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Effects of heavy water on mitochondrial respiration and oxidative phosphorylation

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Abstract. Studies on the influence of heavy water on mitochondrial respiration and oxidative phosphorylation revealed that both isotope and solvent effects, may be responsible for the observed changes. Although the two types of effects could not be totally delineated from each other by the experimental approaches employed, the isotope effect appeared to be primarily responsible for the uncoupling of oxidative phosphorylation, while the inhibition of respiration in the presence of ADP (State 3 respiration) could be a manifestation of the solvent effect.

Keywords. Isotope effect; solvent effect; heavy water.

Introduction

Earlier observations indicated the participation of water or the aqueous phase in biological oxidation-reduction reactions. Heavy water and several organic solvents were found to be 'nonspecific' inhibitors of the respiratory chain activity and the inhibitory action could not be attributed to solvent viscosity (Tylor and Estabrook, 1966). Shibata and Watanbe (1949) found that D_2O inhibited several oxidizing enzymes including mushroom cytochrome *c* oxidase (EC 1.9.3.1).

The respiration of both intact mitochondria and of non-phosphorylating particles [in the presence of ADP+ phosphate, or of DNP (Baum and Rieske, 1966; Margolis *et al*., 1966; Tylor and Estabrook, 1966)] were inhibited by D_2O. It also inhibited the transfer of electrons between cytochromes *b* and *c*, in complex III (Baum and Rieske, 1966). The steady-state reduction of nicotinamide nucleotide and the cytochromes in a D_2O-inhibited system and in water (Tylor and Estabrook, 1966) were similar. Heavy water also inhibited the energy linked nicotinamide nucleotide transhydrogenase (EC 1.6.1.1) and the Mg^{2+} stimulated ATPase, (EC 3.6.1.3), but had little effect on the ATP-Pi exchange reaction or on DNP-induced ATPase (Margolis *et al*., 1966). The P : O ratio of phosphorylating

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Abbreviations used: cyt, cytochrome; ATPase, adenosinetriphosphatase; RCI, respiratory control index.

submitochondrial particles was markedly greater (Margolis *et al.*, 1966) and of the intact mitochondria slightly greater in D_2O than in H_2O. The decrease of the P : O ratio brought about by uncouplers was not affected by D_2O (Baum and Rieske, 1966; Laser and Slater, 1960).

The present study was undertaken to examine, in detail, the effects of heavy water on mitochondrial respiration and oxidative phosphorylation to gain some insight into the relationship of the respiratory chain components to their environment. The results suggest that water plays a major role in the interaction of the components of the electron transport chain during oxidative phosphorylation.

Materials and methods

Adenosine triphosphate, cytochrome c, sodium succinate, glucose, trichloroacetic acid, ascorbic acid, tris (hydroxymethyl) aminomethane, ethylenediaminetetraacetic acids adenosine diphosphate and hexokinase were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Sucrose and other inorganic chemicals of Analar grade were obtained from British Drug House Ltd., Poole, England.

Male albino rats of Wistar strain, weighing around 120-140 g, and maintained on laboratory stock diet, were used in these studies. Rats were killed by cervical dislocation, the livers were quickly removed, cleaned and chilled in 0.25 M sucrose. Livers were homogenised in 0.25 M sucrose to yield 10% homogenates. Mitochondria were isolated according to the method of Hogeboom (1955) and washed once with 0.25 M sucrose.

In one set of experiments, the mitochondrial pellet was suspended in 0.25 M sucrose in H_2O. The mitochondrial suspension was saturated with oxygen by gently bubbling the gas through it. This has been designated as preparation I.

In another set of experiments the mitochondrial pellet was suspended in 0.25 M sucrose in D_2O and was saturated with oxygen. This suspension was prepared just 10-15 min. before starting the reaction. This has been designated as preparation II.

The method of Yost *et al.,* (1967) was used for manometric determination of P/O ratio. The main vessel of the Warburg flask contained: sucrose, 250 µmol; sodium succinate, 40 µmol; potassium phosphate buffer, pH 7.4 (pD 7.8) 36 µmol; cyt c, 0.09 µmol; ATP, .6 µmol; $MgSO_4$, 15 µmol; potassium fluoride, 65 µmol and hexokinase 10 mg in 0.15 M glucose. The central well contained 0.1 ml of 5 N potassium hydroxide along with fluted filter paper strips. The total volume of the reaction mixture was 3.2 ml. The reaction was carried out at 25°C for 30 min. Oxygen consumption was determined manometrically and esterification of phosphate was determined by measuring orthophosphate before and after the incubation (Lowry and Lopez, 1946).

For the Polarographic determination of oxygen uptake and ADP/O ratio,an oxygraph (Gilson Medical Electronics, Wisconsin, USA.) was used. The method followed was that described by Chance and Williams (1955). The reaction medium consisted of 38 mM NaCl; 46 mM KCl; 12 mM KF; 6 mM $MgCl_2$; 16 mM potassium phosphate buffer, pH 7.4 (pD 7.4); 4.8 mM sodium succinate; 0.4 µmol of ADP and 1-1.5 mg mitochondrial protein in a total volume of 2 ml.

The assay of mitochondrial ATPase was carried out by the method of Veldesma-Currie and Slater (1968) with some modifications (Katyare *et al.*, 1971). The reaction system contained 74 mM KCl, 50 mM sucrose, 50 mM Tris-HCl, pH 7.4 0.5 mM EDTA, 8 mM $MgCl_2$, 1.2 to 1.5 mg mitochondrial protein in a total volume of 2.0 ml. The reaction was carried out at 25°C for 15 min and was stopped by the addition of 0.5 ml of chilled 10% trichloroacetic acid (w/v). Appropriate zero time controls were run simultaneously. The tubes were immediately chilled and centrifuged at 3,000 *g* for 10 min. Orthophosphate in the supernatant fraction was estimated by the method of Fiske and Subbarowi (1925).

Results and discussion

From table 1, it could be observed that the oxygen consumption with succinate as substrate decreased progressively with increase in the concentration of D_2O. With

Table 1. Effect of D_2O on mitochondrial (preparation I) respiration and P/O ratio.

D_2O (%)	Mitochondrial respiration (μ atoms/h /mg protein)	% Control	Pi esterification (μ moles/h /mg protein)	% Control	P/O
0	1.6±0.5	100	2.9±0.04	100	1.8
20	1.2±0.5	77	2.3±0.01	79	1.9
40	1.2±0.8	75	2.3±0.07	79	2.0
60	1.0±0.4	65	1.9±0.01	65	1.8
80	1.0±0.3	62	1.6±0.01	57	1.7
100	0.8±0.1	49	1.4±0.02	48	1.8

Mitochondrial pellet (approx. 6-8 mg protein) suspended in an incubation mixture (total volume of 3.2 ml) containing the various additions as indicated in text was determined by manometry. The reaction was carried out at 25°C for 30 min. Results are averages of five independent experiments ±SEM.

total replacement of H_2O by D_2O, oxygen consumption was about 50% of the control value. A similar trend was seen with esterification of Pi and at all concentrations of D_2O, the decrease in oxygen consumption was accompanied by a corresponding change in esterification of Pi· As a result, the P/O ratio remained unaltered.

With the mitochondrial preparation preincubated in heavy water 15 min prior to the reaction (preparation II), oxygen consumption even with no D_2O (table 2) in the incubation medium, was significantly lower than that of mitochondrial preparation I (table 1). The preincubated mitochondria also showed a gradual decrease in oxygen consumption with increasing concentrations of D_2O. A 50% decrease in oxygen uptake over control was observed at ≈ 100% D_2O.

The state 3 respiration of mitochondria from preparation I, exhibited an initial enhancement of oxygen uptake with increase in D_2O concentration upto 80%, the increase being maximum at a D_2O level of 40% (table 3). However, at total

Table 2. Effect of D_2O on mitochondrial (preparation II) respiration and P/O ratio.

D_2O (%)	Mitochondrial respiration (μ atoms/h/mg protein)	% Control	Pi esterification (μmol/h/mg protein)	% Control	P/O
0	1.0±0.02	100	1.8±0.01	100	1.8
20	0.8±0.08	78	1.4±0.03	79	1.8
40	0.7±0.02	69	1.3±0.02	71	1.8
60	0.6±0.07	61	1.1±0.01	62	1.8
80	0.6±0.04	60	1.1±0.05	60	1.8
100	0.5±0.01	51	0.9±0.04	50	1.8

Experimental details were as shown under Table 1 with the only exception that the mitochondrial suspension was also prepared in 0.25 M sucrose in D_2O. Results are averages of five independent experiments ± S E M.

Table 3. Effect of D_2O on oxygen uptake and ADP/O, ratios by mitochondria (preparation I).

D_2O (%)	Oxygen uptake (State 3 respiration) (μ atom/min/mg protein)	% Control	Oxygen uptake (State 4 respiration) (μatoms/min/mg protein)	% Control	RCI	ADP/O
0	0.1±0.008	100	0.04±0.016	100	3.6	1.8
20	0.2±0.007*	137.6	0.05±0.019	148	3.3	0.9
40	0.2±0.075	143.8	0.04±0.012	119	4.3	0.9
60	0.2±0.099	118.1	0.03±0.009	76	5.5	0.9
80	0.1±0.072	106.3	0.03±0.010	71	5.4	1.0
100	0.1±0.038	56.3	0.02±0.008	52	3.8	1.8

Mitochondrial pellet (approx. 50-60 mg) was suspended in a total volume of 1 ml of 0.25 M sucrose in H_2O and 0.02 ml of this suspension was used for determining O_2 consumption using an oxygraph. The reaction was carried out at 25°C for 5 min. Results are averages of six independent experiments ±SEM. * $P<0.01$.

replacement of H_2O by D_2O (≈100% D_2O) the State 3 respiration decreased below control values. In the absence of ADP (State 4), mitochondria from preparation I showed an increase in the oxygen uptake with increasing concentrations of D_2O, upto 40% D_2O, but decreased to below control values at 60% D_2O and higher concentrations. The respiratory control index (RCI) was found to increase with increasing concentrations of D_2O, with mitochondria from preparation I, the maximum value being seen at 60% D_2O, (table 3), whereas at total replacement of H_2O by D_2O (100%) the RCI value was not significantly different from that of control. The ADP/O ratio was significantly reduced at all concentrations of D_2O, except at the highest concentration (≈100%) D_2O in the incubation medium, the ADP/O ratio was similar to that of the control mitochondria.

With a mitochondrial preparation preincubated in D_2O (preparation II) there was a significant D_2O concentration dependent reduction in State 3 respiration (table 4). A similar trend was seen in the O_2 uptake in the absence of ADP (State 4).

Table 4. Effect of D_2O on oxygen uptake and ADP/O, with mitochondria suspended in sucrose in D_2O (preparation II).

D_2O (%)	Oxygen uptake (State 3 respiration) (μ atoms/min/mg protein)	% Control	Oxygen uptake (State 4 respiration) (μ atoms/min/mg protein)	% Control	RCI	ADP/O
0	0.08±0.002	100	0.03±0.003	100	2.9	1.8
20	0.07±0.006	86	0.03±0.002	98	2.6	1.7
40	0.06±0.031	77	0.02±0.000	79	2.9	1.7
60	0.06±0.011	76	0.02±0.006	68	3.1	1.9
80	0.06±0.021	73	0.02±0.001*	60	3.5	2.0
100	0.04±0.041	50	0.02±0.040	55	2.6	1.9

Experimental details were as shown under Table 3 with the only exception that the mitochondrial suspension was also prepared in 0.25 M sucrose in D_2O. Results are averages of six independent experiments ±SEM. * $P<0.01$.

In both cases, with 100% D_2O in the incubation medium, the oxygen consumption decreased by 50% as compared to controls. The RCI values of preparation II were less than those of preparation I and was probably indicative of a poorer integrity of the mitochondrial membranes. It would appear that exposing the mitochondrial preparation to D_2O 15 min prior to the reaction, itself caused some structural changes, resulting in the loss of integrity to some extent. However, there did not seem to be any further damage to mitochondrial membranes with increasing concentrations of D_2O, as seen from RCI values. The ADP/O ratio was similar to control value at any concentration of D_2O.

With mitochondria from preparation II, there was considerable decrease in State 3 respiration even in the absence of heavy water in the reaction mixture (0% D_2O), when compared to that of preparation I (table 4 and table 3). A further decrease in oxygen consumption in the presence of ADP, (State 3), was observed at increasing concentrations of D_2O. The same trend was observed with State 4 respiration.

When the data obtained from manometric and Polarographic studies are compared, certain similarities as well as apparent inconsistencies are observed. With preparation I, whereas, mitochondrial respiration was consistently decreased as assessed in the manometric studies State 3 respiration showed a two way variation when determined polarographically. The P/O ratio determined in the experiments also showed a trend that was different from that of the ADP/O ratio in the oxygraph measurement. On the other hand, in the case of preparation II, the trends in oxygen consumption as well as the degree of coupling were similar in the manometric and the Polarographic studies. Thus, while oxygen consumption

showed a decrease with increasing concentration of D_2O, the degree of coupling appeared unaffected.

It seems likely that these discrepancies are more apparent than real. The Polarographic studies are completed within a few minutes, whereas the manometric studies require about 30 min of incubation. Thus while the former may permit a clearer distinction being drawn between the solvent effect (more time-dependent) and the isotope effect (requires D_2O only at the time of reaction), the latter may not. Thus with preparation I, which is not preincubated with D_2O, the Polarographic study could be expected to give a better assessment of the isotope effect than the manometric studies, as the latter necessarily requires a long period of incubation. An analysis of the data, bearing these considerations in mind, indicated that the inhibition of mitochondrial respiration might be a manifestation of the solvent effect. This conclusion is in accordance with an earlier reported observation on the rate of dehydrogenation of dideutero and tetradeutero succinates by succinoxidase (Thorn, 1951). These compounds were found to be dehydrogenated less rapidly than protosuccinate, the extent of inhibition increasing linearly with increasing concentration of deuterium. The Michaelis constant for deuterosuccinate (Thorn, 1951) was 1.5 times that for protosuccinate.

The nature of the influence of water on respiratory chain reaction can at present be discussed only in general terms. There seems to be universal retardation of half-times of various biological electron transfer reactions involving membrane bound electron carriers in photosynthetic bacteria and mitochondria in the presence of heavy water (Kihara and McRay, 1973). Uncoupling of oxidative phosphorylation is essentially an isotope effect of heavy water. It would further appear that where both types of effects may be operative, the former masks the latter.

To understand the possible mechanism of the uncoupling action of D_2O, the activity of mitochondrial ATPase has been studied in the presence of D_2O. The effects of heavy water on mitochondrial ATPase are shown in table 5. D_2O is seen to enhance the basal mitochondrial ATPase activity significantly, when mitochondria from preparation I were used as enzyme source. On the other hand, with mitochondria preincubated in D_2O (preparation II) as the enzyme source, when the solvent effect may be expected to predominate, there was found to be a decrease in the activity of mitochondrial ATPase. Thus, the enhancement of mitochondrial ATPase activity, seems probably, due to the predominance of isotope effect. These results are in accordance with Polarographic studies. However, in presence of Mg^{2+}, both with preparation I and preparation II, there was found to be a marked inhibition in the activity of mitochondrial ATPase. This is in confirmation with the earlier reported data (Margolis *et al.*, 1966). With either DNP alone or along with Mg^{2+} , the effect of D_2O was found to be one of inhibition, both with preparation I and preparation II.

Table 5. Effect of D_2O on mitochondrial ATPase.

Experimental conditions	Sample	ATPase activity (μmol/h/mg protein)	% Control
Basal	Control	0.34±0.083	100
	Preparation I	1.31±0.041	380
	Preparation II	0.27±0.036	79
Basal+ Mg^{2+}	Control	1.69±0.089	100
	Preparation I	0.29±0.005	17
	Preparation II	0.15±0.040	9
Basal+ DNP	Control	5.89±0.489	100
	Preparation I	4.05±0.400	69
	Preparation II	4.83±0.748	82
Basal+ DNP+ Mg^{2+}	Control	8.55±0.374	100
	Preparation I	4.05±0.400	47
	Preparation II	4.00±0.346	47

The incubation was carried out at 25°C for 15 min. In control samples, no heavy water was used, either during homogenisation or during incubation, wherever indicated, DNP was used at a concentration of 100 μmol. Results are averages of six independent experiments ±SEM.

It has also been separately ascertained whether some of the observed effects of heavy water would arise from the difference in the solubility of oxygen in water and heavy water. The oxygen solubility in heavy water was not found to be significantly different from that in water under the experimental conditions employed (Approved Methods for the Physical and Chemical Examination of Water published by the Institute of Water Engineers, 1953).

The mechanism of action of D_2O may be distinguished from the effect of an inhibitor such as malonate that restricts the entry of reducing equivalents into the respiratory chain. Inhibition of respiration by malonate has no effect on the P/O ratio (Van Dam *et al.,* 1966), but increases the NADPH : O ratio of the energy linked transhydrogenase (Lee and Ernster, 1966).

Our data seem to be consistent with the following hypotheses postulated by earlier investigators (Tylor and Estabrook, 1966): 1) Replacement of water by D_2O may alter the tertiary structure of the functional proteins of the respiratory chain, either by influencing the extent of their hydration by water or by inducing changes in hydrogen bonding; the modification of tertiary structure may lead to an alteration in the spatial relationship of the components of the respiratory chain and thereby reduce the efficiency of their interaction. 2) Water is directly involved in the electron transfer process itself and there exists an array of oriented water molecules between carriers of electrons, as cellular water has been shown to be more structural than pure water (Chang *et al.*, 1972) and electron transfer takes place via the effective diffusional transfer of a hydrogen atom through a water bridge.

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