[CONTRIBUTION FROM THE PHARMACOLOGICAL RESEARCH LABORATORY, HYNSON, WESTCOTT & DUNNING, INC.]

Effect of Deuterium Oxide on Action of Some Enzymes[1]

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Ever since the discovery of heavy water much speculation of very diverse character has been advanced in regard to its possible effects on life. The most extreme views are presented by those who claim, without adequate experimental proof, that deuterium oxide is a most powerful destructive physiological agent and those who, basing their conclusions on experiments made with deuterium oxide in dilute concentrations, state that its pharmacological effects are of negligible character.[2] The authors have recently discovered an interesting and striking contrast in the biophysical and biochemical behavior of H_2O and D_2O, which consists of a difference in velocity of reaction shown by certain animal and vegetable enzymes suspended therein. Previous studies on the effect of deuterium on some enzymes have been described by Barnes and his collaborators.[3] In the present investigation, studies were made on the behavior of muscle oxydase from the rat, of oxidative enzymes of fresh, blood-free brain tissue from the cat and of reductase of finely ground seeds of *Lupinus albus*, by the Thunberg method of determining the rapidity with which a standard buffered solution of methylene blue is decolorized in special vacuum tubes. Over fifty experiments on the subject were performed. The method employed is described in detail elsewhere.[4]

Briefly, the technique employed was as follows: Rats were anesthetized with ether and killed by bleeding from the vessels of the throat, and muscle juices or suspensions were immediately prepared from the fresh tissues. Carefully dissected lean muscle tissue of a given weight was minced with sharp scissors. One cc. of physiological sodium chloride solution (0.9%) for each gram of muscle was added and the mixture was ground up with double its weight of fine clean sand in a mortar for half an hour, when a smooth homogeneous paste was obtained. The mass was then strained through fine linen and in this way was obtained a uniform suspension of finely divided muscle particles in a mixture of muscle juice and physiological saline which could be measured in any desired quantity with a glass pipette. Brain tissue was prepared in a similar manner but in this case it was found more suitable to use 2 cc. of saline and 1 g. of sand for each gram of brain tissue. One cc. of suspension is introduced into a Thunberg tube (Fig. 1) and to this are added 2 cc. of Thunberg solution, consisting of 8 parts of methylene blue, 1:2000, and 6 parts of 0.1 molar solution of acid potassium phosphate. The air is exhausted from the Thunberg tube with a Cenco-Hyvac vacuum pump until the pressure within is less than 10 mm. and the solution begins to boil when the tube is held in the warm hand. It is then placed in a water-bath at 38°, and the time required for complete decolorization of the solution is measured carefully. Other experiments, especially those performed with brain tissue, were made at room temperature. Surprisingly concordant results are obtained with a series of Thunberg tubes containing samples of the same muscle suspensions, provided that the vacuum made in each tube is carefully maintained. This can be accomplished by using carefully ground stoppers lubricated with a special grease containing a small percentage of gutta-percha. A marked difference was found to exist between identical enzymes suspended in H_2O and in the same kind of water to which small quantities of deuterium were added, respectively. This difference was demonstrated not only with concentrations of D_2O, 1:100, but also with concentrations of 1:2000 and less.

Fig. 1

Further studies were made in connection with the influence of deuterium oxide on the activity of another enzyme, catalase. Catalase from fresh rat muscle and from *Lupinus albus* seeds in distilled water, and in water to which small quantities of deuterium oxide were added, also revealed a difference in the speed of evolution of oxygen gas from hydrogen peroxide in the presence of these enzymes. The method employed was a modifica-

(1) Read before the American Physical Society, November 30, 1935, Johns Hopkins University, Baltimore.

(2) Meyer, *J. Tenn. Acad. Sciences*, **IX**, 225 (1934); **X**, 111 (1935); Macht and Davis, THIS JOURNAL, **56**, 246 (1934).

(3) Barnes and Jahn, *Quart. Rev. Biol.*, **9**, 292 (1934); Barnes and Larson, *Protoplasma*, **22**, 431 (1934).

(4) Macht and Bryan, *J. Biol. Chem.*, **110**, 101 (1935); Macht, *Arch. internat. de pharmacodyn. et de thérap.*, **XLIX**, 175 (1934).

tion of that described by Baldwin.[5] Briefly, it consisted of the use of a tube like a Thunberg tube, into the hollow arm of which was introduced a catalase suspension. Into the tube proper were introduced 2 cc. of a solution consisting of a mixture of equal parts of one fifth molar disodium phosphate and one fifth molar diacid potassium phosphate. When the experiment is begun, the catalase suspension is allowed to run into the tube proper and the oxygen gas evolved is collected by displacement of water in a fermentation tube connected with the apparatus.

TABLE I

EXPERIMENTS WITH MUSCLE OXIDASE

Technique: 2 cc. of muscle suspension plus 1 cc. of Thunberg solution introduced into tubes on the water-bath at 38°

Oxydase prepared with H_2O saline

Tube	1	2	3	4	Av.
Decol. time, minutes	180	186	180	180	182

Oxydase prepared with D_2O saline, 1:100

Decol. time, minutes	149	120	115	140	131

TABLE II

EXPERIMENTS WITH RAT MUSCLE OXYDASE

Technique: 2 cc. of muscle suspension and 0.5 cc. of Thunberg solution on water-bath at 38°

Oxydase prepared with H_2O saline (pH 5.7)

Tube	1	2	3	4	5	Av.
Decol. time, minutes	40	43	39	45	48	45

Oxydase made with D_2O saline, 1:5000 (pH 5.7)

Decol. time, minutes	34	35	33	40	36	35.6

TABLE III

EXPERIMENTS ON BRAIN TISSUE EXTRACT WITH H_2O SALINE (NaCl 0.9%)

			Color at times				
Tube	Extract	Evac. at	Time	%	Time	%	
1	H_2O	9:54	10:50	55	2:00	25	3:15 Decol.
2	D_2O, 1:2000	9:52	10:50	45	2:00	Decol.	.. ..
3	H_2O	9:56	10:50	55	2:00	25	3:00 Decol.
4	D_2O, 1:2000	9:59	10:50	45	2:00	Decol.	.. ..
5	H_2O	10:03	11:00	55	2:15	25	3:15 Decol.
6	D_2O, 1:2000	10:01	11:00	45	2:15	Decol.	.. ..

TABLE IV

EXPERIMENTS WITH LUPINUS ALBUS REDUCTASE

Technique: 2 cc. of bean extract plus 0.5 cc. of Thunberg solution, introduced into tubes from which air has been evacuated, placed on water-bath at 38°

Series (6 experiments)	1	2	3
Bean extct. with	H_2O	D_2O	D_2O
Concn.	..	1:500	1:2000
Av. decol. time, min.	294	242	191

(5) Baldwin, *Am. Jour. Bot.*, **22**, 635 (1935).

TABLE V

EXPERIMENTS WITH LUPINUS ALBUS CATALASE

Technique: 1 cc. of H_2O, 3 cc. of H_2O_2 (3%), 1 cc. of KH_2PO_4 (0.2 M) and 1 cc. of NaH_2PO_4 (0.2 M) placed in tube

Series (2 experiments, 0.5 cc.)	1	2
Bean extct. prepd. with	H_2O	D_2O 1:100
O_2 evolved in 15 min., cc.	3.3	5.55

It will be noted that the enzymatic activity was accelerated in the presence of deuterium. In some of the experiments the enzymes exhibited a diphasic activity. This was especially true of more concentrated solutions of D_2O. The enzymes in such experiments, as compared with the controls, exhibited a diphasic activity; that is, there was a primary retardation which was followed by a secondary and more marked acceleration. In all the experiments, however, a definite difference in reaction was noted between H_2O and D_2O media.

These findings have not only a purely theoretical scientific interest but also a practical bearing. It is well known that minute quantities of powerful drugs or poisons are often more readily detected by physiological tests on living animal or plant preparations *in vitro* than by chemical or even physical means.[6] When studying such physiological reactions, it is necessary to employ aqueous solutions of definite composition. Minute solutions of organic or inorganic impurities present in water used for physiological tests may influence profoundly the biological reactions. The writers' work raises a question as to the possibility of differences occurring in various samples of water which may be due to the quantity of the hydrogen isotopes present. When we recall that there are two known isotopes of hydrogen—namely, deuterium and tritium—and that three varieties of oxygen, differing slightly in their atomic weight, have been established, the subject appears to be even more complex than we at first suspected.

Summary

1. Experiments were made with muscle and brain extracts or suspensions prepared, respectively, with H_2O saline with and without small quantities of D_2O, and with extracts of *Lupinus albus* seeds prepared with pure water with and without small quantities of D_2O.

(6) Hunt, *Science*, **72**, 526 (1930); Macht and Anderson, THIS JOURNAL, **49**, 2017 (1927); Hatcher, *American Druggist*, **79**, 22 (1929); Macht, *Am. J. Ophthalmol.*, **14**, 726 (1931).

2. Studies made by the Thunberg method on the reducing power of such suspensions for methylene blue revealed differences in the speed of enzyme action which were dependent on the amount of deuterium oxide in the suspensions employed. As small a quantity of D_2O as 1:2000, added to water, produced a difference in the speed of decolorization of the dye, accelerating the reaction.

3. Similar differences in the speed of catalase activity of both muscle and *Lupinus albus* seed extracts were also noted.

4. The possible physiological importance of the phenomena was discussed.

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Tertiary Alkylbarbituric Acids

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The hypnotic properties of 5,5-dialkylbarbituric acids have been the subject of extensive investigations for more than three decades. A goodly number of these products are now available for therapeutic use, and the number of unknown isomers and homologs with probable hypnotic properties is still greater. In general, those derivatives which have been found most satisfactory have contained one ethyl group and a second hydrocarbon radical with five or six carbon atoms. Within these limits are the well-known drugs Phenobarbital, Phanodorm, Amytal, Pentobarbital and Ortal.

Isomers may differ considerably in potency, duration of effect and therapeutic ratio. A notable instance is the great difference in potency between the isoamyl and the 1-methylbutyl derivatives, where the only variation in structure is a branching at the near or at the far end of the hydrocarbon chain. Further examination to include other isomeric amyl derivatives shows that the difference is a matter of primary *versus* secondary amyl groupings. Thus the primary amyls, *n*-amyl $EtCH_2CH_2CH_2$, isoamyl $Me_2CH\text{-}CH_2CH_2$, and 2-methylbutyl $EtCHMeCH_2$, have practically the same potency, whereas the secondary amyls, 1-methylbutyl $EtCH_2CHMe$ and 1-ethylpropyl Et_2CH, are about twice as effective. In lower homologs the difference is less striking, *e. g.*, propyl *vs.* isopropyl, and butyl or isobutyl *vs.* secondary butyl show less pronounced differences. In the hexyl series adequate data are not available. A comparison between the open-chain hexyls and the secondary hexyls of cyclic structure is hardly valid. With increasing number of carbon atoms the situation becomes increasingly complex. The three primary hexyls, *n*-hexyl, 2-ethylbutyl and 2-methylamyl have about the same potency, but the secondary hexyls are not yet available. Where two branchings occur in the chain, as in 1,2-dimethylpropyl and 1,3-dimethylbutyl, the situation is complicated by a convulsive action of the drug.

The wide difference in potency between the primary and secondary amyl derivatives suggested the possibility of a similar difference between the secondary and tertiary derivatives. No tertiary alkyl barbituric acids are described in the literature. It is hardly likely that no efforts have been made to prepare such derivatives. Probably the poor yields of the intermediate malonic esters have discouraged investigators from pursuing such attempts. Fischer and Dilthey were unable to obtain the diisopropylmalonic ester and attributed their failure to steric hindrance. The same reasoning would apply to esters of tertiary alkylmalonic acids. However, by recourse to other procedures not involving the use of malonic esters, diisopropyl- and diphenylbarbituric acids have been prepared. Their physiological properties were disappointing, aside from the difficulty of synthesis.

Our purpose was to obtain tertiary alkylethylbarbituric acids in quantities just sufficient for physiological testing, regardless of yields, in order to determine whether the increased potency of secondary over primary alkyls would be carried further into the tertiary series. Tertiary butyl- and tertiary amyl-ethylbarbituric acids were thus prepared in small yields and tested for hypnotic action. They differed in melting points from the isomeric primary and secondary butyl and amyl derivatives, all of which are known, hence a possible rearrangement into primary or secondary