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THE EFFECT OF DEUTERIUM OXIDE ON RESPIRATORY-CHAIN PHOSPHORYLATION IN SUB-MITOCHONDRIAL PARTICLES

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SUMMARY

1. The P:O ratio of sub-mitochondrial particles prepared by sonication at pH 8.6 is stimulated by both ^2H_2O and by oligomycin. The stimulations are approximately equal and are additive.

2. The stimulatory effect of ^2H_2O is also additive to that of F_1-X (ref. 9).

3. The P:O ratio of sub-mitochondrial particles prepared by sonication at pH 7.4, which is not affected by low concentrations of oligomycin, is also stimulated by ^2H_2O.

4. The increase in the P:O ratio brought about by ^2H_2O is primarily caused by an inhibition of the oxygen uptake. Phosphorylation is either not affected, or is little affected.

5. The results support the view that the hydrolysis of an intermediate of oxidative phosphorylation is slower in ^2H_2O than in H_2O. If this interpretation of the effect of ^2H_2O is correct, it would seem unlikely that the stimulatory effects of oligomycin and F_1-X are due to inhibition of the hydrolytic reaction that is affected by ^2H_2O.

In 1949 SHIBATA AND WATANABE[1] found that ^2H_2O inhibits the action of several oxidizing enzymes including mushroom cytochrome *c* oxidase, and suggested that "activated water" is concerned not only in hydrolytic enzymes, but also in oxidizing enzymes.

In 1953 SLATER[2] suggested that in non-phosphorylating preparations of the respiratory chain a hydrolytic reaction (Eqn. 2) is required for the release of components of the respiratory chain from bound (high-energy) forms synthesized by an energy-conserving reaction (Eqn. 1).

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2 \quad (1)$$

$$A \sim C + H_2O \rightarrow A + C \quad (2)$$

LASER AND SLATER[3] brought forward as evidence in support of this view the observation that those oxidoreductions that in intact mitochondria are coupled with phosphorylation (supposedly *via* Eqn. 3),

$$A \sim C + ADP + P_i \rightleftharpoons A + C + ATP \quad (3)$$

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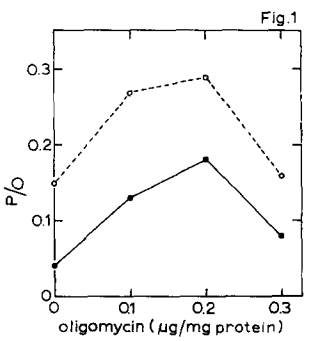
are inhibited by ^2H_2O in sub-mitochondrial particles in which the phosphorylation reaction is lacking. Simpler hydrogen transfers to methylene blue proceed at the same rate in H_2O and ^2H_2O. SLATER[4] concluded that a reaction involving the hydrogen atoms of water is a rate-limiting step of the respiratory chain of non-phosphorylating preparations and that replacement of H by ^2H slows down this reaction.

The effects of ^2H_2O have recently been studied by TYLER AND ESTABROOK[5], BAUM AND RIESKE[6] and MARGOLIS, BAUM AND LENAZ[7]. The results in these and the earlier studies[3,4] may be summarized: (1) The respiration of both non-phosphorylating particles and of intact mitochondria (in the presence of ADP + phosphate, or of dinitrophenol) is inhibited[3,5–7]. (2) The P:O ratio of phosphorylating sub-mitochondrial particles is markedly[7] and of intact mitochondria slightly[5] greater in ^2H_2O medium than in H_2O. (3) The steady-state reduction of nicotinamide nucleotide and the cytochromes is the same in a ^2H_2O-inhibited system as in water[5]. (4) ^2H_2O also inhibits electron transfer between cytochromes *b* and c_1 in Complex III (ref. 6). (5) Glycerol[5,6] and other organic solvents[5] and a high pH (ref. 6) inhibit the oxidation reactions in the same way as ^2H_2O. The degree of inhibition by organic solvents is correlated with the decrease of the concentration of water[5]. (6) ^2H_2O inhibits the energy-linked nicotinamide nucleotide transhydrogenase (without any effect on the NADPH:O ratio) and the Mg^{2+}-stimulated ATPase, but has little effect on the ATP–P_i exchange reaction or on the dinitrophenol-induced ATPase[7]. (7) The decrease of the P:O ratio brought about by uncouplers is not affected by ^2H_2O (refs. 3, 7).

Although the stimulation of the P:O ratio by ^2H_2O is in agreement with the mechanism of action of ^2H_2O proposed by LASER AND SLATER[3,4], other effects of ^2H_2O described, in particular the inhibition of respiration of intact mitochondria and



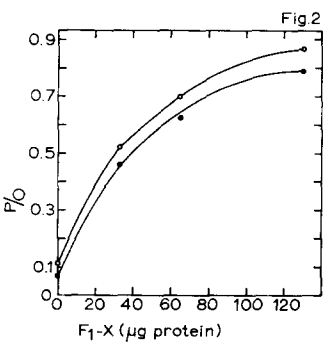


Fig. 1. Effect of oligomycin concentration on the P:O ratio of sub-mitochondrial particles in 96.7% ^2H_2O and in water. The reaction mixture contained 25 mM Tris–HCl buffer (pH 7.4), 0.14 M sucrose, 20 mM phosphate, 1 mM ADP, 32 mM succinate, 20 mM glucose, 3 mM $MgCl_2$, 1 mM EDTA, 0.1 μg rotenone, 39 units (μmole/min) hexokinase, 2% ethanol and 1 mg particles in a total volume of 1 ml. The sub-mitochondrial particles were obtained by sonication at pH 8.6. O---O, in 96.7% ^2H_2O; ●—●, in H_2O.

Fig. 2. Effect of the concentration of coupling factor (F_1-X) (ref. 9) on the P:O ratio of sub-mitochondrial particles in 98% ^2H_2O and H_2O. 0.5-mg particles were preincubated at room temperature in 0.3 ml of a solution containing 2 μmoles $MgSO_4$, 2 μmoles ATP, 30 μmoles potassium phosphate (pH 7.4) and various amounts of F_1-X. After 5 min the reaction was started by adding 0.2 ml of a solution containing 1 μmole $MgSO_4$, 0.5 μmole ATP, 16 μmoles glucose, 2.5 μmoles Tris–sulphate buffer (pH 7.4), 0.25 μmole EDTA, 10 μmoles sodium succinate, 0.5 mg bovine serum albumin, 30 units of hexokinase and 300000 counts/min carrier-free $^{32}P_i$. The sub-mitochondrial particles were obtained by sonication at pH 8.7. O—O, in 98% ^2H_2O; ●—●, in H_2O.

the inhibition of the energy-linked transhydrogenase in sub-mitochondrial particles are not. For this reason further studies were carried out.

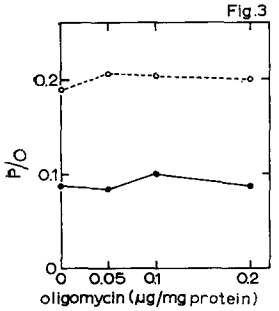
Fig. 1 shows the effect of 96.7 % ^2H_2O on the P:O ratio of sub-mitochondrial particles, prepared by sonication at pH 8.6, at various concentrations of oligomycin. The stimulatory effect of ^2H_2O is about the same as, and is additive to, that of oligomycin[8], and the optimal concentration of oligomycin is about the same in ^2H_2O as in H_2O. The stimulatory effect of ^2H_2O on the P:O ratio is also additive to that of the coupling factor F_1-X (ref. 9), as shown in Fig. 2.

Dinitrophenol (0.1 mM) was found to have no effect on the inhibition by ^2H_2O of succinate oxidation by these particles. Uncoupling by dinitrophenol was also the same in ^2H_2O as in H_2O (*cf.* refs. 3, 7).

The P:O ratio of sub-mitochondrial particles prepared by sonication at pH 7.4, which is not affected by low concentrations of oligomycin[8], is also stimulated by ^2H_2O (Fig. 3).

The experiments described in Figs. 1–3 were carried out under conditions of constant buffer ratio, without any correction for the effect of ^2H_2O on p*L* (where *L* includes both H^+ and $^2H^+$). The reaction mixtures gave about the same reading with a pH meter (glass electrode). This means that the actual p*L* of the mixture in ^2H_2O was about 0.4 higher than that in H_2O (ref. 10). An increase of pH of 0.4 in H_2O caused a decline of the P:O ratio by 28 %. Thus the stimulatory effect of ^2H_2O on the P:O ratio is underestimated in these experiments.

In the experiment described in Fig. 4, the p*L* was adjusted to 7.4 by the addition of increasing amounts of HCl as the concentration of ^2H_2O increased. In this experiment, 80 % or more of ^2H_2O caused inhibition of the oxygen uptake without any effect on the phosphorylation, with a consequent increase in the P:O ratio. In a second experiment (not shown) both the oxygen uptake and the phosphorylation



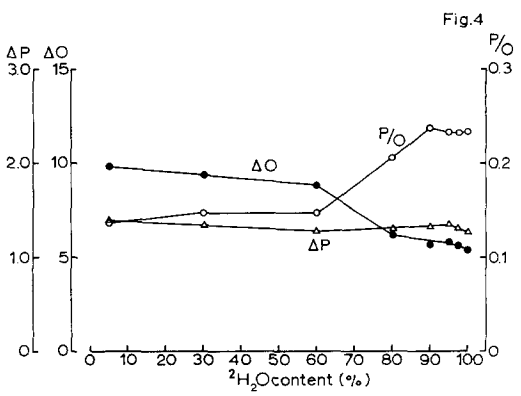


Fig. 3. Effect of oligomycin concentration on the P:O ratio of sub-mitochondrial particles in 96.7 % ^2H_2O and H_2O. The reaction mixture was the same as in Fig. 1. 1.0 mg particles was used. The sub-mitochondrial particles were obtained by sonication at pH 7.4. O---O, in 96.7 % ^2H_2O; ●—●, in H_2O.

Fig. 4. Effect of concentration of ^2H_2O on the oxidation of succinate by sub-mitochondrial particles and on oxidative phosphorylation. The reaction mixture was the same as in Fig. 1, except that it was adjusted to a constant p*L* of 7.4 (see text). 1 mg sub-mitochondrial particles obtained by sonication at pH 7.4 was used. ●—●, oxygen (μatoms) consumed; △—△, phosphate (μmoles) esterified; O—O, P:O ratio.

were inhibited by high concentrations of ^2H_2O, but the phosphorylation was inhibited less than the oxygen uptake, resulting also in an increased P:O ratio. The concentration of ^2H_2O required to inhibit the oxygen uptake also varied from preparation to preparation.

The much greater effect of ^2H_2O on oxidation than on phosphorylation supports the view[3,4] that the hydrolysis of an intermediate of oxidative phorphorylation (A ~ C) is slower in ^2H_2O than in H_2O. Many hydrolytic reactions are slower in ^2H_2O than in H_2O (ref. 11). If this interpretation of the effect of ^2H_2O is correct, it would seem unlikely that the stimulatory effects of oligomycin[8] and F_1-X (ref. 9) on the P:O ratio are due to the inhibition of the same hydrolytic reaction, since the effects of ^2H_2O are additive with those of F_1-X or oligomycin. Moreover, the P:O ratio of particles prepared by sonication at pH 7.4 is stimulated by ^2H_2O but not by oligomycin. The effects of oligomycin and F_1-X are not additive with one another[9].

The mechanism of action of ^2H_2O may also 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[12], but increases the NADPH:O ratio of the energy-linked transhydrogenase[7,12,13]. Inhibition of respiration by ^2H_2O causes a stimulation of the P:O ratio but has no effect on the NADPH:O ratio[7]. VAN DAM AND TER WELLE[12] have shown that the supply of A ~ C is not the limiting factor in the energy-linked transhydrogenase reaction.

Inhibition by ^2H_2O of both respiration and phosphorylation in intact mitochondria[5], with only a slight effect on the P:O ratio, cannot be explained by an inhibition of the hydrolysis of A ~ C, since this reaction is not thought to take place in tightly coupled mitochondria. It is possible that the effects in intact mitochondria represent an inhibition of the interaction of the components of the respiratory chain with one another, as envisaged by TYLER AND ESTABROOK[5] and BAUM AND RIESKE[6]. It is also possible that the different effects on intact mitochondria and sub-mitochondrial particles have a common basis, that is at present obscure.

EXPERIMENTAL

Sub-mitochondrial particles. These were prepared from beef-heart mitochondria[14] by sonication in the presence of 2 mM EDTA (ref. 15), either at pH 8.7 (ref. 8) or at pH 7.4. The particles were washed in 0.25 M sucrose dissolved in ^2H_2O and suspended in the same solution.

Oxidative phosphorylation. Oxygen uptake was followed in differential manometers for 30 min at 25°. The reaction was stopped by the addition of 0.5 ml of 40 % (w/v) trichloroacetic acid. The amount of esterified phosphate was measured enzymically[16,17] or by the incorporation of ^{32}P into the hexose monophosphate[18].

The reaction media were prepared by lyophilizing the aqueous reaction mixture and dissolving in water or in the appropriate mixture of ^2H_2O and water. The percentage of ^2H_2O in the final reaction mixture refers to the final concentration after the addition of the sub-mitochondrial particles suspended in ^2H_2O.

F_1-X was isolated as described by VALLEJOS, VAN DEN BERG AND SLATER[9].

The ^2H_2O, containing 99.7 atom% ^2H, was obtained from Philips-Duphar. Oligomycin, kindly donated by the Upjohn Chemical Co., and rotenone obtained from

S. B. Penick and Co. were added in ethanol. Hexokinase was obtained from Boehringer und Soehne and dialysed against 1 % glucose, 40 mM Tris–HCl buffer (pH 7.4) and 1.25 mM EDTA.

Protein was determined by the biuret method as applied to mitochondria by CLELAND AND SLATER[19].

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