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EFFECT OF DEUTERIUM OXIDE (HEAVY WATER) ON BIOLOGICAL SYSTEMS

E. L. Bennett, M. Calvin, O. Holm-Hansen, A. M. Hughes, K. K. Lonberg-Holm, V. Moses, and B. M. Tolbert*

INTRODUCTION

Twenty-five years ago G. N. Lewis observed the effect of deuterium oxide (D_2O) upon tobacco seeds, flatworms and a mouse.¹ Following the first commercial availability of D_2O , intensive investigations were made of its effects on various biological systems. Primarily because of the high cost and limited availability of deuterium at that time, these studies were generally restricted to small-scale investigations and were mainly concerned with the responses of biological systems to increasing but low concentrations of D_2O . Whereas most biological systems were not markedly affected by D_2O in concentrations up to about 20%, some processes were reported to be stimulated by the presence of much smaller amounts of D_2O . As the concentration of D_2O was increased to levels between 30% and 90%, growth usually was completely inhibited. The earlier literature has been reviewed by Chance and Allen² and Thorn.³ At the present, the price of nearly pure (better than 99%) D_2O is \$60 to \$120 per kg. As a result of this increased availability, relatively large-scale investigations are now possible of the effects of D_2O upon a variety of biological systems.

Different isotopes of an element do not possess identical chemical and biochemical properties. These divergencies in reaction rates and equilibria are caused by differences in mass of the different isotopes, and are large between hydrogen and deuterium because of their high mass ratio. A principal feature of the structure of such biologically important macromolecules as proteins and nucleic acids is the maintenance of their secondary and tertiary structure by participation of many hydrogen bonds.^{4,5} The multiplication that would occur in a macromolecule of even a small difference between a proton bond and a deutron bond would certainly have some effect upon the structure of the macromolecule. Differences in behavior between systems based on protium and those based on deuterium should eventually result.

The work to be reported in this paper is primarily concerned with the effect of deuterium on the growth, reproduction and metabolism of algae, ascites tumor cells, and mice.

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EFFECT OF DEUTERIUM ON ALGAE

Growth effects. The effect of deuterium oxide upon the metabolism and growth of Chlorella pyrenoidosa has been studied.⁶ The alga, when cultured in media containing D_2O , but having no previous history of deuterium, shows a moderate inhibition of growth in D_2O concentrations up to 30% to 40%. Above about 35% D_2O , the growth of Chlorella--as measured by optical density, dry weight, number of cells and the volume of the packed cells--shows a very sudden decrease. At higher concentrations of D_2O there is an increase in the average cell volume up to ten times the normal at 70% D_2O . The increase in cell size in the presence of D_2O has been observed by Weinberger and Porter⁷ and indicates that whereas cell enlargement can continue in the presence of D_2O , one or more vital stages in cell division is inhibited. However, by serial subculture in media containing increasing quantities of D_2O , the cells can be adapted to grow well (enlarge and divide) when the concentration is about 60%, and even show some growth at 99% D_2O . Adapted cells, unlike unadapted cells, show no sudden inhibition of growth with increasing D_2O concentrations, but show a fairly steady drop over the whole range from 0% to 99%. In addition, adapted cells show a marked increase in the average cell volume only when the D_2O concentration exceeds 70%.

Preferential Uptake of Protons or Deuterons from the Medium. Unadapted cells preferentially select protons from the medium, while the reverse is true for cells adapted to grow in 60% D_2O . For example, with unadapted cells cultured in a medium containing approximately 23.5% D_2O , the D_2O concentrations in the medium after growth, in the free intracellular water (obtained by lyophilization of the packed cells), and in the water produced by combustion of the dried cells, were 21%, 24% and 13%, respectively. With cells adapted to grow in about 60% D_2O , on the other hand, the corresponding values were 63.7%, 63.4% and 69.8%, respectively. The samples were analyzed for protons (and hence for deuterons by difference) by nuclear magnetic resonance on a Varian V-4300B spectrometer against a series of standard H_2O - D_2O mixtures. The experimental values were obtained by interpolation.

Effect of Heavy Water on Photosynthesis (1). Unadapted cells. The distribution in a number of compounds of C^{14} incorporated from $C^{14}O_2$ during photosynthesis has been investigated by the use of adapted and unadapted cells suspended in distilled water of varying D_2O concentrations.

The unadapted cells showed a decrease in the total quantity of $C^{14}O_2$ fixed by photosynthesis with increasing D_2O concentration, together with a decrease 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, whereas 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 other sugar phosphates.

There is thus a tendency for the $C^{14}O_2$ fixation to shift from the characteristic pattern of photosynthesis to that found in the incorporation

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of $C^{14}O_2$ 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 $C^{14}O_2$ 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 that may then be diverted to the ornithine-citrulline route.⁸ 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.

(ii) Adapted cells. The effects on photosynthesis of increasing the deuterium concentration in the environment of cells grown in 60% D_2O show many aspects similar to the effects on unadapted algae. Thus, with increasing D_2O concentration there was a fall in over-all activity fixed, a fall in activity fixed into the ethanol-insoluble fraction, a rise in the C^{14} 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 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. Whereas 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 distributions similar to those in 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 C^{14} into citrulline. The total incorporation of $C^{14}O_2$ in this experiment showed no consistent decrease 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 $C^{14}O_2$ fixed with increasing D_2O concentrations in the two previous experiments reported is not clear.

(iii) Discussion. 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 consequent utilization of relatively large amounts of deuterium for general

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synthetic purposes. The lowered rates of the total incorporation of $C^{14}O_2$ by both adapted and unadapted cells shows evidence that this effect is largely due to a deuterium environment.

The presence of a larger pool of radioactive phosphoglyceric acid in adapted than in unadapted cells 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 that in phosphoglyceric acid appears to be affected more by the D_2O concentration present during ^{the} photosynthesis experiment rather than by that during growth. This suggests that D_2O may interfere with an early stage in CO_2 absorption. 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. The incapacity of unadapted cells for rapid protein synthesis leads to an accumulation of some amino acids and organic acids.

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 decrease in the production of storage products.

EFFECT OF DEUTERIUM UPON SURVIVAL OF MICE

The inhibition of cell division in algae cells by moderate concentrations of D_2O suggests that the cell division in higher biological systems, particularly rapidly dividing systems, might also be inhibited by D_2O . The effect of chronic administration of D_2O in the drinking water on the survival of young adult male and female C_{57} mice inoculated with Ehrlich's mouse-ascites tumor cells has been studied.⁹ The experimental animals were maintained on 25%, 30%, or 40% D_2O in the drinking water for two days prior to inoculation with ascites tumor cells. The mice were then maintained on a similar concentration of D_2O in the drinking water for the duration of the experiment. The survival times for the experimental animals on D_2O were compared to those for control animals inoculated at the same time, and with the same inoculum, but maintained on H_2O instead of on water enriched with D_2O . The mice maintained on 25% and 30% D_2O had mean survival times approximately 40% to 50% longer than the control mice (12 to 14 days for the control mice compared with 18 to 19 days for the experimental animals). The mice maintained on 40% D_2O in the drinking water had a survival curve very similar to that obtained for the control mice, and the mean survival time was slightly decreased. Other experiments have indicated that 50% D_2O in the drinking water is fatal to mice within several days, whereas mice can be maintained on 40% D_2O for several months. However, at the end of this period gross physiological effects caused by D_2O , such as emaciation, epilation, and blindness can be observed. The additive effect of two stresses--high D_2O and ascites tumor cells--probably explains the decreased survival of mice at 40% D_2O compared to mice maintained on 25% and 30% D_2O . Barbour and Allen reported significant inhibition of carcinoma and lymphosarcoma growth in mice maintained on 40% D_2O in the drinking water, but the survival time of the mice was shortened.¹⁰ Katz et al have shown that D_2O inhibits the growth of Krebs-2 ascites tumor

in CF-1 mice, and prolongs the life of DBA/2 mice inoculated with lymphatic leukemia.¹¹

PRODUCTION OF STERILITY BY DEUTERIUM OXIDE

Since cell division plays a major role in the reproductive processes of higher organisms, the effect of D_2O in the drinking water of mice upon their fertility has been investigated.¹² C₅₇ and Swiss male and female mice were maintained on 5%, 20% or 30% D_2O in the drinking water for 8 weeks. At the end of this period, D_2O administration was discontinued and each mouse was individually mated (caged continuously) with a normal mouse of the same strain. At the same time, normal mice of each strain were individually mated as controls. Offspring were counted and sexed two weeks after birth. Those pairs which did not produce a litter during a 28 day period after the initial mating were considered to be a sterile pair. This period is one week longer than the average 21 day gestation period for mice.

The results are summarized in Table I. The data indicate that 30% D_2O causes 100% sterility in both C₅₇ and Swiss male mice, and in some C₅₇ female mice. The results of another experiment indicate that 20% D_2O also produces almost complete sterility in C₅₇ male mice. Five percent D_2O in the drinking water of C₅₇ mice appears to produce a degree of sterility comparable with that produced by 20% D_2O in Swiss male mice. No significant difference was found in the litter size or the sex ratio of the litters for the experimental animals and for the controls. This is in contrast to the effect of radiation, which shows, in addition to the sterility effects^{13,14}, reduction in the litter size and a change in the sex ratio.¹⁵

Several physiological mechanisms by which D_2O produces sterility in mice can be suggested. D_2O could interfere with maturation of the ova or sperm, or possibly reduce sperm motility. In addition, D_2O may interfere with the proper development of the fertilized ovum. The known ability of D_2O to inhibit cell division in egg cells¹⁶ and the greater sensitivity of the male mice suggest that the most likely point of susceptibility would be the development of the sperm. It is known that the overall time required for the development of spermatozoa in the seminiferous tubules and their transport through the epididymis is about 40 days in the mouse.¹⁷ Twenty-eight male animals which had received 30% D_2O did not sire any litters which were born during the initial 28 days after D_2O administration was discontinued. These mice sired two litters which were born 48 to 49 days after D_2O administration was discontinued, 18 litters which were born during the 57- to 76-day period following withdrawal of D_2O , and only three litters which were born in the following 5 week period. Females of the five remaining pairs were not pregnant at 105 days. The litters were of normal size but an increased mortality in these litters was observed. When it is remembered that the gestation period for a mouse is 21 days, this result strongly suggests that the spermatogenic process is affected, either by the inhibition of sperm production or by the formation of defective sperm.

Other experiments have indicated that 4 weeks of D_2O administration is sufficient to produce sterility in male mice, and further experiments are

TABLE I

The Effect of D₂O on the Fertility of C₅₇ and Swiss Mice

Sex treated	D ₂ O conc. in drinking water (%)	Number of pairs	% sterile pairs	Av. no. offspring per litter ^a	Av. no. offspring per mating ^b
<u>C₅₇ mice</u>					
Male	5	10	30	5.4	3.8
Female	5	10	30	7.6	3.8
Male	30	19	100	0	0
Female	30	10	40	5.2	2.6
Controls	0	24	17	6.4	4.4
<u>Swiss mice</u>					
Male	20	10	40	9.3	5.6
Female	20	11	0	9.1	9.1
Male	30	10	100	0	0
Female	30	10	0	9.1	7.3
Controls	0	19	5	9.0	8.5

a. Calculated as the total number of offspring surviving two weeks divided by the number of litters containing live offspring at two weeks.

b. Calculated as the total number of offspring surviving two weeks divided by the number of mated pairs.

planned to determine the minimum time required to produce sterility. Histological studies are planned to investigate the effect of D₂O upon sperm production. Craig *et al.* have noted that x-irradiation, triethylenemelamine, and bisulphan act upon different stages of the spermatogenic processes in the rat.¹⁸ Irradiation (500r) produces decreased litter size up to 7 weeks, and then prolonged sterility. Triethylenemelamine (five daily doses) produces immediate sterility followed by recovery 6 weeks later.

On the biochemical level, hydrogen bonding is important for the maintenance of the structure of many biologically important molecules including proteins and nucleic acids. When D replaces H in a H bond one may expect both the energy and dimension to change but for short H bonds between O atoms there is an approximately 2% expansion of the bond's distance upon the substitution of D for H.^{19,20} Aberrations in the structure of these compounds may result in abnormalities in the metabolism of low molecular weight compounds used to form macromolecules. We have investigated the rate of metabolism to CO₂ of glucose-C¹⁴, acetate-2-C¹⁴, formate-C¹⁴, and glycine-2-C¹⁴. No differences were found in the rate of metabolism of glucose or the acids, but glycine-C¹⁴ was metabolized approximately 20% more rapidly over a 2 hour period in mice that had been maintained on 30% D₂O for 2 weeks. Aberrations in the structure of deoxynucleic acids may be of particular importance owing to their role in gene structure and cell division. It is expected that deuterium may be mutagenic, and that in the proper biological system, an increased mutation rate may be found. Such studies are contemplated.

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