration of D_2O to which adapted cells have been adapted. Of all the ^{14}C taken up by unadapted cells photosynthesizing in 66% D_2O, only 0.2% is incorporated into the ethanol-insoluble material. Adapted cells grown in 60% D_2O and subsequently suspended in 66% D_2O fix 4% in this fraction, while in another experiment cells grown and suspended in 60% D_2O converted 11% of the ^{14}C utilized into insoluble material, thus indicating that as the cells become more adapted to D_2O, they become more normal with respect to the proportion of ^{14}C incorporated into the insoluble material.

The presence of a larger pool of radioactive phosphoglyceric acid in all adapted cells, compared with unadapted organisms, indicates that incorporation of deuterium into enzymic material during adaptation may impair the subsequent reduction of this substance. The rise found in the ratio of the radiocarbon in ribulose diphosphate to phosphoglyceric acid appears to be affected more by the D_2O concentration present during the photosynthesis experiment rather than that during growth, suggesting that D_2O may interfere with an early stage in CO_2 absorption. However, except at the highest D_2O concentrations, the sum of the percentages of the total activities in ribulose diphosphate and phosphoglyceric acid are roughly equal in all cases. The inhibitions of phosphoglyceric acid reduction and of CO_2 fixation lead to some drop in the activities in the sugar phosphates as a whole, with fluctuations in individual species, which may probably be ascribed to uncontrolled changes in the experimental environment. The incapacity of adapted cells for rapid protein synthesis leads to an accumulation of some amino acids and organic acids. Adapted

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cells fix appreciable amounts of ^{14}C into citrulline; although activity appears in citrulline in unadapted cells when placed in the presence of high concentrations of D_2O, larger amounts are present at all D_2O concentrations with cells grown in 60% D_2O, and the growth for one subculture in low concentrations of D_2O of cells adapted to 60% D_2O leaves a capacity for incorporating $^{14}CO_2$ into citrulline greater than that of unadapted cells.

Unadapted cells show an abnormally low incorporation of CO_2 into sucrose when placed in 99.5% D_2O but adapted cells exhibit this effect even when placed in H_2O. A possible explanation is the need of adapted cells to utilize the reduced amounts of fixed CO_2 available for synthesis of structural material with a resultant fall in the production of storage products.

SUMMARY

The growth, particularly cell division, of *Chorella pyrenoidosa* is markedly inhibited by concentrations of D_2O in the medium greater than about 30%. At higher concentrations there is an increase in the average cell volume. However, by serial subculture in media containing increasing quantities of D_2O the cells can be adapted to grow well at about 60% D_2O, and even show some growth at 99% D_2O. The relative incorporation into cell material of protons and deuterons from the medium has been determined with both adapted and unadapted cells.

The distribution in a number of compounds of ^{14}C incorporated from $^{14}CO_2$ during photosynthesis has been investigated using unadapted and adapted cells suspended in distilled water of varying D_2O contents. The effect on the $^{14}CO_2$ incorporation patterns of adapting cells to grow in 60% D_2O, and then subculturing them in lower concentrations of heavy water has also been examined.

Certain conclusions have been drawn as to the effect of D_2O on the pattern of $^{14}CO_2$ incorporation, and an effort has been made to distinguish between those effects due solely to the presence of deuterium in the medium and in the exchangeable hydrogen atoms in the macromolecules, and those effects resulting from a utilization of deuterium for general synthetic processes.

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