OBSERVATIONS ON THE EFFECT OF D_2O ON ENERGY-LINKED REACTIONS OF THE MITOCHONDRION[1]

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In this communication we provide evidence that using D_2O instead of water as the solvent medium selectively inhibits certain processes in mitochondrial energy transfer. The mechanism of this inhibition may as yet be obscure but it is considered that any conditions which selectively inhibit portions of a complex pathway such as oxidative phosphorylation are useful in the study of that pathway.

It is known that the respiration of intact mitochondria, of submitochondrial particles, and of isolated complexes of the electron transfer chain is inhibited in D_2O (1,2,3). It has also been reported (1) that the degree of this inhibition in D_2O, in the case of phosphorylating particles, is decreased by uncouplers.[5] This finding, (together with observations on the effect of a D_2O medium on the pattern of spectral changes during electron transfer in isolated

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[5]In the case of certain preparations of mitochondria where the rate of State 3 respiration is much lower than that in the presence of uncouplers, it has been found that the former respiratory rate is not inhibited by D_2O. This is in spite of the fact that both State 4 and uncoupled respiration are considerably inhibited. It is possible that in this case State 3 respiration is not limited by electron transport but by some transfer process across the outer mitochondrial membrane; Baum, H., and Ozawa, T., unpublished observations.

complexes of the electron transfer chain (2)), appeared to relate the inhibition of respiration to the sites of energy transduction in the electron transfer chain. We have therefore studied the effects of using D_2O as the solvent medium on both the rate and the efficiency of two energy utilizing processes, namely energy linked transhydrogenation between pyridine nucleotides and oxidative phosphorylation. Results of these studies are presented here.

Materials and Methods

Fresh heavy beef heart mitochondria (HBHM[6]) were prepared by the method of Hatefi and Lester (4) or by use of the proteolytic enzyme, Nagarse (5) (N-HBHM) and ETP_H were prepared by the method of Hansen and Smith (6). Respiration during oxidative phosphorylation was measured at 30° in an oxygraph (Gilson Medical Electronics, Middleton, Wisconsin) equipped with a 2 ml cell. Phosphorylation was estimated on samples removed from the oxygraph cell by the isotope distribution method described by Lindberg and Ernster (7). Transhydrogenation was assayed either spectrophotometrically (by following the increase in absorbancy at 340 mμ) or by enzymic determination of the TPNH formed by the method of Lee and Ernster (8). The ATPase activity was determined by measuring the liberation of inorganic phosphate (9) and the ATP-P_i exchange was determined by measuring the incorporation of ^{32}P into organic phosphate (7). The media were prepared by lyophilizing measured amounts of aqueous solutions of sucrose, $MgCl_2$, EDTA, BSA (when indicated) and Tris-acetate buffers (when indicated), and dissolving the residues in the appropriate volumes either of water or deuterium oxide. In all experiments the final deuterium oxide concentration was 92-96%. The pH was measured with a Beckman pH meter and corrected according to the method of Lumry et al. (10). The composition of the various media used and the conditions of incubation are described in the legends of the tables. EDTA was routinely added to the media, because some samples of D_2O were contaminated by

[6] The following abbreviations will be used: HBHM, heavy beef heart mitochondria; ETP_H, phosphorylating submitochondrial particles from HBHM; BSA, bovine serum albumin; EDTA, ethylene diamine tetraacetic acid; ADH, yeast alcohol dehydrogenase; DNP, 2,4 dinitro-phenol; pF_3CO-CCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

traces of heavy metals.

Results and Discussion

When the nonenergy-linked transhydrogenase activity of ETP_H was studied in H_2O and in D_2O (under conditions in which pH equalled pD) no inhibition of the reaction was observed. This was not surprising in view of the fact that the transhydrogenation involves a direct transfer of hydrogen with no known involvement of water molecules or protons in the reaction mechanism (11). However, it was found (cf. Table I) that the energy-linked transhydrogenase activity of these particles was substantially inhibited in D_2O, whether the reaction was driven by oxidation of substrate (succinate) or by ATP. Since the terminal step(s) in transhydrogenation, both energy-linked and nonenergy-linked, are prob-

TABLE I

The Effect of D_2O on Transhydrogenase Activities in ETP_H

Additions	Rate of Transhydrogenation (mμmoles TPNH/min/mg) H_2O	D_2O
Oligomycin + Antimycin A	12.7	12.3
Succinate (10 μmoles) + Oligomycin	222	115
ATP (10 μmoles) + Antimycin A	227	111

The incubation medium contained, in 1 ml, 250 μmoles of sucrose; 60 μmoles of Tris-acetate, pH 7.8 (H_2O) or pD 7.8 (D_2O)*; 10 μmoles of $MgCl_2$; 0.25 μmoles of EDTA; 0.2 μmoles of DPNH; 0.092 mg of ETP_H protein; 0.1 mg of ADH; 2 μg of rotenone; 2 μg of antimycin A, and/or 2 μg of oligomycin; 0.2 μmoles of TPN, and BSA at a final concentration of 0.1%. Absorbancy at 340mμ (1 ml cuvette) was measured every 30 sec in a Beckman DU spectrophotometer at 37°; the rate of TPNH production was calculated from the linear portion of the curve.

*The pH of the buffer used in the reaction mixtures was adjusted in H_2O before lyophilization so that the pD of the reaction mixture subsequently obtained by dissolving the lyophilized material in D_2O was equal to the pH of the corresponding aqueous reaction mixture (10). Under these conditions the rates of nonenergy linked transhydrogenase in each solvent were optimal and identical.

ably catalyzed by the same enzyme (11,12), the site(s) of inhibition in D_2O are presumed to involve the generation and/or transfer of the high energy intermediate which ultimately drives the energy-linked reaction.

The generation of high energy intermediates by the electron transfer chain is not considered to be a rate-limiting process in transhydrogenation, because inhibition of respiration does not initially inhibit the former process but merely increases its apparent efficiency (8). Thus, the inhibition in D_2O of substrate-driven transhydrogenation could not be due simply to the known parallel inhibition of respiration. This point is illustrated in Table II. In the case of inhibition of respiration by malonate, transhydrogenation in water

TABLE II

The Effects of D_2O and Malonate on the Efficiency of Succinate-driven Transhydrogenation in ETP_H

Medium	Malonate*	Rate of transhydrogenation**	Respiratory Rate***	$\frac{(TPNH)}{O}$†
H_2O	-	200	345	0.58
D_2O	-	102	160	0.64
H_2O	+	189	183	1.06
D_2O	+	88	87	1.02

The incubation medium contained per ml: 250 μmoles of sucrose; 60 μmoles of Tris-acetate pH 7.8 (H_2O) or pD 7.8 (D_2O);† 10 μmoles of $MgCl_2$; 0.25 μmoles of EDTA; 0.1% BSA (final concentration); 0.092 mg of ETP_H: 1 μg of rotenone; 1 μg of oligomycin; .05 mg of ADH; 0.2 μmoles of DPNH; 0.5 μmoles of semicarbazide; and 5 μmoles of succinate. The reaction mixture was preincubated for 3 min in the oxygraph cell; the reaction was started by the addition of 0.2 μmoles of TPN per ml, and was stopped with 0.2 ml of 10 N H_2SO_4 after it had been incubated for 4 minutes at 30°. The rate of transhydrogenation was determined by the method of Lee and Ernster (8).

* 0.55 μmoles per ml.

** mμmoles of TPNH per min per mg of protein.

*** mμatoms of oxygen per min per mg of protein.

† Ratio of rates in previous two columns.

† See footnote to Table I.

was energized by oxidation of succinate with an increased efficiency (as measured by the TPNH:O ratio). On the other hand, when the same degree of respiratory inhibition was imposed by using a D_2O medium, the rate of transhydrogenation decreased in parallel; consequently the TPNH:O ratio did not increase. Table II also shows that, in the D_2O system, further inhibition of respiration by malonate had little effect on the rate of transhydrogenation, with a concomitant increase in the TPNH:O ratio.

One possible interpretation of these findings is that the inhibition of respiration in D_2O was quite unrelated to the inhibition of energy-linked transhydrogenation. This would imply that the latter inhibition was the result of an effect of the D_2O medium on some process involving (directly or indirectly) the utilization of the first high-energy intermediate, rather than its formation by coupled respiration. An alternative interpretation is that the inhibition of respiration in D_2O was a specific process which was related to the nature of the primary energy-conserving step; that is, the first high energy intermediate formed in D_2O differed in some way from that formed in water. The disposition of such an intermediate (e.g. transhydrogenation) would thus be affected to the same extent as was its formation (coupled respiration).

In Table III are summarized data relating to the inhibition in D_2O of certain reactions involving ATP. The ATP-P_i exchange reaction is believed to reflect the terminal span of the pathway of oxidative phosphorylation and to involve the site at which ATP reacts in the case of ATP energized reactions in mitochondria. Under conditions corresponding to those of the transhydrogenase assay, this exchange reaction was inhibited in D_2O only to a marginal degree. Indeed, in a modified assay system (incubation for 10 instead of 5 min) the inhibition was negligible. On the other hand, the Mg^{++}-dependent ATPase activity of the system was substantially inhibited in D_2O. Presumably, this activity relates to the breakdown of a high energy intermediate generated by ATP. The inhibition of the ATPase, therefore, could have been due either to an inhibition of the rate of generation of this intermediate or of

TABLE III

The Effect of D_2O on ATP-P_i Exchange and ATPase in ETP_H

Reaction	Activity		Inhibition by D_2O
	H_2O	D_2O	
ATP-P_i** exchange	41	36	12%
ATPase (Mg^{++})	954	416	56%
ATPase (Mg^{++} + DNP)	1875	1162	---
DNP-stimulation of ATPase*	921	746	18%

The ATPase reaction mixture contained in a total volume of 1 ml: 250 µmoles of sucrose; 60 µmoles of Tris-acetate buffer, pH 7.8 (H_2O) or pD 7.8 (D_2O)†; 10 µmoles of $MgCl_2$; 0.25 µmoles of EDTA; 0.1% BSA (final concentration); 0.274 mg of ETP_H protein and 100 mµmoles of DNP (when indicated). The reaction was started by the addition of 5 µmoles of ATP, was incubated for 3 min at 30° and was stopped with 3.5 ml of silicotungstate solution (7). The ATP-P_i exchange reaction mixture was identical except for the addition (per ml) of 20 µmoles of $^{32}P_i$ (potassium salt) (134×10^3 cpm per µmole) and 0.548 mg of ETP_H protein. The reaction was started by the addition of enzyme; following incubation for 5 min at 30°, 0.2 ml of the reaction mixture was added to 4.3 ml of silicotungstate solution (7).

* mµmoles of P_i liberated per min per mg of protein.

** mµmoles $^{32}P_i$ exchanged per min per mg of protein.

† See footnote to Table I.

its rate of breakdown. The finding (cf. Table III) that the DNP-stimulated ATPase was much less sensitive to the use of a D_2O medium than was the unstimulated, (Mg^{++}-dependent), ATPase indicated that the rate of generation by ATP of nonphosphorylated high energy intermediates was not significantly inhibited in D_2O. Thus, this conclusion localized the site of inhibition in D_2O of ATP-energized transhydrogenation as the point of utilization of some nonphosphorylated high energy intermediate.

Three processes relating to high energy intermediates appeared to be inhibited in D_2O; the generation of the high energy intermediates (respiration); their utilization (transhydrogenation); and their breakdown (Mg^{++}-dependent

TABLE IV

The Effect of D_2O on Respiratory Rates and the Efficiency of Oxidative Phosphorylation

Particles	Medium	Respiratory Rate (μatoms O/min/mg)		P/O		
		H_2O	D_2O	Assay	H_2O	D_2O
N-HBHM	Sucrose	115	72	AMP/O*	1.85	2.04
N-HBHM	Mannitol	114	48	AMP/O	1.95	2.5
HBHM	Sucrose	341	186	^{32}P/O**	2.28	2.72
ETP_H	Sucrose	275	191	^{32}P/O	0.67	0.98

One ml of the sucrose medium contained 250 μmoles of sucrose; 10 μmoles of $MgCl_2$; and 0.25 μmoles of EDTA. The mannitol medium was identical to that described by Chance and Hagihara (13).

*For the determination of AMP/O ratios each ml of medium also contained: 10 μmoles of potassium phosphate, pH 7.4; 0.67 mg of HBHM protein; and 1.5 μmoles each of pyruvate and malate. The respiratory control was released by the addition of 300 mμmoles of AMP; P/O ratios were calculated from the number of moles of AMP added and the atoms of oxygen consumed during State 3 respiration (14).

**For the P/O ratios, each ml of the sucrose medium also contained: 1.67 μmoles of ADP; 33 μmoles of glucose; 0.033 mg of hexokinase; 10 μmoles of potassium phosphate (^{32}P) (100,000 cpm per μmole) pH 7.4;and either 0.67 mg of HBHM protein or 0.61 mg of ETP_H protein. The reactions were initiated with 3 μmoles each of pyruvate and malate (HBHM); or with 25 μmoles of succinate (ETP_H).

ATPase). Two processes appeared not to be affected: the ATP-P_i exchange segment of the pathway of oxidative phosphorylation and the nonenergy linked transhydrogenase. It was, therefore, of interest to re-examine the effect of D_2O on oxidative phosphorylation. In agreement with Tyler and Estabrook (3) we found that phosphorylation in D_2O was not inhibited to the same extent as was respiration in D_2O. Thus, under a variety of conditions (cf. Table IV) the P/O ratio observed in D_2O was increased towards, but never in excess of, the "theoretical" ratio. Presumably, these findings represented a summation of the decrease in the rate of generation of high energy intermediates (in parallel with the decrease in the rate of coupled respiration) plus a decrease in

their rate of breakdown, (in confirmation of the inhibition of the Mg^{++}-dependent ATPase, Table III).

We have previously reported (15) that uncouplers of oxidative phosphorylation, such as pF_3CO-CCP, have a very high affinity only for high energy intermediates; therefore, the amount of uncoupler required for a given degree of uncoupling was proportional to the rate of generation of such intermediates. Thus, when respiration was inhibited by malonate, rotenone, or cyanide, HBHM became progressively more sensitive to uncoupling by pF_3CO-CCP. Fig. 1 shows that this was not the case when respiration was inhibited by the use of a D_2O medium. The simplest interpretation of this finding was that, although the rate of generation of high energy intermediates was reduced in D_2O, the rate of uncoupler-mediated breakdown was also decreased to a similar extent.

We have thus shown that in a D_2O medium three processes related to the energy conservation pathways of mitochondria are inhibited to a similar degree. These processes may be summarized as the formation, utilization and breakdown of high energy intermediates. It is, therefore, tempting to speculate that the inhibitions which we have described are not independent phenomena but have a common basis in the nature of the primary energy-conserving process in mito-

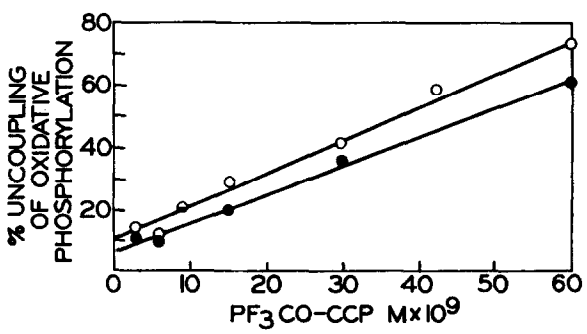

Fig. 1. The effect of deuterium oxide on the uncoupling of phosphorylation by pF_3CO-CCP in HBHM. The symbol O represents the results of experiments performed in D_2O; ● those performed in water. One ml of medium contained: 250 μmoles of sucrose; 10 μmoles of $MgCl_2$; 0.25 μmoles of EDTA; 1.67 μmoles of ADP; 0.05 mg of hexokinase; 10 μmoles of ^{32}P potassium phosphate, pH 7.4 (111,000 cpm per μmole); 0.67 mg of mitochondrial protein and varying amounts of pF_3CO-CCP. The reaction was started with 3 μmoles each of pyruvate and malate. The temperature was 30°.

chondria. There would then be several possible explanations of inhibitions of the magnitude we have described. One would be an effect of D_2O on the conformation of some protein(s) involved in the primary coupling process. Another would be that, in water, an unsolvated proton is involved in the respiration-coupled formation of the first "high energy intermediate".

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