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

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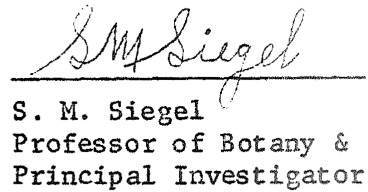
Submitted by: 

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Introduction

The three inhibitor studies presented here have little in common save that they all inactivate substances existing in nature. The first is organic, a plant product and unique to an ancient group of plants of basic evolutionary significance. Further, for a specialized natural product, a content of nearly one per cent by weight of psilotin is exceptional. The second paper continues earlier work on Mn-toxicity and is addressed to the question of enzymatic Mn-detoxification and its significance relative to other, related elements.

The third article presents new data on an old problem — the mechanism of D_2O toxicity. The novel feature of the present work is in the demonstration of differential effects of moderate D_2O levels on the akinetics.

Each one of these subjects could by itself form a starting point for an extended program of research. Thus the properties of psilotin suggests that it might be of ecological importance in reducing competitive pressures in stands of the plant species bearing it and the existence of mechanisms for precipitation of MnO_2 may determine species distribution in Mn-rich soils. The experiments with D_2O involve higher concentrations than those that are ever to be found on this planet. Nevertheless, some novel effects of partial deuteration are made quite evident.

Biological Activity in Psilotin, a Phenolic Glucoside from the Psilotales

Psilotin, 6-[4'-β-D-glucopyranosyloxyphenyl]-5,6-dihydro-2-oxo-2H-pyran, was first isolated by McInnes, Yoshida and Towers in 1965 from *Psilotum nudum*[1]. Subsequently, Tse and Towers isolated the compound from *Tmesipteris tannensis* but reported it to be absent from lycopods[2]. McInnes, *et al.* called attention to the common structural relationship between psilotin parasorbic acid and massoilactone, namely the αβ-unsaturated δ-lactone ring. Evidence for growth inhibitory activity in such structures[3] led to their suggestion that psilotin, too, might be biologically active. We can now report that psilotin is in fact an active inhibitor toward a variety of organisms and processes.

Experimental materials used in the survey included seeds of turnip, lettuce and onion; cultures of *E. coli* and *Penicillium notatum*; cysts of the brine shrimp *Artemia salina*; and local collections of the crustacean *Daphnia* and the millipede *Oxidus*. Seed and seedling tests were carried out on moist filter paper; *E. coli* plates were tested using antibiotic disc assay; response of *Penicillium* was tested by radial growth on nutrient plates; excystment of *Artemia* was followed on salt water agar. Protozoans were examined microscopically for locomotory changes or loss of cell integrity after treatment with psilotin.

Seed germination, seedling growth; locomotory activities in *Daphnia* and *Oxidus*; and excystment of *Artemia* were all inhibited to some degree by psilotin, whereas *Penicillium*, *E. coli*, and the protozoa showed no apparent response, even at 10 mmoles/1 (Table 1).

Comparing ID_{50} values obtained by interpolating the 50 per cent point in dose-response plots, the effectiveness of psilotin upon the aquatic animals appear greater than on the millipede (Table 2). It should also be noted, however, that *Daphnia* is far more sensitive than *Artemia*. The greater apparent resistance of *Oxidus* may relate to a lower rate of uptake of psilotin from the moist filter paper substratum.

Plant responses were also given more detailed attention. Seed germination was similarly inhibited in all three species (Table 3). Seeds held for 4-6 days after peak control germination failed to yield additional seedlings, hence were considered dead or in a state of extreme dormancy.

At a concentration of 10 mmoles/1, psilotin inhibited turnip and lettuce germination completely and limited onion to only 12 per cent.

In general, root length was more sensitive to psilotin than other growth parameters in each species, and inhibition of linear growth differed more among species than overall fresh weight. Differential organ sensitivity was most evident in turnip (Fig. 1)

Table 1. A Survey of Inhibitory Effects of Psilotin

Organism	Process	Lowest Effective Concentration[a] (mmoles/l)	Observation Period (hrs)
Seeds	germination	1.0	24-72
Seedlings	growth	0.1-1.0	168
Penicillium	growth	In.[b]	72
E. coli	growth	In.	24
Ameba	locomotion	In.	3
Paramecium	locomotion	In.	3
Oxidus	locomotion	1.0	24
Artemia	excystment	1.0	48
	locomotion (adults)	1.0	48
Daphnia	locomotion	0.1	26

[a]For inhibition > 10 per cent.

[b]Compound completely inactive at 10 mmoles/l, highest concentration used routinely.

Table 2. Sensitivity of Invertebrates to Immobilization by Psilotin

Organism	Exposure (hrs)	ID_{50} [a] mmoles/l
Daphnia	16	0.4
	26	0.3
Artemia	24	1.4
	48	1.4
Oxidus	12	40.0
	24	4.4

[a] ID_{50} = concentration required to immobilize 50 per cent of the test population determined by graphical interpolation of dose-response data.

Table 3. Inhibition of Seed Germination and Seedling Growth by Psilotin

Species	ID_{50} [a]			
	Germination	Seedling growth (7 days)		
		fresh wt.	root length	shoot length
Turnip	5.2 (24)[b]	2.2	1.6	2.8
Onion	1.6 (48)	3.1	0.8	1.6
Lettuce	1.6 (72)	3.4	0.3	0.9

[a]See footnote a, Table 2.

[b]Figure in parenthesis gives time in hours for maximum germination.

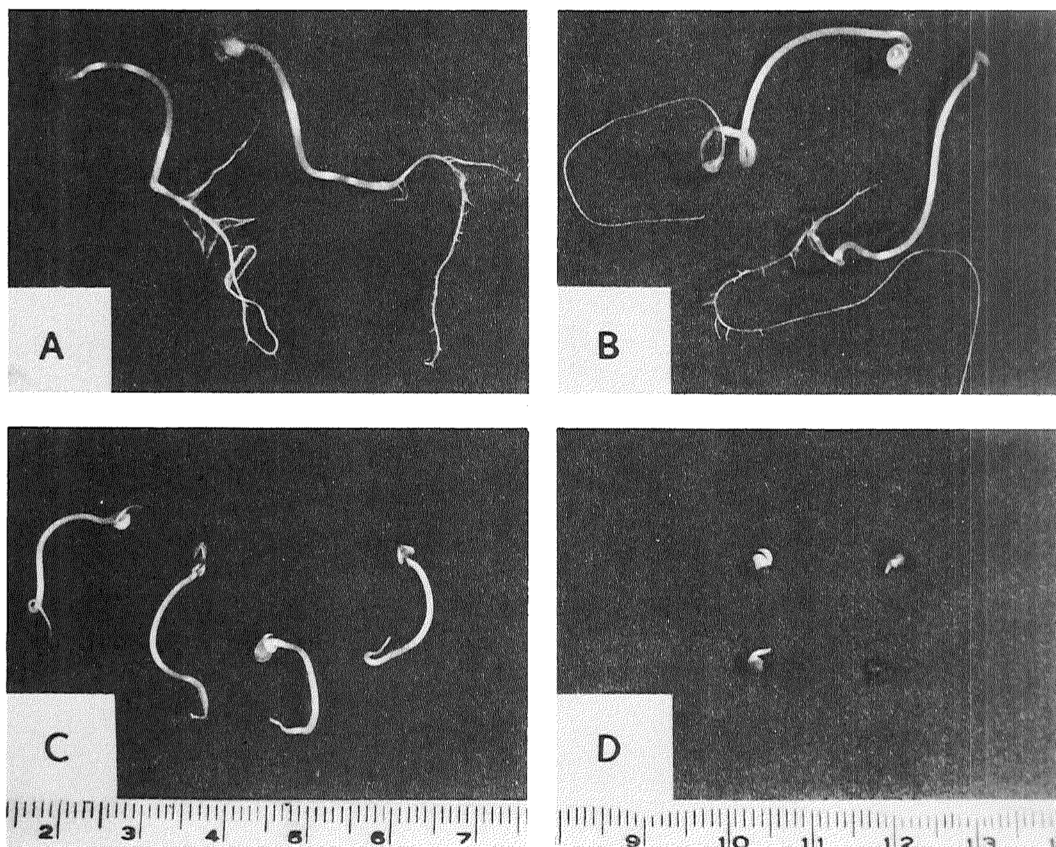

Fig. 1. Turnip seedlings after 7 days. Cultured at 25°C in (A) water, or psilotin at 1.0 (B), 3.0 (C) and 10.0 (D) mmoles/l.

which shows, with increasing psilotin level, a far greater suppression of the root and its appendages than of the shoot.

The reported psilotin content of 0.13-1.22 per cent (fresh weight)[1,2] marks this compound as a major natural product in the Psilotaceae. The existence of its wide spectrum of inhibitory activities may be entirely fortuitous. However, a role in ecological or biotic relationships of the group certainly is worthy of consideration.

The authors express their thanks to Dr. G. H. N. Towers, Department of Botany, University of British Columbia, for a generous sample of psilotin.

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Toxicology and Metabolism of Mn (II)

At the conclusion of the previous report on manganese (Hawaiian Botanical Science Paper, No. 20, July 1970, pp. 9-15) it was noted that seeds germinated in low concentrations of p-chloromercuri-benzoate (PCMB), sodium azide and fluoroacetate (FLA) had appreciably longer roots than the controls. We have endeavored to repeat this result and have not succeeded in confirming the result using either pea or cucumber seeds (Tables 4, 5 and 6). We have been unable to ascertain what was the reason for the previously reported result. The experiments using KCN are being repeated.

It has been repeatedly noted by the senior author and others that there is a correlation between the ability of the peroxidase enzyme to catalyse the oxidation of various substrates and the phylogeny of the chlorophyte line of plants.

This prompted an investigation of the capability of various plants to oxidize Mn^{++}. This work was separated into two parts. Firstly, an investigation using flowering plants and secondly using other plant material.

Seeds of cucumber, pea, turnip, red beet, cabbage, tomato, onion, lettuce, portulaca and phlox when grown in 10^{-2}M $MnCl_2$ all show deposition of MnO_2 in various parts of the seedlings. The seeds of winter rye and kidney bean show no deposition of MnO_2

Table 4. The percentage germination of pea seeds after 5 days growth in various concentrations of metabolic inhibitors in the presence or absence of 10mM $MnCl_2$.

Metabolic inhibitor	Concentration (M)					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
a. No $MnCl_2$						
PCMB	—	87	76	79	76	82
IAA	1	64	81	81	79	74
DNP	—	58	83	76	82	81
NaN_3	0	59	76	74	80	81
FLA	42	71	79	73	91	72
Water						92
b. 10mM $MnCl_2$						
PCMB	—	73	75	71	79	82
IAA	0	61	80	77	82	74
DNP	—	50	80	78	79	81
NaN_3	0	61	73	76	82	81
FLA	47	68	79	78	75	72
Water						89

Table 5. The length of the longest root of pea seedlings grown for 5 days in various concentrations of metabolic inhibitors.

Metabolic inhibitor	Concentration (M)					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
PCMB	—	27	26	24	25	25
IAA	0	14	27	27	21	27
DNP	—	13	24	26	23	28
NaN_3	0	13	27	25	26	28
FLA	9	16	27	26	27	25
Water						28

Table 6. The percentage germination and length of the longest root of cucumber seeds grown for 5 days in various concentrations of metabolic inhibitors.

Metabolic inhibitor	Concentration (M)					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	0
a. Germination						
PCMB	—	98	98	98	96	100
IAA	89	98	99	100	98	99
DNP	—	100	100	100	100	100
NaN_3	0	7	97	97	99	100
FLA	98	99	99	99	100	100
KCN	99	99	99	100	99	96
Water	99	100	100	100	100	99
b. Root length (mm.)						
PCMB	—	55	59	55	59	58
IAA	1	2	19	33	31	30
DNP	—	1	32	38	38	44
NaN_3	0	1	47	52	51	52
FLA	8	15	26	32	32	30
KCN	50	55	52	52	50	51
Water						59

in their walls. However, when tissues slices of

mustard cabbage stalks

edible ginger rhizomes

yellow squash fruits

"daikon" radish roots

lotus rhizomes

carrot roots

potato tubers

eggplant fruit

were cultured in 10^{-2}M $MnCl_2$ only the eggplant and mustard cabbage stalk slices showed black MnO_2 deposits. The deposits were always associated with the vascular bundles. The failure of some tissue slices to oxidize the manganese is thought to be due to their low metabolic activity rather than their total inability to carry out the reaction. This problem is being further investigated.

To study the phylogenetic argument, plants of the following species were cultured in 10^{-2}, 10^{-3}, and 10^{-4}M $MnCl_2$:

Gymnosperms	*Araucaria*
Ferns	*Azolla filiculoides* (Salvineaceae)
	Pleopeltis thumbergiana (Gramniitidae)
	Nephrolepis exaltata
Psilotales	*Psilotum nudum*
Mosses	*Leucobryum glaucum*
	Rhizogonium

Algae	*Ulva lactuca*
	Codium edule

Of the above species only *Azolla filiculoides* formed any black deposit of MnO_2. Thus it appears that here we have another example of a phylogeny in the oxidation potential of peroxidase. The anomalous result with *Azolla filiculoides* does not detract from this argument. The Salvineaceae are heterosporous ferns of very uncertain affinities. This is probably an example of parallel evolution. The inability of the one gymnosperm studied to carry out the oxidation was not expected as it was thought that all seed plants were capable of converting $MnCl_2$ to MnO_2.

However, the reliability of the above interpretations are suspect when the following results are considered.

When cucumber tissues are macerated and separated into filtrate and the supernatant, and then mixed with 10^{-2}M $MnCl_2$, no deposition of MnO_2 occurred. Even the addition of H_2O_2 and/or guaiacol did not result in MnO_2 formation. However, both the supernatant and filtrate turned brown in the presence of H_2O_2 and guaiacol indicating that peroxidase was present and active.

Similar experiments using filter papers impregnated with solutions of Sigma Chemical Company crude peroxidase enzyme gave indentical results with those above.

Both the above results prompted the question as to whether or not isolated organs were capable of carrying out the oxidation.

Cucumber seedlings were separated into cotyledons, hypocotyls and roots and each was incubated separately with 10^{-2}M $MnCl_2$ for three days. Of these organs only the cotyledons had deposits of MnO_2 in their tissues after treatment. The roots and hypocotyls did not form MnO_2 even when incubated for seven days, yet when part of an intact plant, they form deposits of MnO_2 in their tissues. Perhaps something is wrong with the maceration technique but various modifications of buffer pH, concentration of MnO_2, concentration of H_2O_2 or tissue to incubating medium have had no effect. There is no immediate explanation for the different responses by the tissues. However, the above results do make the angiosperm tissue slice experiments more comprehensible.

Manganic manganese will dissolve in pyrophosphate at neutral pHs to yield a stable manganipyrophosphate. However, manganese dioxide is insoluble in neutral pyrophosphate unless there are free manganous ions present. Kenten and Mann noted that in the above situation the tissue remained brown colored. The brown color is readily confused with the end products of polyphenol oxidase activity. It is interesting to note at this point that in all the experiments where no MnO_2 was formed the tissue or macerate was always brown or yellow after treatment with $MnCl_2$. The above explanation might explain why the tissue extracts were unable to form MnO_2 though inactivation of the enzymes is still a possibility. However, why

organs which as part of the intact plant are able to synthesize MnO_2 but are not able as isolated organs is not readily explained.

Anomalous Deuterium Isotope Effect on Selected Enzymes

For many years, high concentrations of deuterium oxide (D_2O) have been known to inhibit the processes of many biological systems. Katz, *et. al*[4], however, has recently been successful in culturing a variety of microorganisms in completely deuterated environments. In attempts to discover the molecular basis for the effects of deuterium at the organismic level, many investigators have studied the effect of heavy water on the activity of various enzymes. The results of these studies suggest that the substitution of D_2O for H_2O generally retards enzymatic reactions; for example, see Colman[5]. The amount of inhibition is directly proportional to the amount of isotopic substitution. However, no work has been done on deuterated enzymes in aqueous media. This paper presents evidence for a stimulatory deuterium effect on certain enzymes in aqueous and deuterated environments.

The commerically available enzymes used in this study were: crystalline peroxidase, lactate dehydrogenase (LDH), α-amylase and β-glucosidase. The activity of each enzyme was assayed according to standard procedures: peroxidation of guaiacol, LDH catalyzed reduction of ferricyanide, α-amylase catalyzed hydrolysis of starch, and the β-glucosidase catalyzed hydrolysis of salicin. The crystalline enzymes were dissolved in both H_2O and D_2O and allowed to equilibrate for at least forty eight hours[6]. Following this exchange

period, four activity determinations for each enzyme were carried out: (1) water control; (2) complete deuteration of enzyme, substrate and environment; (3) deuterated enzyme in an aqueous environment; and (4) non-deuterated enzyme in a deuterated environment.

Primary and secondary isotope effects were distinguished in the following way. Thermal-inactivation experiments using peroxidase (Fig. 2) indicated that complete substitution of deuterium for protium "lowers" the temperature-environment of an enzyme, thus increasing its thermal stability and lowering its overall rate of reaction. Parallel Km determinations, in agreement with Kaiser[7], showed no significant differences between the H- and D- enzymes. Therefore, it was concluded, the primary effect of deuterium oxide on enzymes was a "cooling" one, manifested in a lower overall rate of catalysis and increased thermal stability when compared to its water control. Any other deuterium-rate effects thus, are assumed to be secondary in nature.

The effect of varying concentrations of D_2O on the activity of peroxidase is presented in Table 7. The V_D/V_H ratios for this enzyme are assumed to be the approximate norm for enzymes which exhibit only a primary deuterium effect.

The data presented in Table 7 demonstrates a previously unreported secondary isotope effect of deuterium on enzymes. This effect causes an enhancement of the overall rate of reaction of

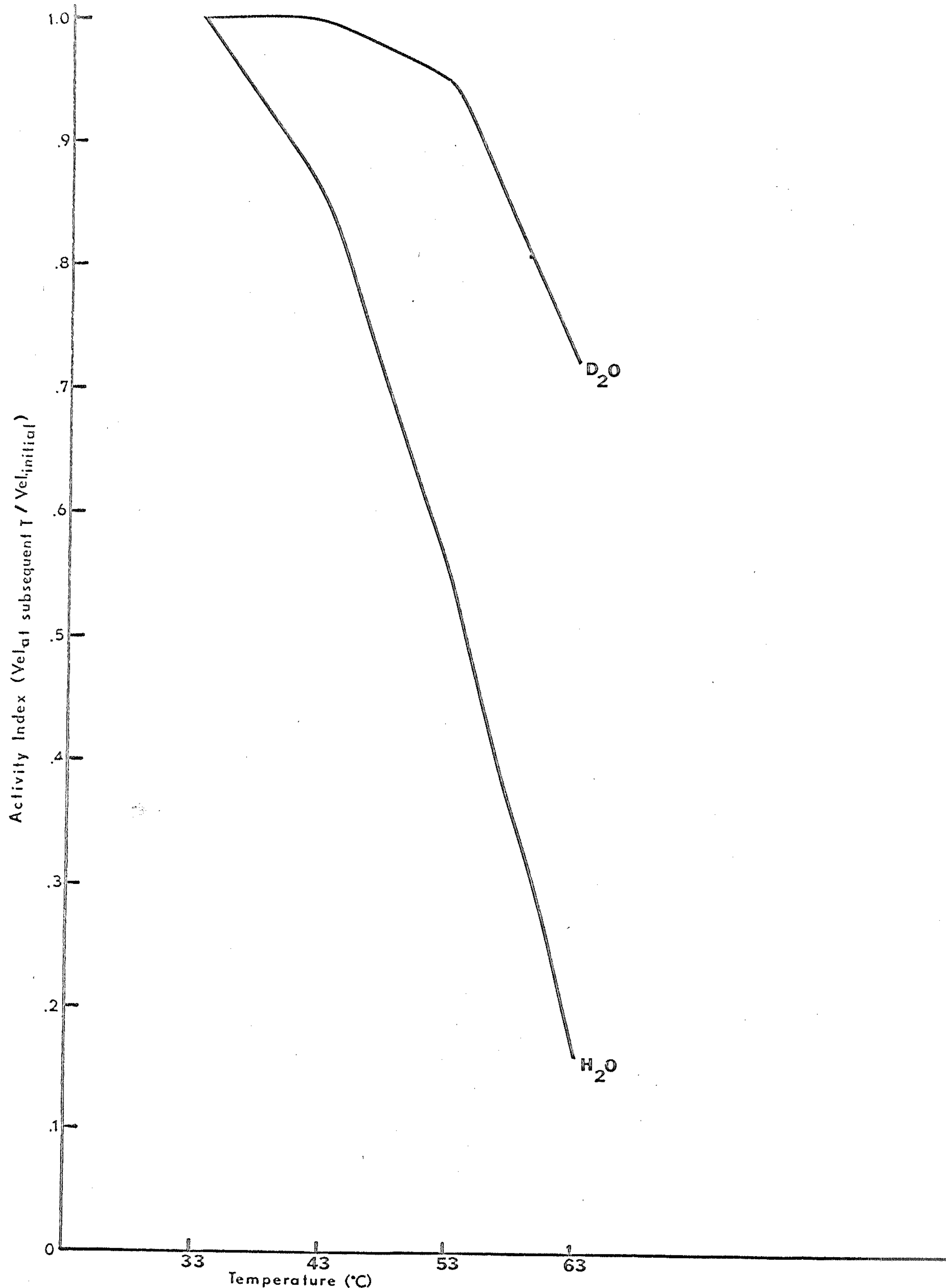

Fig. 2. Effect of D_2O on the Thermal Stability of Peroxidase.

The activity of peroxidase was determined over a range of temperatures from 33° to 63°C with the activity index expressed as ratio of observed velocity at some higher temperature divided by initial velocity at 33°C. Initial velocity in H_2O = .13±.01 ΔO.D./min. and D_2O = .07±.01 ΔO.D./min.

Table 7. Activity of Enzymes in D_2O.

Enzyme	Velocity in D_2O/Velocity in 100% H_2O			
	Composition of reaction media			
	100% H_2O	80/20 H_2O, D_2O	50/50 H_2O, D_2O	100% D_2O
Peroxidase	1.00	0.90±.021	0.81	0.54±.03
α-Amylase	1.00		0.947±.02	
β-Glucosidase	1.00	≃1.00±.02		≃1.00
Lactic acid dehydrogenase	1.00			1.67±.121

the deuterated enzymes when compared to their water controls. Using the V_D/V_H ratios of peroxidase as a criterion, α-amylase exhibits a marked enhancement of its activity in a 50 per cent deuterium environment (Table 7). LDH activity is also stimulated, with the completely deuterated system running 1.67 times faster than its protium analogue (Table 7). β-glucosidase also seems to exhibit this effect, however, more determination must be made with this enzyme before any definite conclusions about it are reached (Table 7).

The explanation of this effect is not immediately obvious. As previously mentioned, the Km of the enzymes remains constant irrespective of solvent. Apparently, then, the enhancement effect cannot be explained on the basis of substrate-enzyme affinity, or enzyme-substrate complex dissociation. It has been reported by Tomita and Rich[8] that complete deuteration extends the α-helix of a protein and increases the length of the hydrogen bonds. It seems reasonable to propose then, that the stimulatory effect deuteration has on some enzymes could be attributed to this type of conformational change around the active site of the enzyme, thus enabling substrate molecules to more easily enter into reactive complexes with the enzyme. On the basis of thermodynamic data, Osaki and Walaas[9] have used this argument to explain the inhibitory effect of D_2O on ferroxidase. Although the effects are different, the mechanism could be the same, and will subsequently be investigated.

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