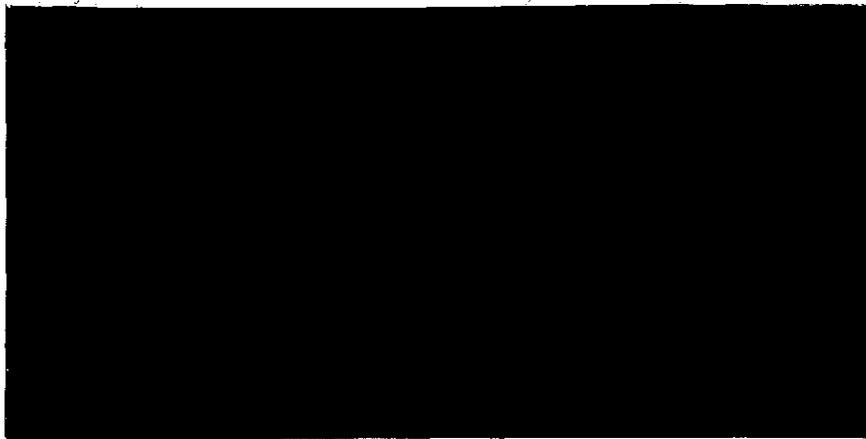


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THE PERFORMANCE AND CAPABILITIES OF TERRESTRIAL ORGANISMS IN EXTREME AND UNUSUAL GASEOUS AND LIQUID ENVIRONMENTS



(NASA-CR-138616) TOXICOLOGY: MECHANISMS
OF DEUTERIUM OXIDE ACTION, PART 2 ⁵¹
Semiannual Report (Hawaii Univ.) 52 p
HC \$5.75 CSCL 06T
N74-26593
G3/04 Unclass
41305

S. M. SIEGEL

BOTANY DEPARTMENT, UNIVERSITY OF HAWAII

Prepared under
Grant No. NGL 12-001-042
with the
NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

SEMI-ANNUAL REPORT

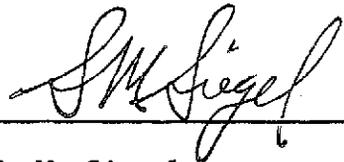
Toxicology: Mechanisms
of Deuterium Oxide Action
Part II

December 1973

University of Hawaii
Hawaii Botanical Science Paper
No. 35

*Informal technical report, neither to
be cited nor credited as a publication.*

Submitted by:



S. M. Siegel
Professor of Botany &
Principal Investigator

NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

Grant No. NGL 12-001-042

*This dissertation is part of a research program
supported by National Aeronautics and Space
Administration Research Grant NGL 12-001-042.*

THE EFFECT OF D₂O ON PROTEIN SYNTHESIS, ULTRASTRUCTURE
AND PERMEABILITY IN HIGHER PLANTS

Part II

*A Dissertation submitted to the Graduate Division of
the University of Hawaii in partial fulfillment
of the requirements for the degree of*

Doctor of Philosophy

in

Botanical Sciences

By

Jack Waber

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THE EFFECT OF GROWTH IN 99.6% D₂O ON
THE METABOLISM OF WINTER RYE

Abstract

The metabolism of winter rye seedlings (*Secale cereale*, L. cv. Winter) cultured in 99.6% D₂O was investigated. Compared with water grown seedlings, the protein content was much lower in the D₂O cultured seedlings and the incorporation of ³H-leucine and ³H-phenylalanine into medium to high molecular weight proteins was partially blocked. The synthesis of the enzyme peroxidase was also reduced in the D₂O plants. Seedlings cultured in D₂O incorporate ³H-thymidine into DNA, but do not take up ³H-uridine. These results suggest that some of the toxic effects of D₂O culture on higher plants can be attributed to a partial block of protein synthesis.

Introduction

Though interest in the effects of D₂O culture on angiosperms dates back to 1933 (10), none have yet been induced to grow vigorously in 99.6% D₂O. Seed plants generally show more complex responses to D₂O than do microorganisms and therefore are very refractory to culture in concentrations of D₂O greater than 70% (2,3). The grass winter rye, however is capable of growth, albeit to a limited

extent, in a 99.6% D₂O environment (13) without prior adaptation (8). The observed growth however, is indeed limited and while the rye is partially immune to the well documented toxic effects of D₂O (e.g. 2, 3 and 8) it is not completely resistant to them.

An earlier investigation of the ultrastructural consequences of growth in D₂O was not successful in finding any major differences between the H₂O- and D₂O-grown plants that suggested a toxicological mechanism for heavy water. It was concluded, therefore, that D₂O must be acting, at least initially, on a level not observable with the electron microscope.

The present study seeks to examine aspects of the physiology and biochemistry of D₂O-grown winter rye to determine what effect, if any, D₂O has on the metabolism of the plant.

Methods and Materials

Culture Method:

Seeds of the grass, winter rye (*Secale cereale*, L. cv. Winter) were surface sterilized by soaking for 5 minutes in 70% ethanol followed by 5 minutes of soaking in 6% sodium hypochlorite. The seeds were then rinsed with sterile water, patted dry, and then sown in petri dishes on sterile filter paper moistened with either sterile H₂O or D₂O. The dishes were then covered and sealed with tape to prevent any isotopic exchange. The plants were harvested

at a time of morphological similarity between the two types of tissues, approximately two days for the H₂O controls and nine days for the D₂O-grown seedlings.

³H-Thymidine and ³H-Uridine Uptake Studies:

Plants were grown in 125 μ Ci of either thymidine (methyl-³H) or uridine-5-³H for approximately 2 (H₂O) and 9 (D₂O) days. After this time, root tips were removed and fixed for 5 minutes in 10% formaldehyde and 20% acetic acid. The tips were then squashed on a subbed slide. The slide was then coated with Kodak N.T.A. liquid emulsion and stored in the cold for 13 days. The radioautographs were then developed and stained with 0.05% toluidine blue-0. The slides were viewed and photographed with a Leitz microscope and camera system.

The incorporation of thymidine into DNA was studied by using a trichloroacetic acid extraction technique. Tissue grown in labeled thymidine was washed with trichloroacetic acid (T.C.A.) according to the following procedure:

1. Plants grown in the presence of 100 μ Ci of thymidine (methyl-³H) were rinsed in H₂O, chopped up and placed in 10 ml of cold (23°C) 6% T.C.A.
2. After gentle mixing for 1 hour, 0.1 ml aliquots were taken from the vessel for radioactivity determinations. The remaining solution was removed by aspiration and

10 ml of fresh T.C.A. added. This same procedure was repeated twice more.

3. After the final cold (23°C) T.C.A. wash, 10 ml's of fresh T.C.A. were added and then heated to 80°C. After 30 minutes at this temperature with gentle mixing, 0.1 ml aliquots were taken from the vessel for radioactive determinations.

In total, this procedure amounted to three cold (23°C) and one hot (80°C) T.C.A. washings.

³H-Leucine and ³H-Phenylalanine Studies:

H₂O and D₂O plants grown in the presence of 100 µCi of l-leucine (4,5-³H) or l-phenylalanine-³H(GL) were homogenized in 5 ml of a 0.1 M potassium phosphate buffer, pH 6.1, and centrifuged at 30,000X g for 30 minutes at 4°C. The resulting supernatant was placed in a dialysis bag and dialyzed against 400 ml of 0.01 M, pH 6.1, phosphate buffer for 48 hours at 7°C with four changes of solution. The contents of the bag were then rinsed out and made up to a volume of 10 ml. To this solution was added ammonium sulfate to bring the concentration up to 25% saturation. This solution was then mixed for one hour. After mixing, the solution was centrifuged for 30 minutes at 30,000X g. The resulting supernatant was decanted and made up to 50% ammonium sulfate and mixed for one hour. The precipitate was redissolved in 10 ml of buffer and subsequently counted; this was designated

the 0-25% protein fraction. The 25-50%, 50-75% and 75-100% protein fractions were obtained in the same manner. The samples were counted on a Beckman LS-100 liquid scintillation counter. The cocktail was Beckman Fluoralloy in dioxane.

Peroxidase Assay:

Five seedlings were homogenized in 5 ml of a 0.1 M potassium phosphate buffer (pH 6.1) and centrifuged at 30,000X g for 30 minutes at 4°C. The resulting supernatant was analyzed for peroxidase activity according to the following scheme:

Substrate: 0.1 M potassium phosphate buffer (pH 6.1) containing 5×10^{-3} M guaiacol and H_2O_2 .

Procedure: To three ml of substrate was added 0.1 ml of the enzyme preparation and the increase in O.D. with time at 470 nm was recorded. Activity was expressed as Δ O.D. units/minute. The μ gm of peroxidase present in a plant was calculated by comparing the observed activity of the extracted enzyme to a standard curve generated with known amounts of horseradish peroxidase.

Protein Determinations:

The protein content of the cell-free preparations was determined according to the method described by Lorwy *et al.* (11).

Quantitative Electron Microscopy:

Electronphotomicrographs were analyzed for ultrastructural indications of the physiological state of the H₂O- and D₂O-grown seedlings. Standard E. M. techniques were used to fix, stain, view and photograph the tissue.

Results

The results of the ³H-Thymidine (T) and ³H-Uridine (U) uptake studies are presented in Figure 9. Figures 9A and 9C clearly indicate that, as would be expected, the water grown tissue takes up both T and U, with the U label being generally distributed throughout the cell and the majority of the T being restricted to the nucleus. Figures 9B and 9D, however, illustrate that while the D₂O tissue does take up the T and sequester it in the nuclei of its cells, it does not take up appreciable amounts of U. The effects of three cold (23°C) and one hot (80°C) T.C.A. washings, presented in Figure 10, illustrate that the 23°C washings rinsed out the unincorporated T and the hot wash released into the bulk solution the labeled T that had been incorporated into DNA.

The pattern of amino acid incorporation into protein in both H₂O- and D₂O-grown plants is presented in Figures 11 and 12. The counts/minute reflect the incorporation of ³H-leucine (2) or ³H-phenylalanine (3) into a nondialyzable, macromolecule that

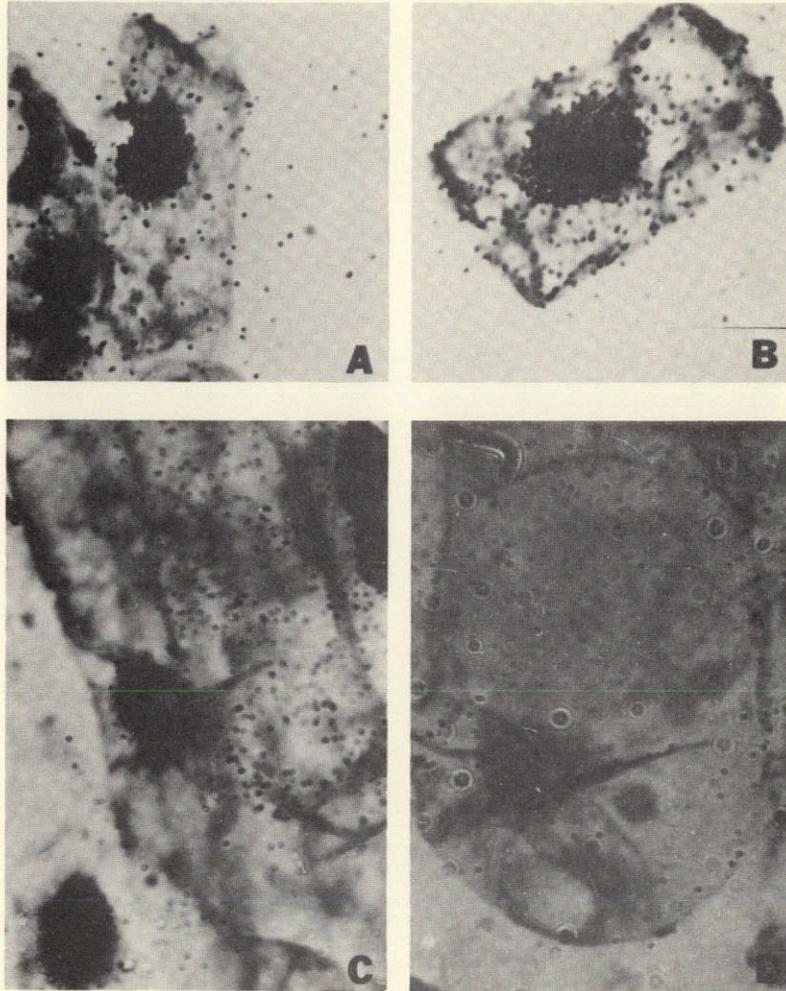


Figure 9. A and B: Radioautograph of root cells cultured for approximately 2 days (H_2O , A) and 9 days (D_2O , B) in the presence of 3H -thymidine. After development of the autographs, the cells were stained with a 0.05% solution of toluidine blue-0. The vast majority of the thymidine activity is restricted to the nuclear area of both cell types.

C and D: Radioautograph of root cells cultured for approximately 2 days (H_2O , C) and 9 days (D_2O , D) in the presence of 3H -uridine. After development of the autographs, the cells were stained with 0.05% toluidine blue-0. The uridine label is generally distributed through the cell of the H_2O cultured plant. The D_2O cell, on the other hand, has only background quantities of the label.

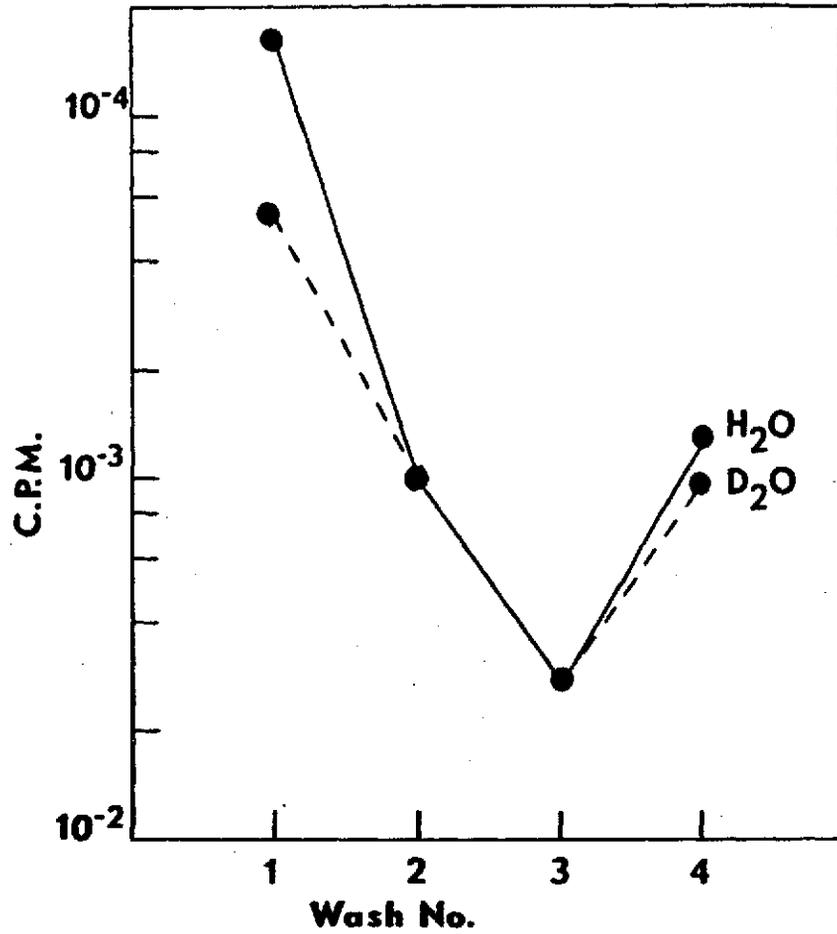


Figure 10. The effect of three cold (23°C) followed by one hot (90°C) TCA wash on H_2O and D_2O cultured tissue grown in the presence of ^3H -thymidine. Counts per minute (C.P.M.) of ^3H activity present in 0.1 ml of the washing solution are plotted against the number of sequential washes.

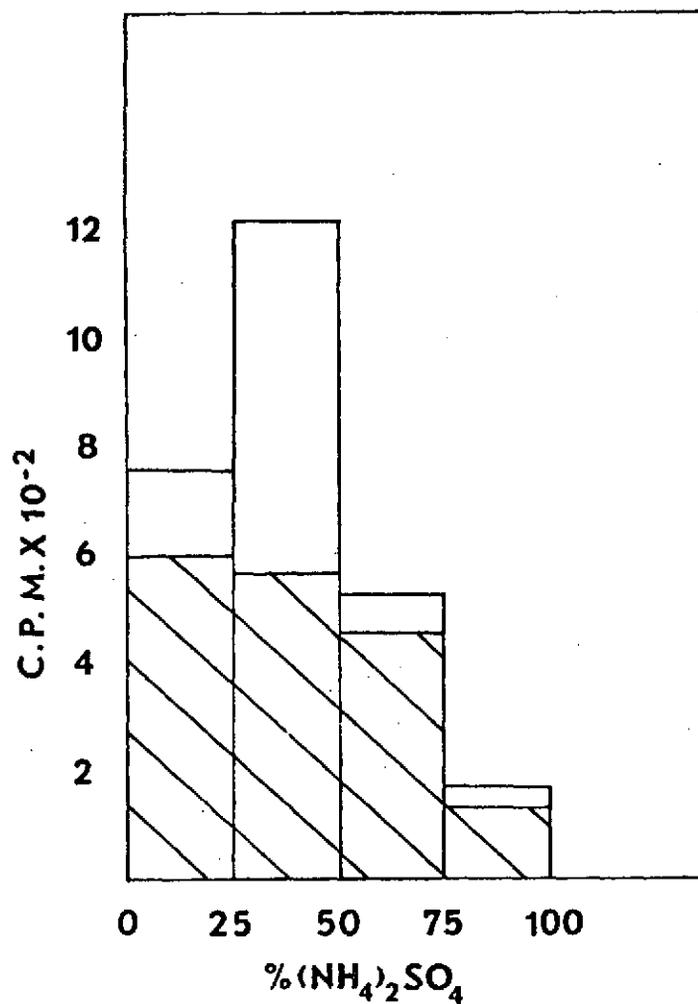


Figure 11. The incorporation of ³H-leucine into various protein fractions is plotted. The fractions were obtained by precipitating the proteins from the original solution with (NH₄)₂SO₄. The activity in these fractions (C.P.M.) was determined by redissolving the precipitated protein in 10 ml of buffer and then counting 0.1 ml aliquots of this solution. The incorporation peak present in the H₂O (□) cultured material is not present in the D₂O (▨) cultured plants.

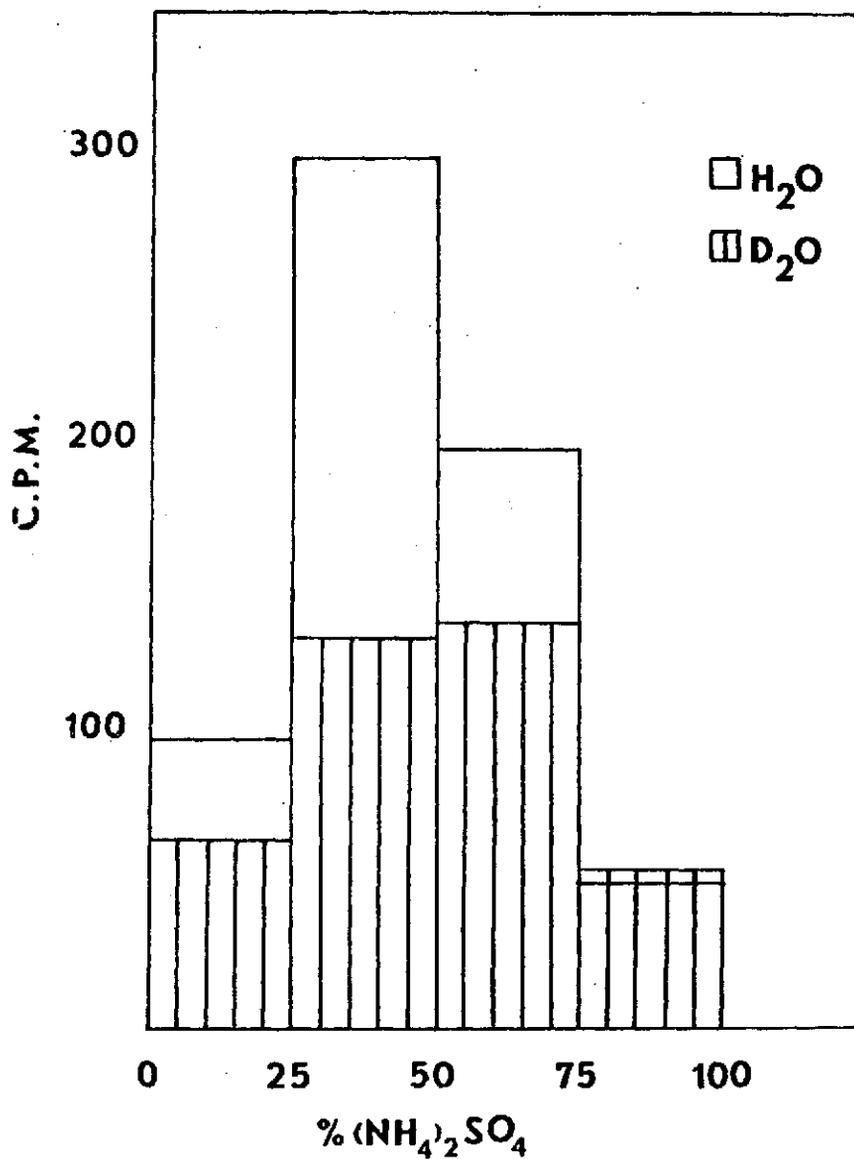


Figure 12. The incorporation of ^3H -phenylalanine (GL) into various protein fractions is illustrated. The fractions were obtained by precipitating the proteins from the original solution with $(\text{NH}_4)_2\text{SO}_4$. The activity in these fractions (C.P.M.) was determined by redissolving the precipitated protein in buffer and then counting 0.1 ml aliquots of this solution. The incorporation peak present in the H_2O (\square) cultured material is not present in the D_2O (\blacksquare) cultured tissue.

precipitates from solutions of the indicated % saturation with ammonium sulfate, presumably protein. As these figures illustrate, the pattern of incorporation for the two types of seedlings is very different. The D_2O plants lack the peak of incorporation in the 25-50% fraction that is exhibited by the H_2O -grown tissue. D_2O -grown tissue did not seem to incorporate a substantial portion of the labeled leucine or phenylalanine into any particular fraction. A calculation of the % inhibition of both leucine, phenylalanine and some preliminary results with proline incorporation into protein by D_2O is presented in Table 4.

The above results could be interpreted in terms of an interference in protein synthesis by D_2O . Therefore, a comparison of the peroxidase activity and protein content of dry embryos, H_2O - and D_2O -grown tissues was done to determine whether an effect on protein synthesis could be detected in the amount of protein and peroxidase activity of the various tissues. The results of this comparison are presented in Table 5. From this table it can be seen that the specific activity of peroxidase does not vary from tissue to tissue. However, if this activity is converted to μgm of peroxidase/plant or μgm of peroxidase/ μgm of protein, the lower amount of peroxidase in the D_2O cultured tissue is very evident. From these data it can be seen that the protein content of the D_2O -grown tissue is also much lower than the control value. The percent inhibition of Lorwy positive protein synthesis caused by D_2O culture is presented in Table 4.

Table 4

Inhibition of protein synthesis
in D₂O cultured plants

	% Inhibition
Lowry Protein	65
³ H-Leucine	60
¹⁴ C-Proline	49
³ H-Phenylalanine	50

Table 5

Peroxidase activity in H₂O- and D₂O-grown tissue

	Dry Embryo	H ₂ O-Rye	D ₂ O-Rye
Δ O.D./min./ μ gm \pm S.E.M.	$5.02 \pm 0.87 \times 10^{-2}$	$3.9 \pm 0.3 \times 10^{-2}$	$1.72 \pm 0.6 \times 10^{-2}$
μ gm/plant	44	78	16
μ gm peroxidase/ μ gm protein	3.4×10^{-1}	3.8×10^{-1}	1.4×10^{-1}
μ gm protein/plant \pm S.E.M.	130 ± 0.4	316 ± 11	111 ± 6.5

Nucleolar volume and the number of cisternae/dictyosome have recently been reported to reflect protein synthesis inhibition (4,9). A lower number of cisternae/dictyosome and an abnormally small nucleolar volume have been correlated with the inhibition of protein synthesis. In conjunction with an extensive electron microscopic analysis of H₂O- and D₂O-grown rye, the nucleolar volume and the number of cisternae/dictyosome for H₂O- and D₂O-grown seedlings was determined. In the D₂O-grown tissue the nucleolar volume and the number of cisternae/dictyosome were smaller than the comparable values for the water grown controls (Table 6).

Discussion

Most higher organisms, both plant and animal, cannot tolerate high concentrations of D₂O in their environment (3). The basis for this toxic effect of high concentrations of D₂O has been the object of many investigations over the years (e.g. 2,3,5 and 8). These investigations have resulted in two models that attempt to explain the biological effects of essentially complete (99.6%) deuteration. The first of these is the so-called "kinetic isotope theory", which explains the effect of D- for H-substitution in terms of the difference in E_a of corresponding H- and D-compounds. Support for this theory can be found in the large number of papers that report the magnitude of this effect on several enzymes, and it is

Table 6

Comparison of number of cisternae/dictyosome and nucleolar volume in cells from H₂O- and D₂O-grown plants

	Cisternae/Dictyosome (P = 0.005)	Nucleolar Volume (P = 0.08)
H ₂ O-grown tissue	5.6 ± 0.2	8.8 ± 1.8 μ ³
D ₂ O-grown tissue	4.0 ± 0.4	5.5 ± 2.4 μ ³

true as Katz states that "so complex are the interrelationships of the reactions that proceed concurrently and sequentially in living systems that even relatively minor perturbations in reaction rate may have far-reaching consequences for an organism" (8). However, the adaptability of biological systems should not be minimized. The fact that no enzyme has been found to be completely inhibited (*in vitro*) in D_2O leaves open the possibility that an organism could adjust to growth in a deuterium rich environment if the only problem to be overcome was one of kinetics.

The alternate theory that explains the biological effects of D_2O is less well defined than the former and has not been thoroughly investigated. This hypothesis suggests that D_2O in some way affects the normal operation of the DNA-RNA-Protein complex (5,6), and that this may explain the inability of most organisms to tolerate deuterium rich environments.

The data presented in this paper supports the second theory, and suggests a new answer to the question of the mechanism of D_2O toxicity in higher plants. The hypothesis proposed is that the replacing of H_2O with D_2O affects the DNA-RNA-Protein complex within the organism with the result that protein synthesis is partially inhibited. This hypothesis is supported by four pieces of evidence.

First, the low protein content of the seedlings grown in D_2O plus their failure to incorporate substantial amounts of either leucine or phenylalanine into any particular protein fraction is

indicative of a defect in the protein synthesizing machinery. Indeed, the average % inhibition of protein synthesis in the D₂O tissue as measured by both labeled amino acid incorporation and Lowry determinations is 56 ± 4%.

The T- and U-uptake studies suggest a possible mechanism for the observed inhibition. The lack of substantial U-uptake in D₂O-grown tissue suggests a low demand for this compound. A low level of RNA synthesis in the D₂O tissue is thus implied, and a low level of RNA synthesis could explain the lack of protein synthesis in the D₂O-grown seedlings. It is possible, however, that the U is not entering the cells. But T and U are both pyrimidines, and there is no reason to believe that the cells are permeable to one and not the other, although this is a possibility that cannot be rigorously excluded. However, it is felt that U is not being taken up because it is not needed, due to a low level of RNA synthesis in the D₂O-grown tissue. Thymidine, on the other hand, is readily incorporated into DNA. Indicating that the synthesis of DNA is not blocked in winter rye as it apparently is in onion roots cultured in 90% D₂O (1).

The peroxidase activity and protein content measurements also support the hypothesis that protein synthesis in D₂O is partially inhibited. The D₂O cultured tissue has deficiencies in the amount of peroxidase and Lowry positive protein suggesting that protein synthesis is at least partially inhibited in the D₂O-grown plants.

Finally, the quantitative evidence from electron microscopy also supports the idea that protein synthesis is inhibited. The lower number of cisternae/dictyosome and the smaller nucleolar volume in D_2O -grown tissue correlates well with the results obtained by other workers using synthetic protein inhibitors such as cycloheximide (4,9). Although the relatively small sample size resulted in a calculated probability of approximately 0.08 for the nucleolar volume difference, the observed difference is an indication that, in D_2O -grown cells, the nucleolar is abnormally low.

It is interesting to note, that while the number of cisternae/dictyosome and the nucleolar volume do support the protein synthesis hypothesis, the overall ultrastructural appearance of the D_2O -grown tissue is, except for some small differences, normal. This normal appearance is even more remarkable when it is considered that only about 40% of normal amount of protein is present in the D_2O -cultured plants.

The accumulated evidence presented here, when taken together, suggests an explanation of the toxicity of D_2O to winter rye. That explanation being, that high concentrations of D_2O affect the functioning of the DNA-RNA-Protein complex of winter rye with the result that protein synthesis is partially blocked. The exact position and effectiveness of this block is not known at this time. The T and U uptake studies suggest that RNA metabolism is in some way

involved, as previously suggested by Henderson (5,6) although it has been reported (7) that the *de novo* synthesis of isocitrate lysase occurs in watermelon cotyledons grown in 80% D₂O.

In conclusion, culture in 99.6% D₂O partially inhibits protein synthesis in winter rye. It is suggested that this type of inhibition at least partially explains the toxicity to higher life forms of high concentrations of D₂O.

Literature cited

1. Bal, A. K. and P. R. Gross. 1964. Suppression of mitosis and macromolecule synthesis in onion roots by heavy water. *J. Cell Biol.* 23:188-193.
2. Bhandarkar, M. K., S. Bhattacharya (nee Bose) and B. K. Gaur. 1971. Physiological studies with heavy water. II. Germination and growth inhibition of barley by D₂O. *Physiol. Plant.* 24: 517-521.
3. Flaumenhaft, E., S. Bose, J. L. Crespi and J. J. Katz. 1965. Deuterium isotope effects in cytology. *Int. Rev. Cytol.* 18: 313-361.
4. Fleckinger, C. H. J. 1971. Alteration in the golgi apparatus of amebae in the presence of an inhibitor of protein synthesis. *Exp. Cell Res.* 71:381-387.
5. Henderson, T. R. 1962. Differential inhibition of beta-galactosidase induction and synthesis by deuterium oxide. *Biochem. and Biophys. Res. Commun.* 9:240-245.
6. Henderson, T. R. and M. R. Lamonds. 1966. Effects of D₂O on citric acid and pyruvate carboxylase formation by *Aspergillus niger*. *Arch. Biochem. Biophys.* 115:187-189.
7. Höck, B. 1970. Die zytliche Dauer der isocitrate-lysase synthese in kotyledonen von Wassermelonenkeimlingen. *Planta.* 93:26-38.
8. Katz, J. J. 1960. Chemical and biological studies with deuterium. *Amer. Sci.* 48:544-580.
9. King, B. and J. M. Chapman. 1972. The effect of inhibitors of protein and nucleic acid synthesis on nucleolar size and enzyme induction in Jerusalem Artichoke tuber slices. *Planta.* 104: 306-315.
10. Lewis, G. N. 1933. The biochemistry of water containing hydrogen isotope. *J. Amer. Chem. Soc.* 55:3503-3504.

11. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
12. Siegel, B. Z. and A. W. Galston. 1966. Biosynthesis of deuterated isoperoxidases in rye plants grown in D₂O. *Proc. Natl. Acad. Sci. U.S.A.* 56:1040-1042.
13. Siegel, S. M., L. A. Halpern and C. Giunarro. 1964. Germination and seedling growth of winter rye in D₂O. *Nature* 201:1244-1245.

THE EFFECT OF D₂O ON THE PERMEABILITY OF
BEET ROOT MEMBRANES TO BETACYANIN

Abstract

The effect of D₂O on the permeability of beet root membranes to betacyanin was investigated. The rate of pigment efflux was significantly greater in a 99.6 percent D₂O environment than in H₂O. The enhanced rate of leakage, evident at all D₂O concentrations greater than 65 percent, was temperature sensitive and Ca⁺⁺ retardable. Cycloheximide and puromycin had similar effects on pigment efflux. It was concluded, therefore, that the disruption of the beet root membranes in D₂O was due to a blockage of membrane protein turnover caused by at least a partial inhibition of protein synthesis in the D₂O incubated tissue.

Introduction

The discovery of deuterium oxide (D_2O) in 1932 stimulated much research on the biological consequences of substituting D_2O for H_2O , which suggested that life processes were inhibited in essentially 100 percent D_2O . However, in 1960, Chorney *et al.* (2) reported the successful culturing of a number of species of green algae in 99.6 percent D_2O . Since that time, a number of other "lower" organisms such as bacteria, fungi and protozoa have been cultured in 99.6 percent D_2O environments (5). Higher plants, however, appeared incapable of tolerating D_2O concentrations of greater than approximately 70 percent, until 1964, when Siegel *et al.* (12) reported that seeds of the grass winter rye were able to germinate and grow in 99.6 percent D_2O . The growth was not vigorous and indeed the plants died shortly after germination. Thus winter rye was only partially successful in overcoming the toxic effect of high concentrations of D_2O . This toxicity is the subject of the present paper.

With the exception of O'Brien's report (8) on potassium, phosphate and glucose uptake in yeast cells placed in D_2O , the effect of D_2O on an intact, functional biological membrane has not been investigated. Since the strength of intermolecular hydrogen and hydrophobic bonds is greater in D_2O than in H_2O (7), the activity of a membrane in D_2O could be quite different from that of a membrane in an H_2O medium.

Therefore, this study was initiated to determine if there was a difference in membrane activity in D_2O , and if this difference could partially explain the toxic effects of D_2O on biological systems.

Methods and Materials

Washed root sections of the red beet (*Beta vulgaris* L.) can be maintained under simple experimental conditions for several days with little leakage of the vacuolar pigment betacyanin. The rate of betacyanin efflux from the washed sections has been demonstrated by Siegel and his co-workers (10, 11) to reflect the status of the beet root membrane. This system has proven useful in the study of permeability regulators and responds classically to monohydric alcohols. Because this system has been extensively characterized, and is technically convenient, it was chosen as the object of this investigation.

Roots of the red beet were cut and sliced into discs approximately 2 mm x 10 mm and then washed in running tap water overnight. For experiments using D_2O , the washed sections were equilibrated in D_2O for a total of 60 minutes, with fresh D_2O being added after the first 30 minutes. Equilibrium was assumed to have occurred, when the beet root discs sank in the D_2O . For the H_2O experiments, the washed discs were used directly without equilibration.

The experimental system consisted of 5 or 10 ml of distilled water (control) or D_2O with 5 or 10 beet discs respectively.

Betacyanin efflux was followed using a B/L Spectronic 20 spectrophotometer set at 540 nm. Optical density (O.D.) readings beyond the range of the instrument were obtained from suitably diluted portions of the media. Absolute O.D. readings varied from experiment to experiment since the efflux from the control tissue varied from root to root. However, in each experiment the treatments were duplicated or triplicated and repeated at least four times; although the absolute O.D. readings changed, the relationships between the different treatments did not.

Where noted, the efflux from 15 beet discs in 10 ml of test solution was followed with a Beckman DBG Dual Beam Spectrophotometer if particularly low level measurements were to be made.

The effect of 3 percent butanol and 10 percent propanol, temperature, pH and pD, and Ca ion, on the rate of betacyanin efflux from beet root discs in H₂O and D₂O was tested. The various pH's and pD's were obtained by adjusting solutions of H₂O and D₂O with concentrated HCl.

The effect of cycloheximide (50 µgm/ml) and puromycin (100 µgm/ml) on pigment efflux was also investigated.

Results

Beet root discs incubated in D_2O at $25^\circ C$ immediately began to leak betacyanin at a rate significantly greater than that of the water control (Figure 13). The increased rate of leakage is evident at temperatures ranging from 7 to $45^\circ C$ ($Q_{10} \approx 4.4$). The enhanced rate of betacyanin efflux was found to be Ca^{++} retardable (Figure 14).

Leakage induced by a 10 percent solution of n-propanol was found to be greater in D_2O than in H_2O (Figure 15). A 3 percent solution of n-butanol, however, did not cause a more rapid efflux of the pigment from the D_2O incubated sections until after 40 minutes (Figure 16). Alcohol induced leakage, was always greater in D_2O , although there was leakage in both solvents due to the alcohol itself.

The effect of various pH's and pD's on the rate of pigment efflux in H_2O and D_2O respectively, was also studied (Figure 17). The rate of efflux did not depend on the composition of the bulk solvent, indicating that D_2O did not appreciably alter the ability of the beet root tissue to respond to environments of initially low pH or pD.

The threshold of this D_2O effect, that is the concentration of D_2O which caused an appreciable increase in the rate of betacyanin efflux from the beet tissue was determined. Figure 18 shows that at D_2O concentrations greater than 65% (v/v) pigment efflux from the tissue is appreciably greater than that of the H_2O control. This particular experiment was performed at $35^\circ C$, thus allowing the efflux to be studied over a convenient period of time.

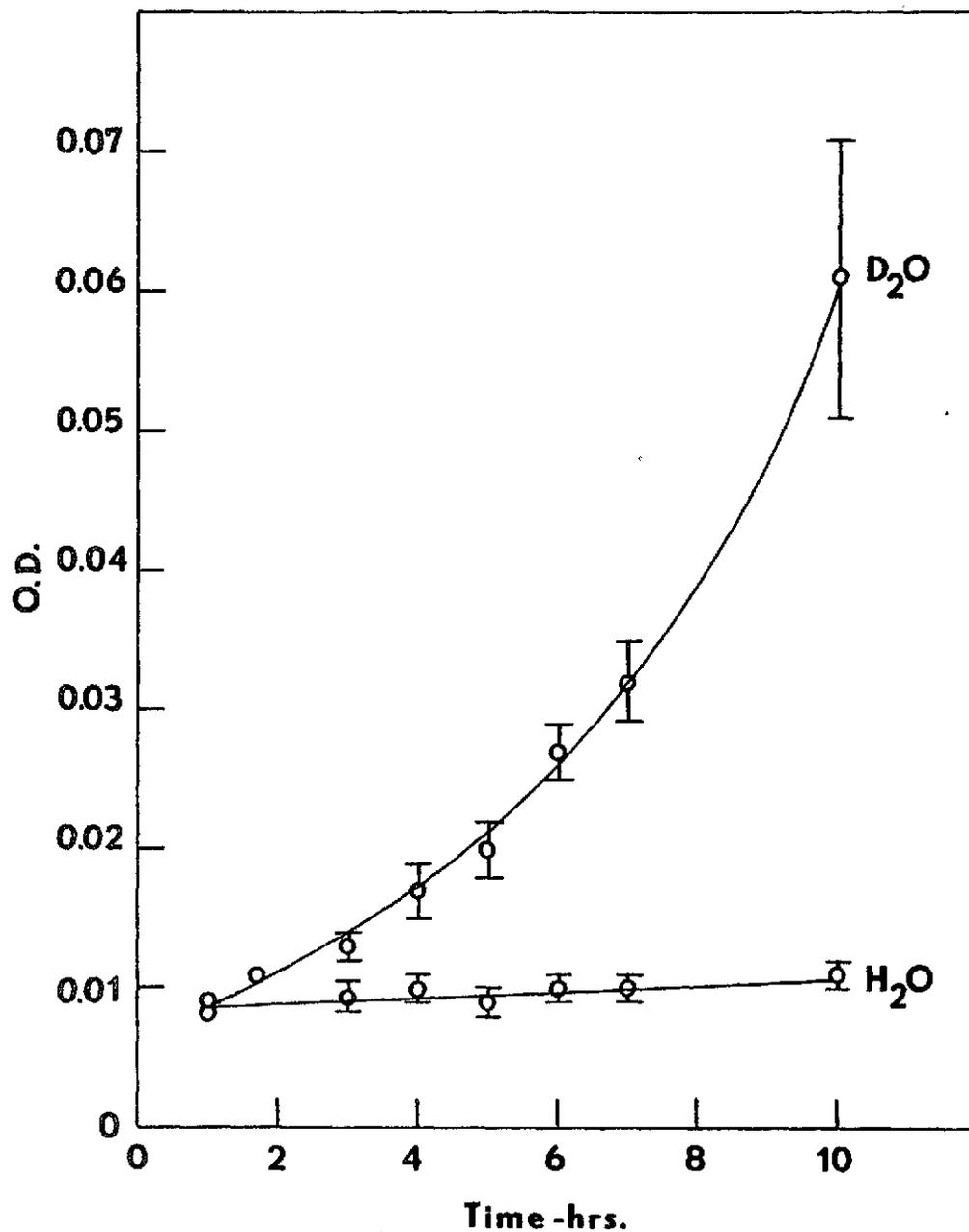


Figure 13. Effect of 99.6 percent D₂O on the permeability of beet root membranes to betacyanin at 25°C. The enhanced rate of pigment efflux is evident at all temperatures below 45°C. Low level measurements were made with Beckman DBG Spectrophotometer. The vertical bars represent the standard error of the mean.

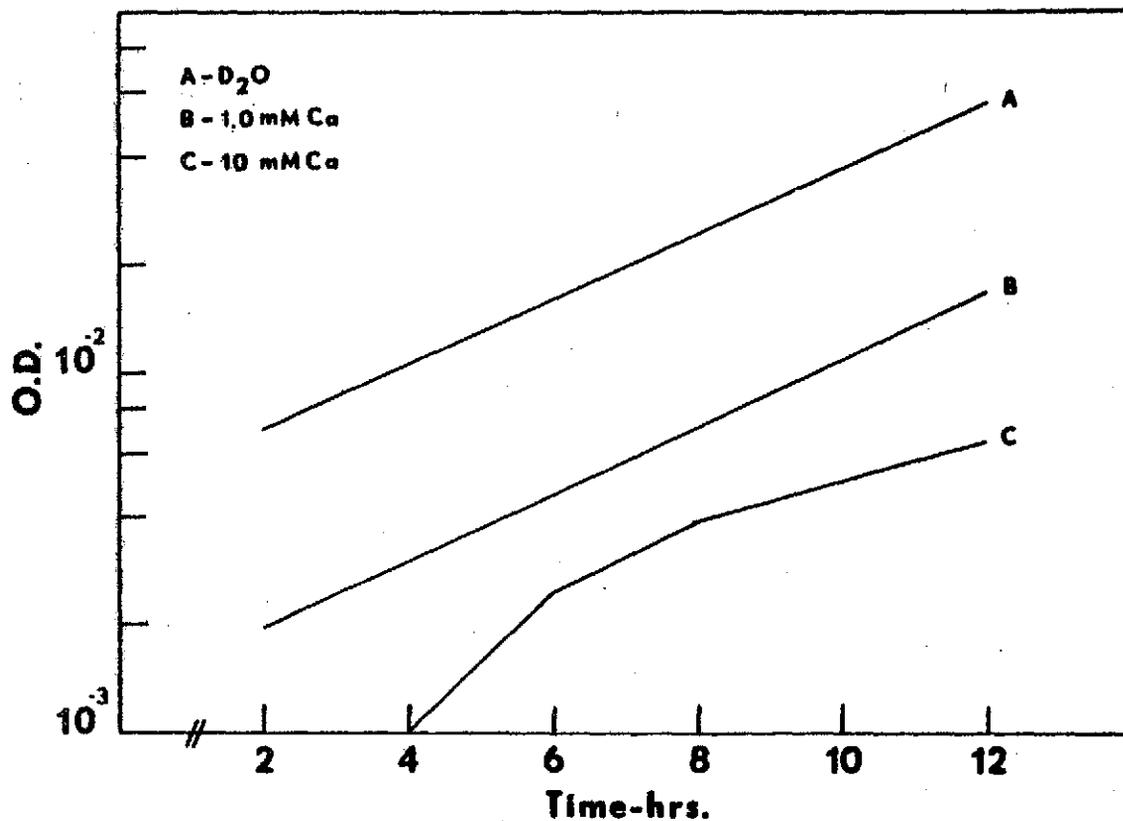


Figure 14. Modification of the D_2O effect (Figure 13) by exogenously supplied Ca^{++} . Standard regression techniques were used to determine the equation of the line for the various treatments. The equation, n value, correlation coefficient and p value for each line are as follows:

$$A: y = 0.0005e^{0.196X}, \quad n = 15, \quad r = 0.92, \quad p < 0.01$$

$$B: y = 0.001e^{0.212X}, \quad n = 15, \quad r = 0.73, \quad p < 0.01$$

$$C: y = 0.001x + 0.0006, \quad n = 15, \quad r = 0.74, \quad p < 0.01.$$

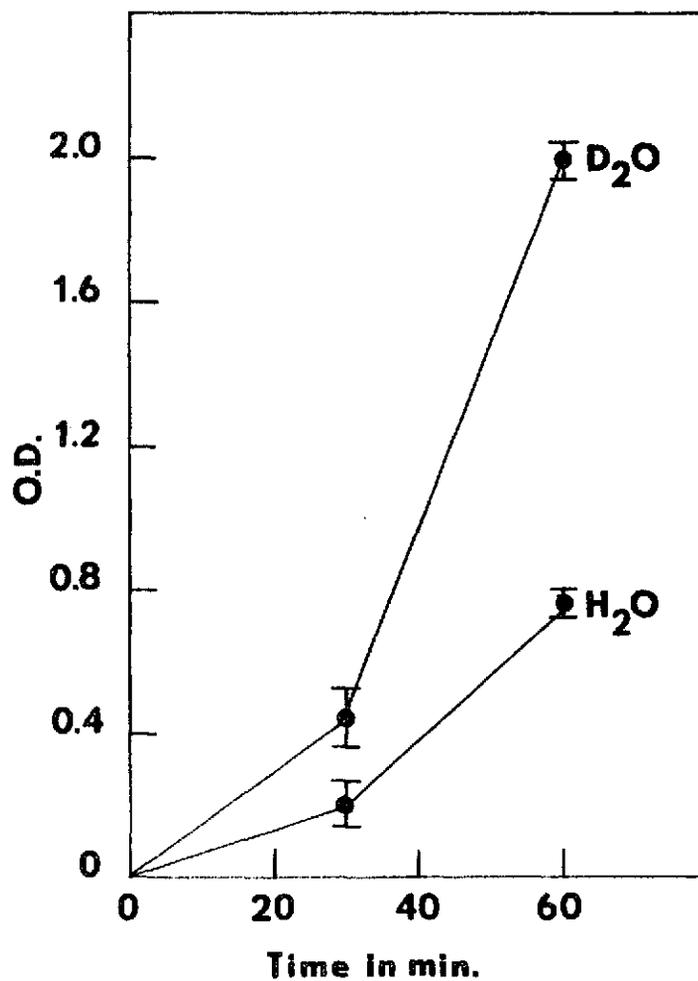


Figure 15. The effect of 10 percent n-propanol on the rate of betacyanin efflux in H₂O and D₂O incubated tissue at 25°C. Leakage was always greater in D₂O, although there was leakage in both solvents due to the alcohol itself. The vertical bars represent the standard error of the mean.

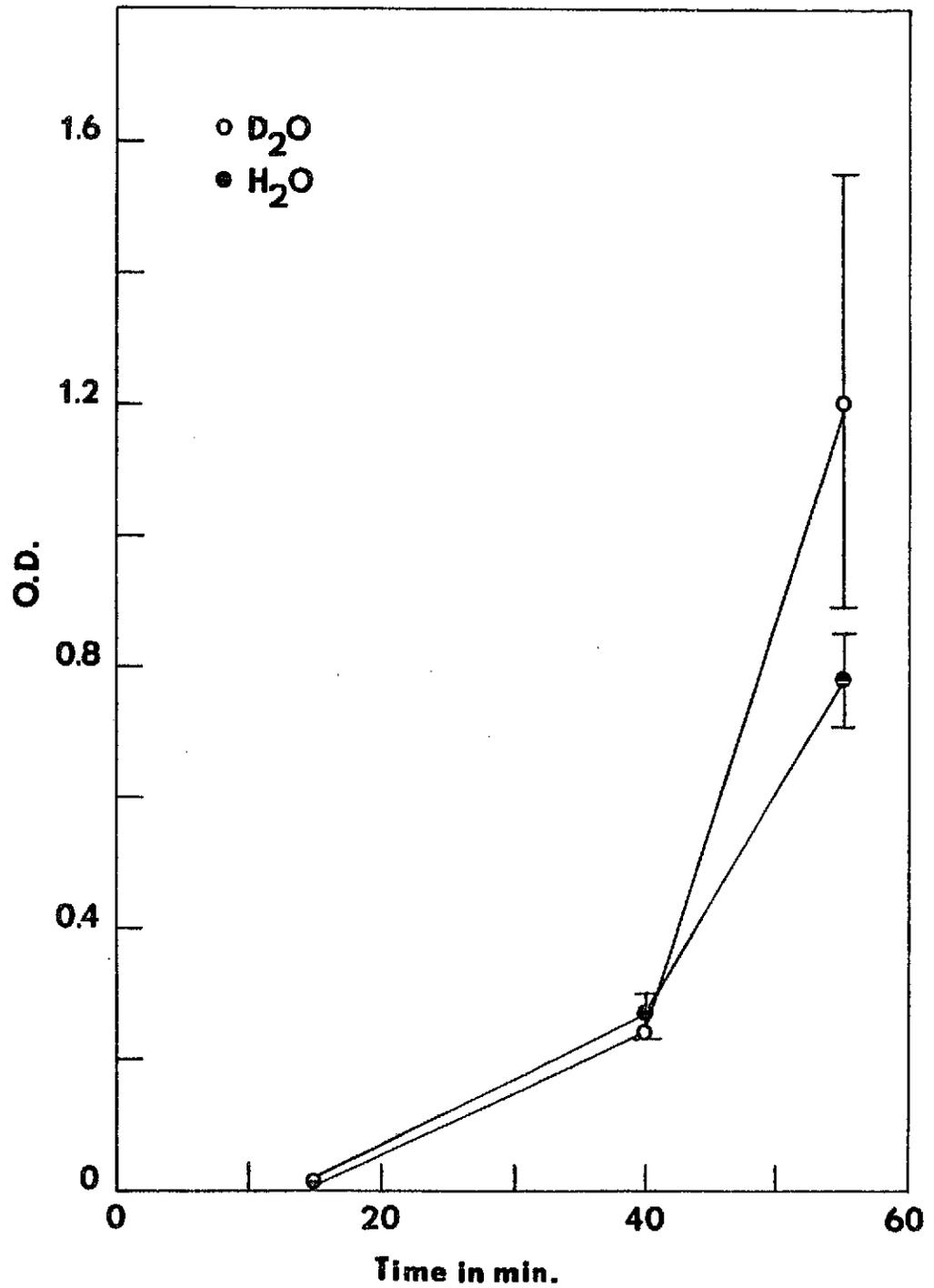


Figure 16. The effect of 3 percent n-butanol on the rate of betacyanin efflux in H₂O and D₂O incubated tissue at 25°C. Leakage was always greater in D₂O, although there was leakage in both solvents due to the alcohol itself. The vertical bars represent the standard error of the mean.

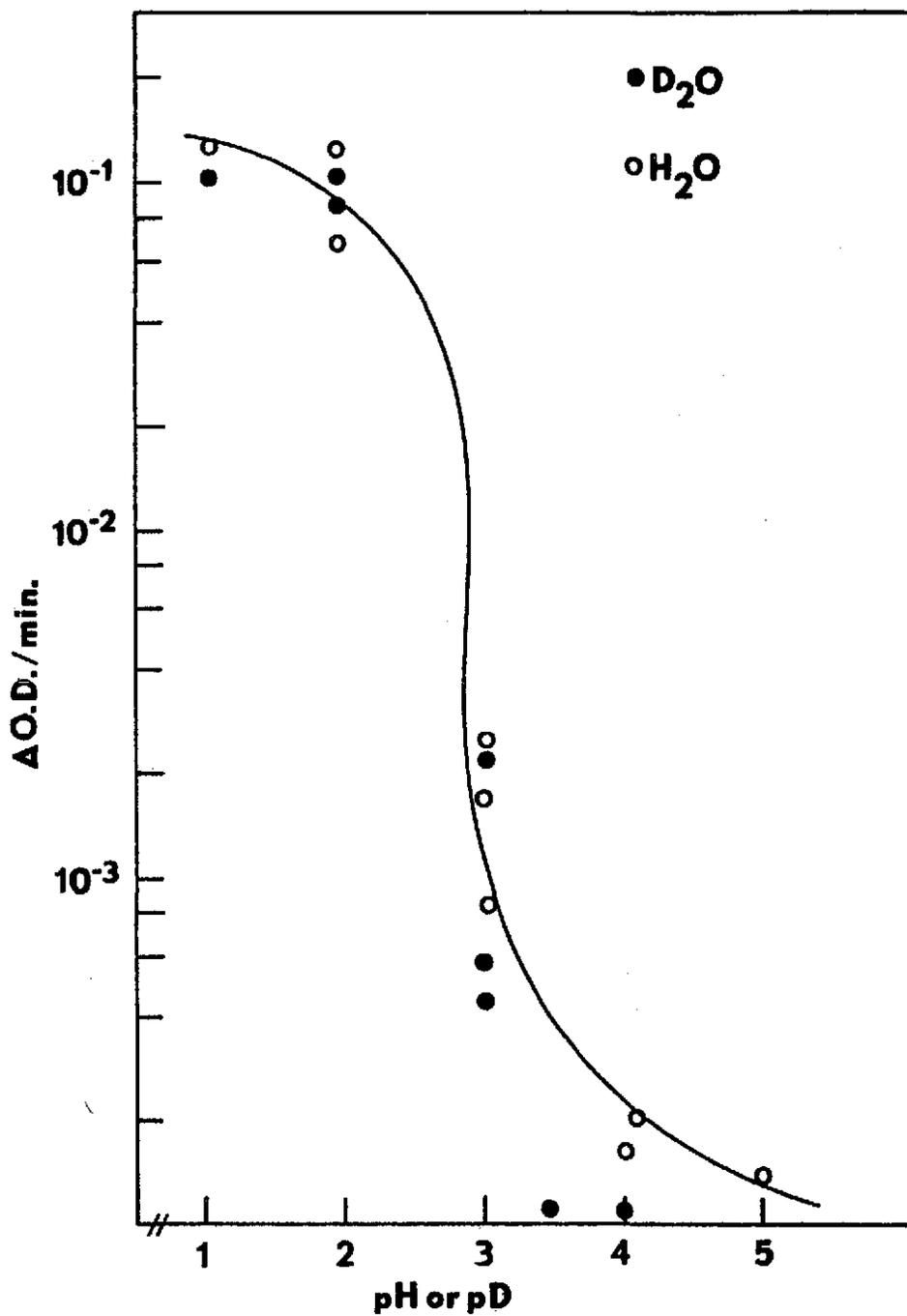


Figure 17. The effect of various pH's and pD's, obtained by the addition of concentrated HCl, on the rate of betacyanin efflux from beet root tissue maintained at 25°C. The rate of efflux did not depend on the composition of the bulk solvent.

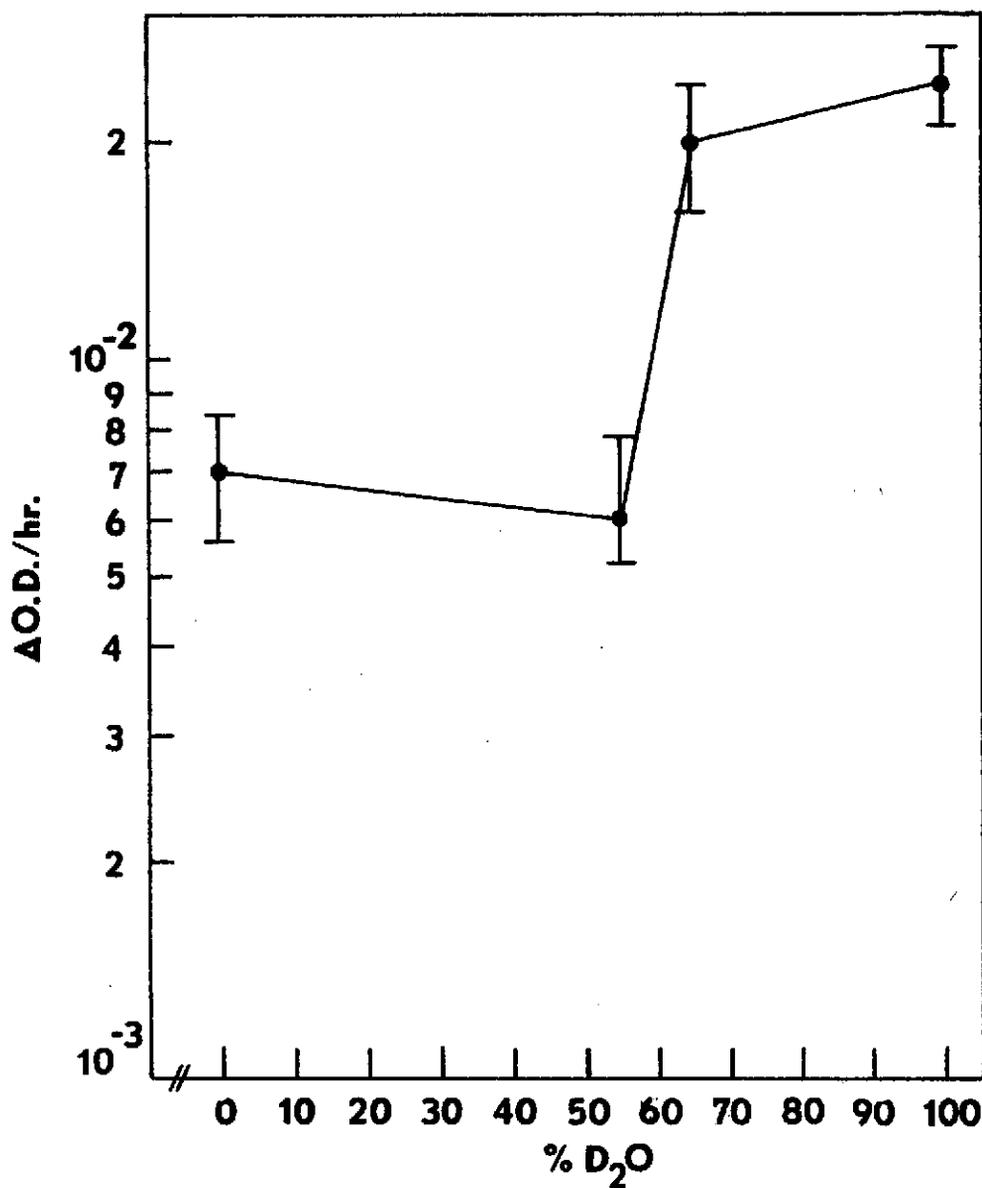


Figure 18. The effect of 0, 55, 65, and 100 percent D₂O on the rate of betacyanin efflux from beet discs maintained at a temperature of 35°C. At D₂O concentrations greater than 65 percent, pigment efflux from the tissue is appreciably greater than that of the water control. Incubation at 35°C allowed the experiment to be studied over a convenient period of time. The vertical bars represent the standard error of the mean.

The effect of cycloheximide (50 $\mu\text{gm/ml}$) and puromycin (100 $\mu\text{gm/ml}$) on beet root discs is presented in Figures 19 and 21 respectively. In both cases, the exponential leakage induced by these chemicals was found to be Ca^{++} retardable (Figures 20 and 22), although cycloheximide induced leakage was more sensitive to exogenously supplied Ca^{++} .

Discussion

O'Brien (8), in a study of the uptake of phosphate, glucose and potassium in yeast cells, found that D_2O seemed to be inhibiting the activity of the yeast cell membrane. The present data confirm and expand this theme. The enhanced rate of pigment leakage in D_2O reflects a detrimental effect of D_2O on the beet root membrane.

There are two possible explanations for the effect of D_2O on excised beet tissue. High concentrations of D_2O in the environment may have a direct and adverse effect on the beet root membranes. Alternatively, by affecting some metabolic process essential for membrane maintenance, D_2O could secondarily cause membrane deterioration and hence pigment efflux. Since the strength of intermolecular hydrogen and hydrophobic bonds is greater in D_2O than in H_2O (7), it was initially thought that D_2O was directly and adversely affecting the beet root membranes. The lack of any detectable lag in the efflux from the D_2O incubated tissue (Figs. 13, 14, 15 and 16) suggested that hydrophobic regions of the membrane were affected by D_2O (14). However,

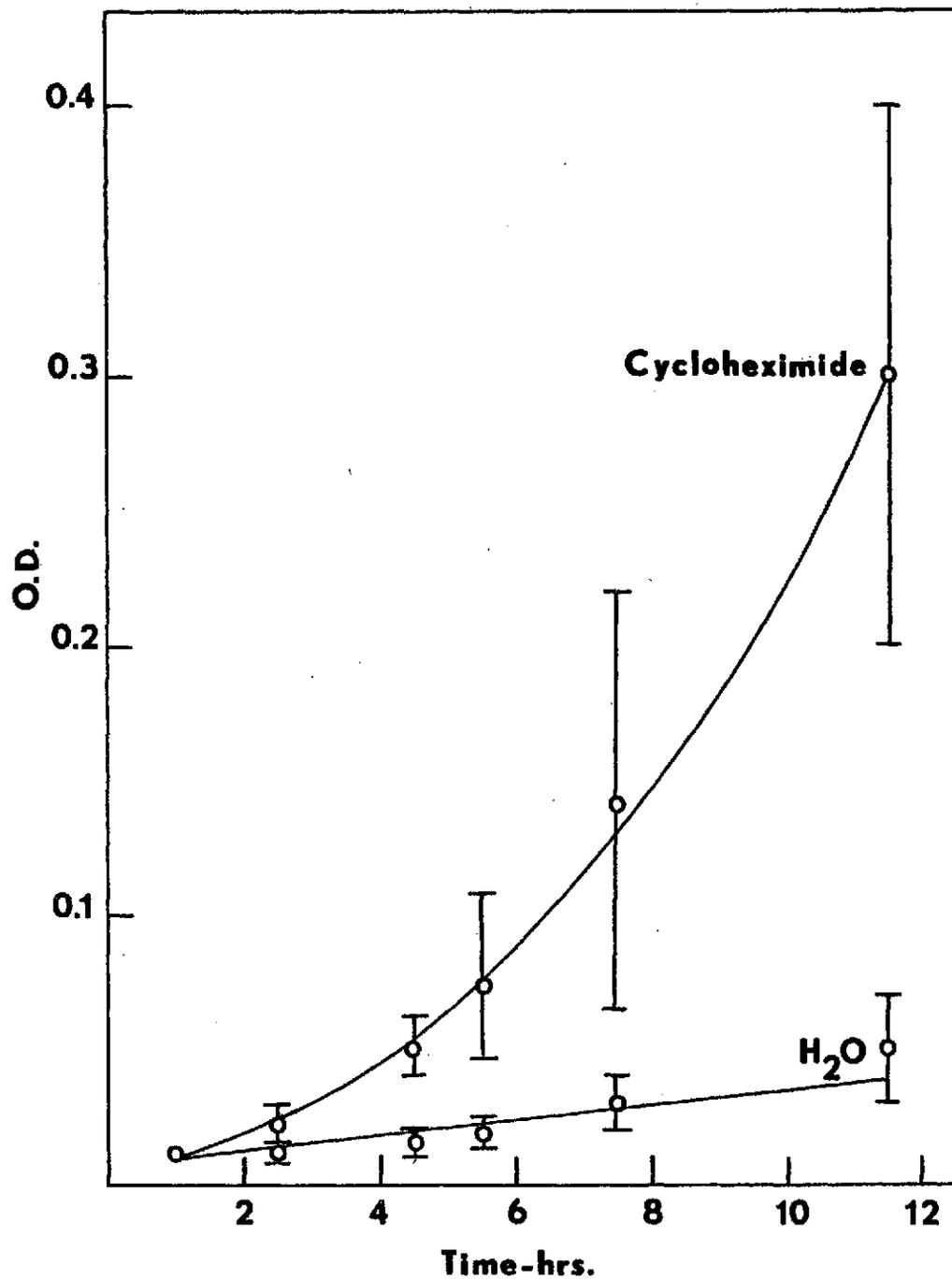


Figure 19. The effect of cycloheximide (50 $\mu\text{g}/\text{ml}$ of H_2O) on beta-cyanin efflux from beet root tissue at 25°C . Low level measurements were made on a Beckman DBG Spectrophotometer. The vertical bars represent the standard error of the mean.

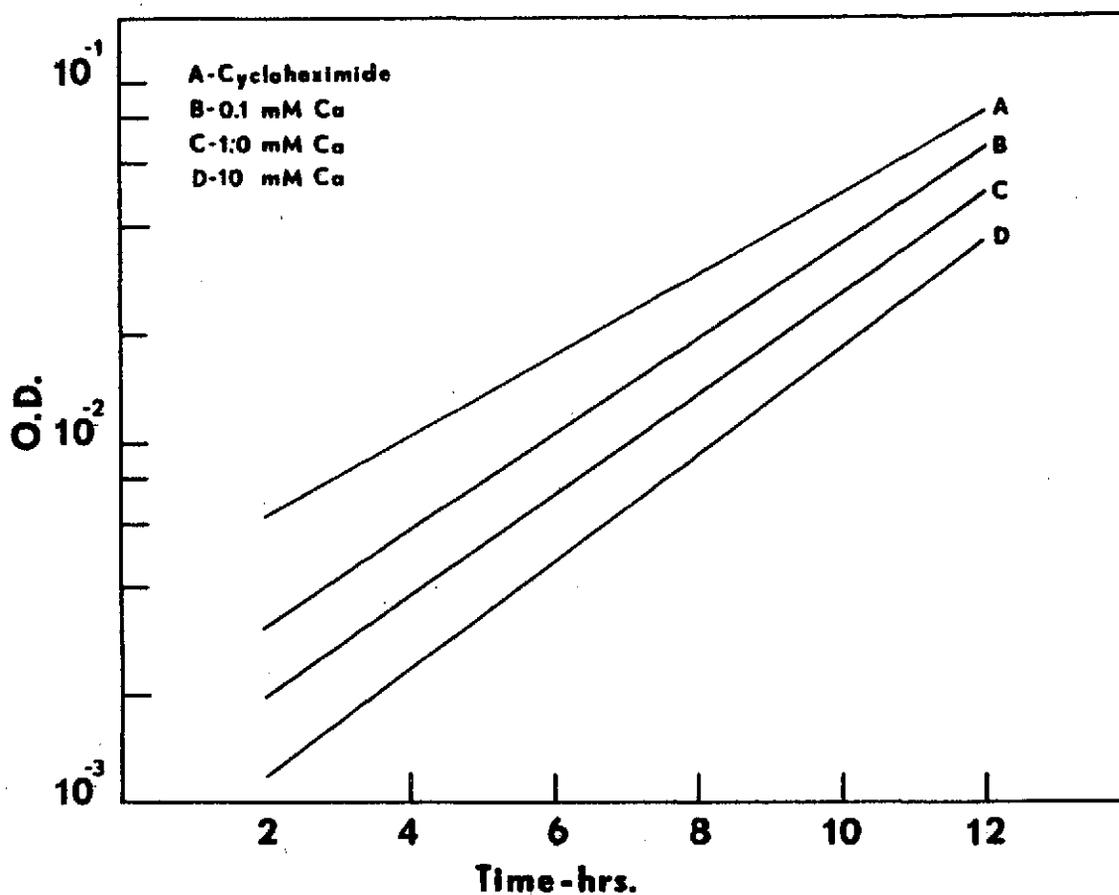


Figure 20. Modification of the cycloheximide effect (Figure 19) by exogenously supplied Ca^{++} . Standard regression techniques were used to determine the equation of the line for the various treatments. The equation, n value, correlation coefficient, and p value for each line are as follows:

A: $y = 0.004e^{0.253x}$, $n = 18$, $r = 0.98$, $p < 0.01$
 B: $y = 0.002e^{0.297x}$, $n = 18$, $r = 0.99$, $p < 0.01$
 C: $y = 0.001e^{0.317x}$, $n = 18$, $r = 0.99$, $p < 0.01$
 D: $y = 0.0006e^{0.342x}$, $n = 18$, $r = 0.99$, $p < 0.01$.

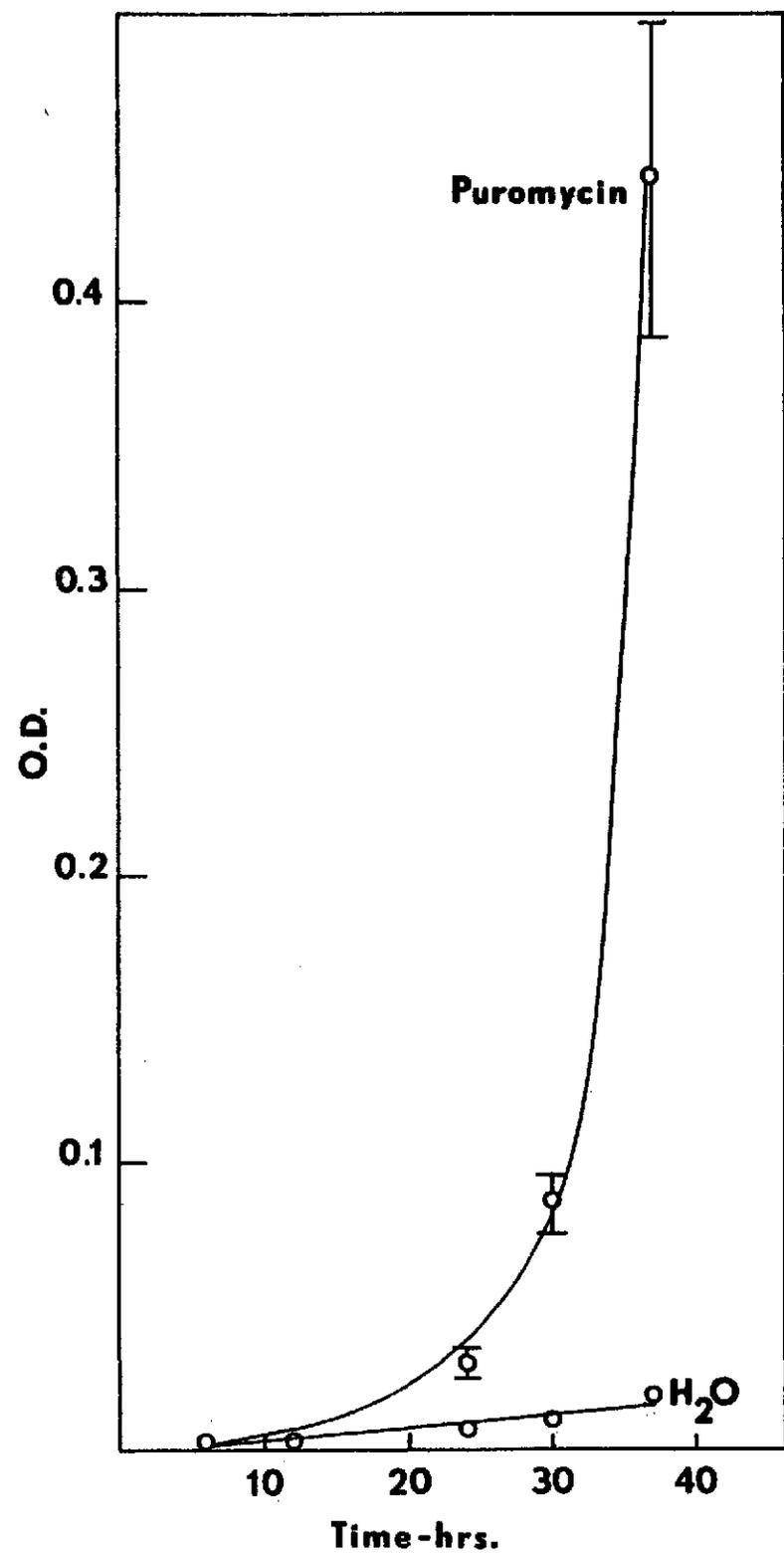


Figure 21. The effect of puromycin (100 $\mu\text{gm/ml}$) on betacyanin efflux from beet root tissue at 25°C. The vertical bars represent the standard error of the mean.

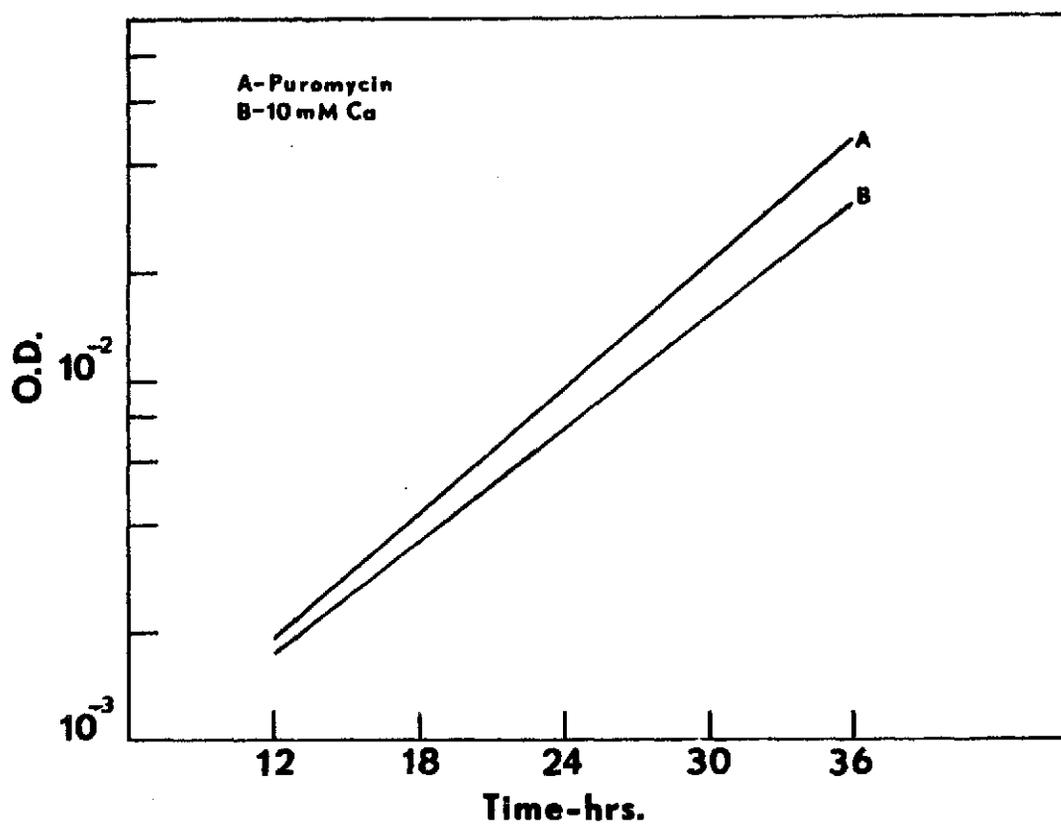


Figure 22. Modification of the puromycin effect (Figure 21) by Ca^{++} . Standard regression techniques were used to determine the equation of the line for the various treatments. The equation, n value, correlation coefficient and p value for both lines are as follows:

$$\text{A: } y = 0.0004e^{0.132X}, \quad n = 16, \quad r = 0.97, \quad p < 0.01$$

$$\text{B: } y = 0.0004e^{0.12X}, \quad n = 16, \quad r = 0.98, \quad p < 0.01.$$

D₂O partially inhibits protein synthesis in winter rye seedlings (Page 50), and therefore, the effect of cycloheximide and puromycin, specific inhibitors of protein synthesis (9), on beet root tissue was investigated. It was found that both compounds cause an exponential efflux of the membrane bound pigment (Figures 19 and 21). As with D₂O, leakage induced by cycloheximide and puromycin is retarded but not inhibited by exogenously applied Ca⁺⁺ (Figures 20 and 22).

Puromycin and cycloheximide are both specific inhibitors of protein synthesis (9), and both inhibitors, especially cycloheximide, have been shown to be extremely fast acting (1, 3, 6). For example, hemoglobin synthesis in rabbit reticulocytes was inhibited 96 percent 30 seconds after the addition of 1.4×10^{-4} M cycloheximide (3). Excised beet root tissue has been shown to be synthesizing protein at an appreciable rate (4) and this synthetic ability is inhibited by both cycloheximide and puromycin (4). Some of this newly synthesized protein is incorporated into the cell membranes (13), thus, the leakage caused by cycloheximide and puromycin may be attributable to an inhibition of protein synthesis within the tissue. This inhibition would stop membrane protein turnover, leading to eventual membrane deterioration and therefore pigment efflux. D₂O is thought to be acting in a similar manner, that is by inhibiting protein synthesis and thereby blocking membrane protein turnover. The position of the D₂O block is not known, however, D₂O is not thought to be a ribosome poison. The retardation of the D₂O effects by Ca⁺⁺ is thought to result from

simple Ca^{++} stabilization of a membrane that is slowly deteriorating, therefore the Ca^{++} is effective for only a short time and eventually the damage becomes so severe that complete death of this tissue follows.

It is possible that the D_2O could have some direct effect on the beet root membrane, however, it is not possible to evaluate this hypothesis with a whole tissue system susceptible to metabolic poisoning.

The effect of D_2O on excised beet root tissue has been found to be one of disruption of the membrane. It is suggested that this effect is the result of a block of membrane protein turnover caused by a partial inhibition of protein synthesis in a 99.6 percent D_2O environment.

Literature cited

1. Burka, E. R. and P. A. Marks. 1964. Protein synthesis in erythroid cells II. Polysome function in intact reticulocytes. *J. Mol. Biol.* 9: 439-451.
2. Chorney, W., N. J. Scully, H. L. Crespi and J. J. Katz. 1960. The growth of algae in deuterium oxide. *Biochem. Biophys. Acta* 37: 280-287.
3. Colombo, B., L. Felicetti and C. Baglioni. 1965. Inhibition of protein synthesis by cycloheximide in rabbit reticulocytes. *Biochem. Biophys. Res. Commun.* 18: 389-395.
4. Ellis, R. J. and I. R. MacDonald. 1967. Activation of protein synthesis by microsomes from aging beet discs. *Plant Physiol.* 42: 1297-1302.
5. Flaumenhaft, E., S. Bose, J. L. Crespi and J. J. Katz. 1965. Deuterium isotope effects in cytology. *Int. Rev. Cytol.* 18: 313-361.
6. Fletcher, J. S. and H. Beevers. 1971. Influence of cycloheximide on the synthesis and utilization of amino acids in suspension cultures. *Plant Physiol.* 48: 261-264.
7. Kresheck, G. C., H. Schneider, and H. A. Scheraga. 1965. Thermodynamic parameters of hydrophobic bond formation in a model system. *J. Phys. Chem.* 69: 1310-1324.
8. O'Brien, R. T. 1964. Effect of D₂O on uptake in yeast. *Proc. Soc. Exp. Biol. Med.* 177: 555-558.
9. Pestka, S. 1971. Inhibitors of ribosome function. *Ann. Rev. Micro.* 35: 487-562.
10. Siegel, S. M. 1970. Further studies on regulation of betacyanin efflux from beet root tissue: Ca ion reversible effects of hydrochloric acid and ammonia water. *Physiol. Plant.* 23: 251-257.
11. Siegel, S. M. and L. A. Halpern. 1965. Effects of peroxides on permeability and their modification by indoles, vitamin E, and other substances. *Plant Physiol.* 40: 792-796.

12. Siegel, S. M., L. A. Halpern and C. Giunarro. 1964. Germination and seedling growth of winter rye in deuterium oxide. *Nature* 201: 1244-1245.
13. Van Steveninck, R. F. M. and M. E. Van Steveninck. 1972. Effects of inhibitors of protein and nucleic acid synthesis on the development of ion uptake mechanisms in beet root slices (*Beta vulgaris*). *Physiol. Plant.* 27: 407-411.
14. Waber, Jack. 1972. Physiological investigation of deuterium oxide toxicity: permeability studies. *Plant. Physiol.* 49 (supplement): 5.

CONCLUSION

The biological effects of deuterium oxide have been the subject of many investigations since Urey *et al.* first discovered the compound in 1932. The vast majority of these studies involved only a cursory recording of the changes that could be observed when the test organism was placed in a D₂O enriched solution (2). The general conclusion reached through these experiments was that high concentrations of D₂O are generally toxic to biological systems, however, the mechanism of this toxicity has remained unclear.

Following the successful culturing of several species of green algae in essentially 100 percent D₂O by Chorney *et al.* (1), the question of D₂O toxicity and its mechanism was extensively studied by Katz *et al.* (10, 11) and many other investigators (e.g., 2, 3, 4, 5). This work suggested that the effects of D₂O on biological systems could be explained by the kinetic isotope theory. That is, the kinetic imbalance created by growth in D₂O would be sufficient to poison the test organism. However, this proposed mechanism cannot sufficiently account for the high toxicity of D₂O to higher life forms.

An alternative explanation of D₂O toxicity has been proposed by Henderson (7, 8, 9) and suggests that high concentration of D₂O interferes with the normal functioning of the DNA-RNA-Protein

complex, thus causing the observed toxicity. The major conclusion of this dissertation is in agreement with this hypothesis. That conclusion being that protein synthesis is partially inhibited in higher plants cultured or incubated in 99.8 percent D₂O and this explains the general toxicity of high concentrations of D₂O to higher life forms, especially angiosperms.

The inhibition of protein synthesis in D₂O cultured winter rye is very marked. Total protein content, as measured by the method described by Lowry (13) is lower and the incorporation of ³H-leucine and ³H-phenylalanine into medium to high molecular weight proteins is severely inhibited. The partial inhibition of protein synthesis results in lower levels of two enzyme α -amylase and peroxidase in D₂O-cultured tissue. In agreement with Henderson, ³H-thymidine and ³H-uridine uptake studies suggest that the block to protein synthesis occurs at the level of transcription.

The demonstration of protein synthesis inhibition in the rye, plus a report by Flaumenhaft *et al.* (2) pointed to the possibility that the ultrastructure of the cells of D₂O-cultured seedlings would appear "less well organized" than that of the cells of H₂O-cultured plants. This expectation was not borne out. Except for a lower number of cisternae/dictyosome, decreased nucleolar volume, and a higher number of cytoplasmic ribosomes, the cells of D₂O-grown seedlings appeared similar to the cells of H₂O-grown plants. The lower number of cisternae/dictyosome, and a decreased nucleolar volume, however,

suggest that protein synthesis in the D_2O -cultured tissue is inhibited (6, 12). The observation of Flaumenhaft *et al.* (2) that the chloroplast structure of a completely deuterated alga (*Scenedesmus*) was "primitive in appearance, less well-differentiated and distinctly less well-organized," could not be confirmed. Indeed, it is the lack of subcellular disruption that is the most interesting result of this study, especially in light of the data which demonstrate that protein synthesis in this tissue is severely inhibited.

The effect of high concentrations of D_2O on intact functional membranes has not been extensively investigated (14, 15). Since the strength of intramolecular hydrogen and hydrophobic bonds are stronger in D_2O , the possibility of a disruptive D_2O -membrane interaction was investigated. Because it lends itself well to investigation of membrane phenomena, excised sections of beet root were used in this portion of the study. In agreement with the preceding sections, protein synthesis seems to be inhibited in excised beet root tissue incubated in 99.8 percent D_2O . This inhibition of protein synthesis apparently blocked the normal turnover of membrane protein, resulting in disruption of the membranes and consequently rapid efflux of the normally vacuolar bound pigment betacyanin. This conclusion was reached after comparing the effect of D_2O on pigment efflux with the effects produced by cycloheximide and puromycin. A recent report by Van Steveninck and Van Steveninck (16) partially substantiates this conclusion by demonstrating that at least some of the proteins

in the beet root membrane are turning over at a rate that is of the right order of magnitude to explain these data and furthermore that this turnover is sensitive to cycloheximide and puromycin poisoning. D_2O could also be acting directly on the membrane, however, it is not possible to fully test this hypothesis with a whole tissue system which is susceptible to metabolic poisoning.

It is concluded then that 99.8 percent D_2O at least partially inhibits protein synthesis in the two plant systems tested, D_2O -cultured winter rye and D_2O -incubated beet root sections. The detrimental and toxic effect of this kind of inhibition, plus the threshold for the detection of this effect in each system (65% D_2O in beet tissue and $\approx 70\%$ in winter rye) suggests that the inhibition of protein synthesis at least partially explains the toxicity of high concentrations of D_2O to higher life forms.

Literature Cited

1. Chorney, W., R. J. Scully, H. L. Crespi, and J. J. Katz. 1960. The growth of algae in deuterium oxide. *Biochem. Biophys. Acta.* 37:280-287.
2. Flaumenhaft, E., S. Bose, H. L. Crespi, and J. J. Katz. 1965. Deuterium isotope effects in cytology. *Int. Rev. Cytol.* 18: 313-361.
3. Flaumenhaft, E., S. M. Conrad, and J. J. Katz. 1960. Nucleic acids in some deuterated green algae. *Science* 132:892-894.
4. Flaumenhaft, E., S. M. Conrad, and J. J. Katz. 1960. Cytochemical studies on deuterated green algae. *Ann. N.Y. Acad. Sci.* 84:634-643.
5. Flaumenhaft, E. and J. J. Katz. 1964. Unpublished results. Cited in: Flaumenhaft, E., S. Bose, H. L. Crespi, and J. J. Katz. 1965. Deuterium isotope effects in cytology. *Int. Rev. Cytol.* 18:313-361.
6. Fleckinger, C. H. J. 1971. Alteration in the golgi apparatus of *Avebae* in the presence of an inhibitor of protein synthesis. *Exp. Cell Res.* 71:381-387.
7. Henderson, T. R. 1962. Differential inhibition of β -galactosidase induction and synthesis by deuterium oxide. *Biochem. Biophys. Res. Commun.* 9:240-245.
8. Henderson, T. R., J. M. Dacus, H. L. Crespi, and J. J. Katz. 1967. Deuterium isotope effects on β -galactosidase formation of *Escherichia coli*. *Arch. Biochem. Biophys.* 120:316-321.
9. Henderson, T. R. and M. R. Lamonds. 1966. Effects of D_2O on citric acid and pyruvate carboxylase formation by *Aspergillus niger*. *Arch. Biochem. Biophys.* 115:187-191.
10. Katz, J. J. 1960. Chemical and biological studies with deuterium. *Amer. Sci.* 48:544-580.
11. Katz, J. J., H. L. Crespi, A. J. Finkel, R. J. Hasterlik, J. F. Thomson, W. Lester Jr., W. Chorney, N. Scully, R. L. Shaffer, and S. H. Sun. 1958. The biology of deuterium. *Proc. Second Internatl. Conf. Peaceful Uses Atomic Energy* 25:173-180.

12. King, B. and J. M. Chapman. 1972. The effect of inhibitors of protein and nucleic acid synthesis on nucleolar size and enzyme induction in Jerusalem artichoke tuber slices. *Planta*. 104:306-315.
13. Lewis, G. N. 1933. The biochemistry of water containing hydrogen isotope. *J. Amer. Chem. Soc.* 55:3503-3504.
14. O'Brien, R. T. 1962. Effects of deuterium on yeast (*Saccharomyces cerevisiae*). U.S. At. Energy Comm. Res. and Devel. Dept. HW-72500: 113-118.
15. O'Brien, R. T. 1964. Effect of D₂O on uptake in yeast. *Proc. Soc. Exp. Biol. Med.* 117:555-558.
16. Van Steveninck, R. F. M. and M. E. Van Steveninck. 1972. Effects of inhibitors of protein and nucleic acid synthesis on the development of ion uptake mechanisms in beetroot slices (*Beta vulgaris*). *Physiol. Plant.* 27:407-411.

Please note correction.

Table 3, page 28 — *Toxicology: Mechanisms of Deuterium Oxide Action* (Part I) should read as follows:

Table 3

Morphological comparison of 2 day old H₂O-grown and 9 day old D₂O-grown winter rye seedlings.

	H ₂ O	D ₂ O
Shoot length	7.7 ± 1.5 mm	5.3 ± 1.7 mm
Root length	1.5 ± 0.4 cm	3.8 ± 1.9 mm
Fresh weight	53.0 ± 15 mgm	59.0 ± 9.9 mgm
Dry weight	20.3 ± 3.6 mgm	19.5 ± 3.6 mgm
# ribosomes/ μ^2 (P = 0.01)	324 ± 60	540 ± 82
Cisternae/dictyosome (P = 0.005)	5.6 ± 0.52	4.0 ± 1

The number (#) of ribosomes/ μ^2 and cisternae/dictyosome were computed by analyzing electronphotomicrographs. Averages are all $\pm \sigma$.