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METABOLISM OF HYDROGEN ISOTOPES BY RAPIDLY GROWING CHLORELLA PYRENOIDOSA CELLS

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METABOLISM OF HYDROGEN ISOTOPES BY RAPIDLY GROWING CHLORELLA PYRENOIDOSA CELLS

By

Daniel Weinberger and John W. Porter

Metabolism Unit
Biology Section

September 1, 1953

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1140274

ABSTRACT

Chlorella pyrenoidosa cells have been grown in the presence of deuterium and tritium oxide, and the quantity of each of these isotopes incorporated into the whole cells, methanol insoluble, ether soluble and methanol soluble, ether insoluble fractions has been determined. The quantity present has been recorded in terms of the per cent expected on the assumption of no isotope effect.

It has been found that the cells incorporate deuterium approximately 50% as rapidly as hydrogen, and that they incorporate tritium 90% as rapidly as deuterium. Further, the extent of incorporation of tritium or deuterium by the enzymes involved in hydrogen transfer in the synthesis of lipids is significantly higher than the extent of incorporation by enzymes involved in the transfer of hydrogen in the synthesis of proteins, nucleic acids and starch (methanol insoluble fraction).

1140275

METABOLISM OF HYDROGEN ISOTOPES BY RAPIDLY GROWING CHLORELLA PYRENOIDOSA CELLS

INTRODUCTION

Studies with isotopes of carbon and hydrogen have demonstrated that isotopes of each of these elements are incorporated to different extents, or at different rates, into algal cells and other organisms. Thus Van Norman and Brown[1] found that $C^{14}O_2$ is incorporated approximately 15% more slowly than $C^{12}O_2$ into photosynthesizing Chlorella cells and barley leaf tissue. Previously Weigl et al.[2] had obtained similar results with barley leaves. Buchanan and Nakao[3] have also reported an isotope effect, 8%, for $C^{14}O_2$ incorporation into algae grown three years in a closed aquarium.

In 1935 Reitz and Bonhoeffer[4] reported that Scenedesmus acutis and Chlamydomonas spp. incorporated deuterium to the extent of only 30-70% of its concentration in the nutrient solution. More recently Thorn[5], working with an isolated system, reported that tetradeuterosuccinic acid is dehydrogenated at 40% of the rate at which succinic acid is dehydrogenated by succinic dehydrogenase. In addition Thorn showed that the activation energy for the deutero compound is approximately 1000 calories more than for succinic acid. Recently we have shown[6] that Chlorella pyrenoidosa cells incorporate only 40% as much tritium as expected on on the assumption that there is no isotope effect.

Comparisons of deuterium and tritium incorporation into algae have not previously been made. With animals, however, Glascock and Duncombe[7] found the ratio of T/D in mammary fat of lactating female rats to be about 0.8 of that of drinking water levels. Thompson and Ballou[8] reported higher values, between 0.9 and 1.0, for whole carcass

1140276

and fractions thereof from rats. Eidinoff et al.[9] found, in a similar study, that deuterium was incorporated 8 and 18% faster than tritium into rat liver glycogen and fatty acids.

In the present paper we are concerned first with the extent of incorporation of deuterium into whole cells, in per cent of theoretical assuming no isotope effect, and then with the extent of incorporation of both deuterium and tritium into Chlorella cells and chemical fractions thereof. These studies give information on the magnitude of the isotope effect in whole cells and in different cellular processes of the algae.

METHODS

Experiments with Deuterium Oxide

Algae were grown and harvested as reported previously[6], except that the algae were carried on agar slants enriched with glucose instead of sucrose, and the cells to serve as inocula were grown in a continuous culture apparatus[10]. The concentration of deuterium oxide in most of the experiments was 23.3 mole per cent. All analyses in these experiments were made on whole cells.

Experiments with Deuterium and Tritium Oxides

Algae were grown in twenty-six 18 x 150 mm tubes which contained a final volume of 20 ml of nutrient solution. Twenty-three and five-tenths mole per cent of deuterium and one mc/ml of tritium were present in these solutions. Two additional tubes were uninoculated. Another two tubes did not contain tritium or deuterium, but were inoculated with Chlorella.

After the growth period the solution in each tube was made to 25 ml. an aliquot taken for light absorption readings, and the remainder centrifuged in a Servall Centrifuge at 8500 rpm for 2 minutes. The supernatant

1140277

solution was discarded. The cells were resuspended and centrifuged twice more. The contents of 25 of the tubes were combined in lots of 5 and each lot was suspended in 25 ml of distilled water.

Algae were removed from each of the lots, dried in tared crucibles, weighed, and then analyzed for hydrogen, deuterium, and tritium contents. The remainder of the suspension was again centrifuged, and the supernatant solution was discarded. Most of the cells were scraped from the centrifuge tube into 40 ml of hot methanol. The remainder were slurried in with 1-2 ml of water.

The methanol solution was refluxed for 20 minutes. Then the methanol insolubles were centrifuged from the soluble fraction. The non-soluble fraction was heated again with methanol and a small amount of ether, centrifuged, washed once more with solvent, and centrifuged. The methanol insolubles were removed as quantitatively as possible and suspended in water to a final volume of 10 ml.

The combined methanol extracts were poured through ethyl ether and into a layer of water which diluted the methanol to 40%. The two phases were shaken in a separatory funnel and then separated. One more extraction was made with ethyl ether. The ether fraction was washed once with water, then evaporated to a small volume, and finally made to a volume of 10 ml.

The ether insoluble fraction was evaporated under vacuum to a small volume, and then diluted to a volume of 10 ml.

Duplicate samples of each lot, except the methanol soluble, ether insoluble fraction (single samples), were pipetted for tritium and deuterium analyses. Single samples were submitted for hydrogen analysis.

1140278

Deuterium and Tritium Analyses

Vacuum dried algae, or fractions thereof, were combusted at 725°C in a micro furnace, and the combustion water was analyzed for tritium as reported previously[6]. Samples for deuterium analysis (separate aliquots) were combusted in a microfurnace also; then the combustion water was converted to hydrogen by passage through heated zinc, as described by Graff and Rittenberg[11]; and finally the deuterium content was determined with a Consolidated-Nier mass spectrometer. Results were reproducible to ± 3% for water samples and ± 5% for samples carried through the entire analytical procedure.

Modifications which we made in the equipment used in the procedure of Graff and Rittenberg[11] for deuterium analyses were as follows: a) 6 mm capillary tubing (i.d. 2 mm) was used for greater structural stability of the system, b) a smaller Toepler pump (40 cm high, 200 ml capacity) was used, and c) a Lab Jack was used to mount and support the mercury reservoir.

Hydrogen Analyses

Hydrogen analyses were made by the conventional micro method on vacuum dried aliquots of whole cells and chemical fractions thereof.

RESULTS

Incorporation of Deuterium into Algal Cells

Fig. 1 shows the incorporation by algae of deuterium, from deuterium oxide, as a function of the number of cell mass doublings. Also plotted is a theoretical curve which was calculated on the following assumptions: 1) the incorporation of deuterium into algae proceeds at the same rate as the incorporation of hydrogen, and 2) the hydrogen of the inoculating cells is not lost in significant amounts through

1140279

metabolism or exchange during the growth and multiplication of the cells. The equation for the theoretical curve is $Y = \frac{2^n-1}{2^n}$, where unity is the value approached as n becomes infinitely large. The equation for the experimental curve is $Y = a\frac{2^n-1}{2^n}$, where a (51%) is the value approached asymptotically as n approaches infinity. The actual curve is that which most closely fits the 10 experimental points. The number of doublings(n) in mass was calculated from the relationship $n = 3.3 \log \frac{b}{B}$, where b = weight at the end of an experiment and B = weight at the beginning of an experiment.

It is obvious that deuterium is incorporated into the algal cells more slowly than hydrogen. At n = 5 the ratio of incorporation of D/H is approximately 50%.

In order to show that the isotope effect is not confined to the level of deuterium oxide used in Fig. 1, algae were grown in the presence of several other concentrations of deuterium oxide. Analyses of the cells for deuterium content were then made. Table I shows that the incorporation of deuterium into algae at these concentrations was the same for each of the concentrations, 40% of theoretical.

A further experiment was performed to determine the extent of growth of Chlorella in concentrations of D_2O of greater than 25 mole per cent. No appreciable growth retardation was observed until the concentration approached 50% D_2O. At levels of 83 and 94% D_2O algae grew only 8 and 6% as rapidly, respectively, as control cells, when growth was measured spectrophotometrically. The number of cells, per the same $\log \frac{I_o}{I}$ (mass) value was, however, only 70 and 50% that of control cells, respectively, for cells grown in 83 and 94% D_2O. These results indicate an enlargement of the cells growing in the higher concentration of D_2O. This result could be brought about by an inhibition of cell division without, or with only a partial, inhibition of growth processes.

1140280

That algae are not permanently damaged by immersion and growth in 83-94% D_2O was shown in an experiment in which cells grown for several days in these concentrations of D_2O were transferred to fresh nutrient solution lacking in D_2O. Growth of these cells, in numbers and mass, over 3 days, was equal to that of control lots of cells.

Incorporation of Hydrogen, Deuterium and Tritium into Chlorella

Table II gives the results, in per cent of theoretical, of the experiment on the relative incorporation of hydrogen, deuterium and tritium into algae. Three facts are evident: 1) a small, but significant, difference exists between the extent of incorporation of deuterium and tritium into the algal cells, the ether soluble, and probably methanol insoluble, cell fractions, 2) a highly significant difference exists in the extent of incorporation of both deuterium and tritium, relative to hydrogen, into lipid and non lipid compounds, and 3) deuterium and tritium contents, in % of theoretical, of the methanol soluble ether insoluble fraction are not significantly different.

It is believed that exchange affected the values we obtained for deuterium and tritium contents of Chlorella and fractions thereof only slightly, for our earlier studies[6] showed that only 6% of the total tritium of algal cells exchanges. Also the total quantitites of deuterium and tritium in each cell fraction when added closely approximated that in the whole cells.

Some differences exist in the amount of deuterium incorporated into Chlorella, in per cent of theoretical, in the data reported in Tables I and II and Fig. 1. These discrepant values do not, however, invalidate any of the conclusions regarding deuterium and tritium incorporation into algae, for comparisons of the extent to which these isotopes are incorporated into algae are made only on data obtained from cells growing in media containing both isotopes.

1140281

DISCUSSION

In the splitting of a C-H bond in an ordinary chemical reaction or in an enzymatically catalyzed reaction, energy must be supplied to effect the split. The quantity of energy that must be supplied to split a C-H, C-D, or C-T bond is a function of the mass of the isotope of hydrogen, other conditions being equal; and the energy required to split the C-T bond is the greatest of the three values. The reason for this is the fact that the zero point energy of the C-H, C-D, and C-T bonds is an inverse function of the effective mass of the oscillating atoms.

In a Chlorella cell water is split photochemically and hydrogen is transferred through an unknown number of steps to a final acceptor substrate compound. The extent to which an isotope effect occurs in the formation of a C-H bond of any compound in a Chlorella cell should depend then upon the amount of energy available for each reaction in the series of hydrogen transfers and the number of hydrogen transfers taking place, in the formation of the C-H bond in question (in proteins, starch, fatty acids, etc.). (Steric hindrance would also be a factor, but we believe a minor one, in determining the quantity of tritium and deuterium incorporated into a compound). If the amount of energy available for each of the reactions involved in the formation of $C\text{-}H^2$ and $C\text{-}H^3$ bonds is well above the activation energy of $C\text{-}H^1$ bonds, and if the number of reactions is small, the isotope effect would be small. If the amount of energy available for each of the reactions involved in the formation of $C\text{-}H^2$ and $C\text{-}H^3$ bonds is only slightly above the activation energy of $C\text{-}H^1$ bonds, and if the number of reactions is large, the isotope effect would be large. Eyring and Sherman[12] calculated that for chemical reactions proceeding at ordinary temperature deuterium would react only 50% as rapidly as hydrogen. Presumably this is a maximal or near maximal isotope effect

That there are differences in the rate at which the enzymes of Chlorella transfer hydrogen, deuterium and tritium in growth and metabolism of the cells is clearly shown in our results. Thus only 50% as much deuterium as protium and only 90% as much tritium as deuterium is incorporated into the cell. Further, and equally significant, there is a large difference in the extent to which enzymes involved in hydrogen transfer in the synthesis of lipids and enzymes involved in hydrogen transfer in the synthesis of proteins, nucleic acids and starch (methanol insoluble fraction) incorporate either deuterium or tritium into these compounds.

It has been shown that either enzymes differ in their ability to break C-T or C-D bonds, or that more hydrogen transfers are involved in the formation of C-H bonds of proteins, nucleic acids, starch, etc. (methanol insoluble fraction) than are involved in the formation of lipids. Further studies will be necessary to elucidate the significance of each of these factors in the overall isotope effect we have shown in these studies.

It is pertinent to state that the isotope effect must be taken into consideration in all tracer experiments utilizing deuterium and tritium, and that all tracer work with these isotopes (including past work) must be carefully scrutinized to make certain isotope effects have not invalidated or distorted results and conclusions.

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1140283

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1140284

TABLE I

Deuterium Content of Algae Growing in Media Containing Different Concentrations of Deuterium Oxide

Results after 5.3 weight doublings

Deuterium In Medium Mole %	Theoretical, Assuming No Isotope Effect Mole %	Experimental Mole %	Per cent of Theory
		Experiment 1	
5.0	4.90	1.91	39
10.0	9.75	3.68	38
25.0	24.3	9.95	41
		Experiment 2	
4.88	4.63	1.94	42
9.76	9.27	3.64	39
24.4	23.2	9.18	40
		Avg. = 40. ± 1.4	

1140285

TABLE II

Tritium and Deuterium Contents of Whole Cells and Fractions Thereof

Cell Fraction	% of Theoretical Tritium[1]	% of Theoretical Deuterium[1]	T/D[2]
Whole Cells[3]	44.9 ± 2.2	50.2 ± 1.8	0.89 ± 0.05
Methanol insolubles[3]	41.5 ± 1.6	44.6 ± 3.7	0.93 ± 0.08
Ether solubles[3]	55.3 ± 5.2	62.8 ± 2.7	0.88 ± 0.08
Methanol soluble, ether insoluble[4]	37.7 ± 8.4	32.3 ± 5.3	1.17 ± 0.23

1. Average ± standard error (95% confidence level).
2. Ratio ± 95% confidence limits.
3. Average of 10 analyses.
4. Average of 5 analyses.

1140286

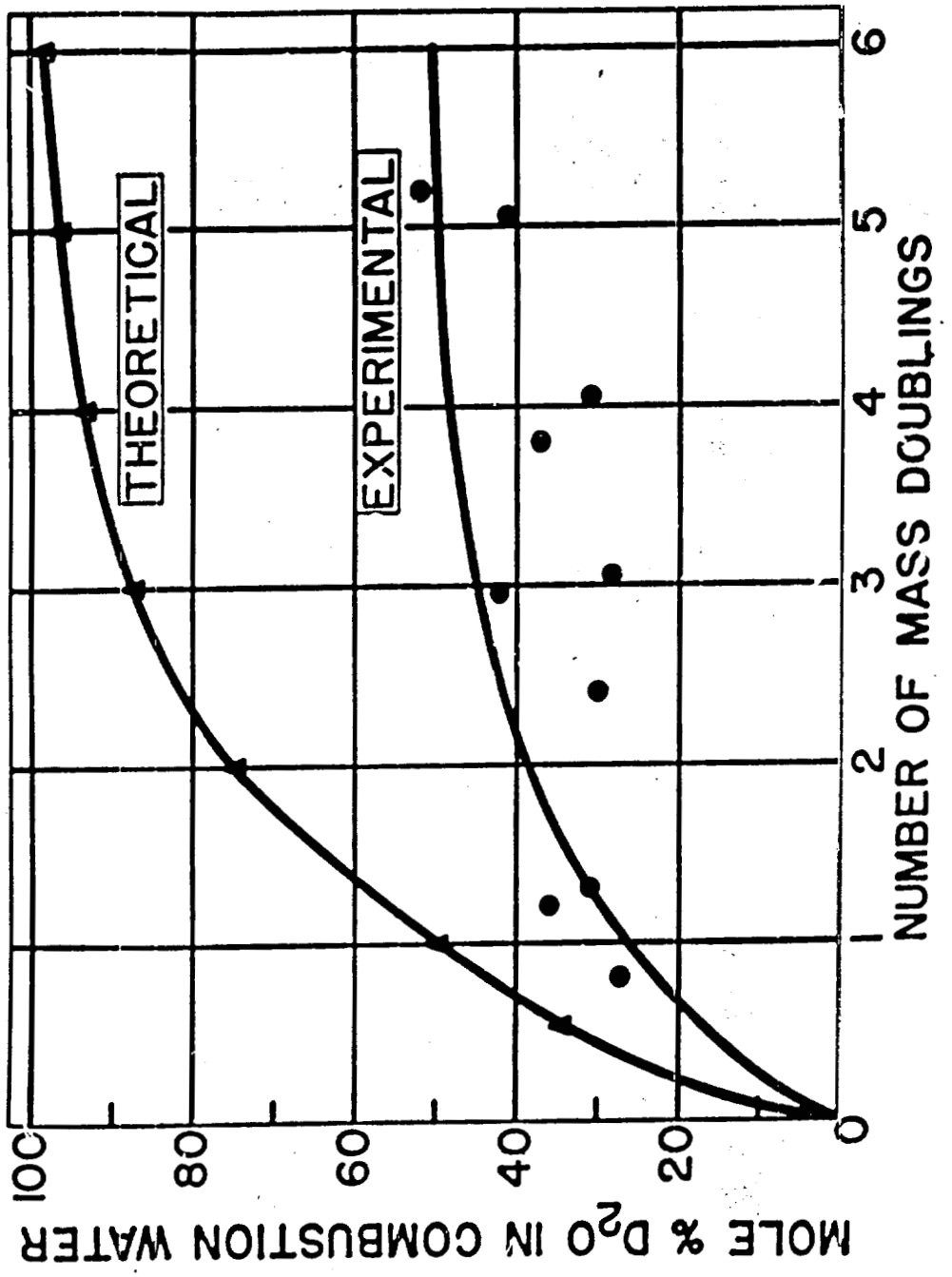

FIGURE 1

THE INCORPORATION OF DEUTERIUM OXIDE INTO RAPIDLY GROWING CHLORELLA CELLS

100 - 23.3 mole % D_2O

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1140287