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SOME EFFECTS OF D_2O *IN VIVO* AND *IN VITRO* ON CERTAIN ENZYMES OF RAT TISSUES*

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Abstract—In animals that had drunk 50% heavy water until about one-third of their body water was replaced by D_2O, changes were observed in the tissue levels of several enzymes. The arginase and oxalacetate transaminase activities of rat liver were significantly increased; alanine transaminase was unchanged. Tryptophan pyrrolase was elevated slightly in livers of unadapted rats; however, there was marked suppression of the increase in activity induced by injection of tryptophan. The activity of glutamic acid decarboxylase of rat brain was depressed by D_2O *in vitro*, but the concentration of this enzyme was not changed by feeding D_2O. Mitochondria prepared from liver and kidney of D_2O-treated rats showed a diminished capacity to oxidize fumarate and α-ketoglutarate, but not succinate; the efficiency of phosphate esterification was unaffected. D_2O (30%) *in vitro* did not affect transaminase activity or oxidative phosphorylation.

ADMINISTRATION of D_2O to an animal represents a situation which is unique in toxicology, since profound changes can be produced in an organism merely by alteration of the relative abundance of different stable isotopes of the same element. The effects of feeding D_2O to mammals were first studied systematically by Barbour[1] and co-workers. However, the types of experiments they could do were limited by the scarcity of heavy water. In recent years, with more adequate supplies of D_2O available, the mechanism of toxic action of D_2O has been reinvestigated.[2] One approach has been to assay tissues of D_2O-treated animals for various enzymes. Preliminary studies showed that the catalase, esterase, and DPN-cytochrome *c* reductase activities of rat liver decreased to about two-thirds of the normal value in animals in which 32 per cent of the body water had been replaced by D_2O. The levels of arginase and uricase were increased, while those of cytochrome oxidase and succinic dehydrogenase were decreased slightly.[2] This report is concerned with assays of some other enzymes in rat tissues.

METHODS

Sprague–Dawley rats, from 4 to 7 months old, were used in these experiments. For the study of the effects of D_2O *in vivo*, they were given 50% D_2O as their drinking water, and were fed Rockland rat diet *ad libitum*. Urine samples were collected periodically and assayed for D_2O to provide an index of replacement of body water by D_2O. Immediately before the rats were killed, a sample of blood was taken by heart puncture, and the D_2O content of the plasma was measured spectrophotometrically.[2] The effects of D_2O *in vitro* were studied on tissues of normal rats.

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Rats were killed by decapitation. The organs were rapidly removed, chilled in cracked ice, then homogenized by means of a Potter–Elvehjem homogenizer in a suspending medium appropriate for the assay of the enzyme under investigation. In different experiments, tissues were assayed for arginase,[3] transaminase,[4] tryptophan pyrrolase,[5] glutamic acid decarboxylase[6] and oxidative phosphorylation.[7] In the last-named assay, mitochondria were prepared from rat liver by gradient centrifugation.[8] Tryptophan pyrrolase was measured in livers both of unadapted rats and of rats injected intraperitoneally 6 hr before they were killed, with 2 g of DL-tryptophan per kg of body weight.

RESULTS

Arginase

Preliminary data on arginase activity in livers of D_2O-treated rats had shown an increase of 18 per cent in rats with about one-fifth of their body water replaced by D_2O.[2] This figure was based on data obtained from only three treated rats and two controls, so that confirmation was necessary. Table 1 shows the results of an experiment which began with six rats in each group; one of the D_2O-treated animals died

TABLE 1. ARGINASE ACTIVITY IN LIVERS OF D_2O-TREATED RATS

	Control	D_2O-treated
Number of rats	6	5
Days on 50% D_2O	—	32
Final plasma D_2O (%)	—	31·5
Range		(31·1–31·7)
Weight change (g)	+27	−9
Range	(+25 to +30)	(−24 to +12)
Arginase activity*	461 ± 21	528 ± 50

* μmoles arginine destroyed per 10 min per mg N, averages and standard deviation.

on the twenty-ninth day, with a plasma D_2O level of 30 per cent. The arginase activity of the livers of the treated rats was about 15 per cent higher than that of the control livers; this difference is statistically significant ($P < 0{\cdot}05$).

Transaminase

Two transaminase reactions were measured in liver homogenates: the formation of oxalacetate from α-ketoglutarate and aspartate, and the formation of alanine from pyruvate and glutamate. *In vitro*, neither was affected by 30% D_2O. Alanine transaminase was inhibited about 15 per cent in 50% D_2O; in three experiments, the inhibitions observed were 12, 16, and 17 per cent. Oxalacetate transaminase was not studied at the 50 per cent level *in vitro*. The *in vivo* changes are shown in Table 2. No effect was observed with alanine transaminase, while the activity of oxalacetate transaminase was increased by nearly 20 per cent ($P = 0{\cdot}05$). In this experiment, the blood glucose and urea were also measured; the level of the former was reduced to about three-fourths of normal, while the latter was more than double the normal value.

Tryptophan pyrrolase

This enzyme was of particular interest since its level in rat liver can vary in different physiological states. Fourteen rats were given 50% D_2O to drink; one rat died after

TABLE 2. TRANSAMINASE ACTIVITY IN LIVERS OF D_2O-TREATED RATS

	Control	D_2O-treated
Number of rats	9	7
Days on 50% D_2O	—	26
Final plasma D_2O (%)	—	31·7
Range		(29·5–34·0)
Weight change (g)	+42	+6
Range	(+22 to +61)	(−13 to +28)
Blood glucose (mg %)	80	62
Range	(61–91)	(45–83)
Blood urea (mg %)	18·9	44·3
Range	(13·8–21·4)	(28·7–58·0)
Transaminase*		
Alanine	24·9 ± 1·1	22·5 ± 1·8
Oxalacetate	67·8 ± 9·9	80·4 ± 9·6

* μmoles product formed per hr per g N, averages and standard deviations.

28 days. An increase in activity was observed in livers of rats killed after 11 days, and after 32 days the tryptophan pyrrolase activity was almost 40 per cent above normal (Table 3). Only four controls were used in this experiment; however, since the values observed in these rats corresponded well with those obtained on fifty-four rats used in other experiments, additional controls were considered unnecessary.

TABLE 3. TRYPTOPHAN PYRROLASE ACTIVITY IN UNADAPTED RATS TREATED WITH D_2O

Days on D_2O	Plasma D_2O, ave. % and range	Ave. weight loss (g)	No. rats	μmoles kynurenine formed per hr/g dry wt.		
				Ave.	σ	*P*-value
0	—	—	4	7·4	0·6	—
			54*	7·7	1·5	—
11	13·6 (13·0–14·2)	4	4	8·8	0·5	0·05
21	26·8 (24·3–27·8)	24	4	8·3	0·9	0·2
32	30·3 (29·2–32·9)	36	5	10·2	1·8	0·03

* Control data from earlier experiments.[9]

The adaptive response to intraperitoneal injection of 2 g of DL-tryptophan per kg was also studied in D_2O-treated animals. Two series of experiments were carried out. In the first experiment, animals were killed after 17, 24 and 31 days; since the values for plasma D_2O were very close for the first two groups, the data for 17 and 24 days have been pooled. For the same reason, the results of the second experiment, in which the rats were killed on the seventh and twelfth days, have been similarly treated.

The rats used in this second experiment were younger and lighter than those used in the first experiment, and accumulated D_2O more rapidly.

It is clear that deuteration markedly reduced the capacity of the rat to respond to the injection of tryptophan (Table 4). Instead of the usual twelve- to fifteen-fold increase in activity seen in normal rats, the activity was increased less than three-fold in rats with 30 per cent replacement. This change is detectable even at 18 per cent

TABLE 4. EFFECT OF FEEDING D_2O ON INDUCTION OF TRYPTOPHAN PYRROLASE IN RAT LIVER BY INTRAPERITONEAL INJECTION OF TRYPTOPHAN

Days on D_2O	Plasma D_2O, ave., % and range	Ave. weight loss (g)	No. rats	μmoles kynurenine formed per hr/g dry wt.	
				Ave.	σ
0	—	—	4	101	16
			12*	121	18
17–24	26·7 (23·5–28·8)	31	6	41	14
31	34·6 (34·0–36·2)	53	4	36	7
0	—	—	4	100	15
7–12	18·1 (15·9–19·8)	1	7	80	19

* Control data from earlier experiments.[9]

replacement, when the animals are essentially free of signs of toxicity, and becomes very marked in moribund animals. One rat among those injected with tryptophan on the thirty-first day failed to survive the 6-hr period after injection.

Glutamic acid decarboxylase

In vitro, 30% D_2O effected a reduction of glutamic acid decarboxylase activity of rat brain to about 85 per cent of normal. The individual values of three experiments were 88, 87 and 78 per cent. However, the glutamic acid decarboxylase activity of brains of D_2O-treated rats did not differ significantly from the controls. The average value for seven treated rats, with an average plasma D_2O of 29·2 per cent, was 45·0 μl CO_2 formed per hr per mg N, while for the controls it was 46·8. The assays were carried out in ordinary water.

Oxidative phosphorylation

Mitochondria isolated from liver and kidney of rats drinking D_2O were tested for their capacity to esterify inorganic phosphate. Three different substrates, succinate, fumarate and α-ketoglutarate, were used in these experiments. Four rats were designated for the experiment, but one died before it could be used. The average value for plasma D_2O of the other three was 32·0 per cent. Although the numbers of animals employed were small, the results seem clear-cut (Table 5).

In no case was the efficiency of phosphorylation impaired; the P/O ratios for all three substrates with both liver and kidney mitochondria were normal. However, the rates both of oxygen consumption and of phosphate esterification were slowed from 15 to 25 per cent with either fumarate or α-ketoglutarate as substrates. The inhibition was considerably less with succinate.

In vitro, with fumarate as the substrate, 30% D_2O had little effect on the reaction. At 60% D_2O, oxygen consumption was decreased by 25 per cent and phosphate esterification by 30 per cent.

DISCUSSION

One of the features of D_2O intoxication in rats is the evident involvement of the adrenals. There is an adrenal hypertrophy, the weight of the glands nearly doubling

TABLE 5. OXIDATIVE PHOSPHORYLATION IN LIVER AND KIDNEY MITOCHONDRIA OF NORMAL AND D_2O-TREATED RATS

	Substrate					
	Succinate	Fumarate	α-Keto-glutarate	Succinate	Fumarate	α-Keto-glutarate
	Normal liver			*Normal kidney*		
No. rats	12	25	7	3	3	5
μ moles P esterified/ 20 min per mg N	19·5 ±3·0	19·2 ±3·1	19·2 ±2·8	46·1 (42·6–50·8)	45·5 (40·4–52·0)	43·0 (41·0–49·2)
μ atoms O consumed/ 20 min per mg N	10·5 ±1·7	6·8 ±0·9	5·2 ±0·8	26·2 (26·0–26·4)	15·9 (15·0–16·3)	12·6 (12·2–12·8)
P/O ratio	1·9 (1·5–2·0)	2·8 (2·6–3·0)	3·6 (3·4–4·1)	1·9 (1·8–2·0)	2·9 (2·9–3·0)	3·6 (3·2–4·0)
	D_2O *liver*			D_2O *kidney*		
No. rats	3	3	3	3	3	3
μ moles P esterified/ 20 min per mg N	17·8 (17·0–18·8)	14·4 (13·0–16·6)	15·9 (15·0–16·5)	47·4 (37·6–50·2)	33·8 (24·9–39·6)	35·7 (28·4–39·9)
μ atoms O consumed/ 20 min per mg N	9·0 (8·1–10·1)	5·1 (4·9–5·4)	4·4 (4·3–4·7)	25·8 (24·1–28·2)	11·9 (9·9–13·6)	11·0 (8·8–12·9)
P/O fatio	2·0 (1·9–2·1)	2·8 (2·6–3·1)	3·5 (3·1–3·9)	1·8 (1·5–2·1)	2·8 (2·6–3·0)	3·2 (3·1–3·4)

Data are expressed as averages with ranges or averages ± standard deviations

at one-third replacement of body water by D_2O.[2] Hypophysectomized rats are much more susceptible to the lethal action of D_2O than are unoperated controls, presumably because of their impaired capacity to adapt to the altered internal environment. Kidney function is depressed,[10] but since the capacity of slices of kidney from D_2O-treated rats to esterify *p*-aminohippuric acid (PAH) *in vitro* is unimpaired,[11] the depression of PAH clearance in the intact animal may be attributed to disturbed adrenal function. The blood picture in moribund D_2O-treated rats resembles that seen in adrenalectomized animals, with high NPN, urea, and lactic acid, and low glucose and plasma protein.

It has been demonstrated that the arginase and transaminase activities of rat liver decrease after adrenalectomy.[9, 12] Since a decrease was not seen in these experiments, the concept that the deuterated rat behaves as if it had been adrenalectomized is untenable, even though some functions of the adrenal are apparently suppressed; the production of adrenal hormones does not seem to be inhibited. It is possible that the rat treated with D_2O may have a higher requirement for adrenal hormones than could be met even by a hypertrophic adrenal cortex, or that the metabolic processes of the rat are so affected by deuteration that no additional amount of adrenal hormones could benefit the animal.

The increase in activity of tryptophan pyrrolase in D_2O-treated rats is consistent with the idea that adrenal activity is unimpaired or even somewhat increased, since the level of this enzyme can be raised by administration of hydrocortisone and to a lesser extent cortisone.[13] However, it is also possible that this increase in activity may result from an increased concentration in the body of products of protein breakdown, since a number of amino acids and their derivatives are known to produce an elevation of tryptophan pyrrolase in the normal rat.[5, 14]

It is particularly interesting to note that the adaptive response of this enzyme to the injection of tryptophan was strongly inhibited. Although the data of Gros *et al.*[15]

indicate that the increase of tryptophan pyrrolase produced by administration of its substrate involves actual synthesis of additional protein, some recent work[16, 17] indicates that *de novo* synthesis may not be the complete explanation for the tryptophan-induced augmentation of tryptophan pyrrolase. Hence, it is difficult to state that these data demonstrate an inhibition of protein synthesis, although such an interference would not be inconsistent with other effects of D_2O.

Although the concentration of glutamic acid decarboxylase was not measurably decreased in the brains of rats D_2O, 15 per cent inhibition was produced *in vitro* by D_2O. Thus, while the level of the enzyme was not affected, its physiological activity *in vivo* could be diminished by replacement of tissue fluids by heavy water. Such an inhibition might explain some of the effects of D_2O on the central nervous system, since a decreased level of γ-aminobutyric acid is associated with hyperexcitability.[18]

The data on oxidative phosphorylation by mitochondria prepared from tissues of D_2O-treated rats showed that with the substrates which are dehydrogenated via DPN, i.e. fumarate and α-ketoglutarate, there was an appreciable reduction in oxidative activity that was not seen with succinate as substrate. Earlier data had demonstrated a decrease in DPN–cytochrome *c* reductase in liver; the levels of this enzyme in kidney were not measured in rats with plasma D_2O values above 20 per cent. Conceivably a deficiency of this enzyme could account for the inhibition of oxidative phosphorylation, although it has not been established whether DPN–cytochrome *c* reductase is lost from mitochondria, microsomes, or both. Furthermore, the significance of the decrease is questionable, since there is no indication of the importance of the influence of D_2O on these reactions in the intact animal. Whether a 35 per cent decrease in DPN–cytochrome *c* reductase would disturb the economy of the rat is not known. Since the size of the liver is increased by as much as 50 per cent in heavily-deuterated rats,[2] the decrease of an enzyme to two-thirds of the normal level would still entail no loss in *total* organ activity.

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