Short Communications

The Inhibition of Phosphate Entry into Rat Liver Mitochondria by Organic Mercurials and by Formaldehyde

By D. D. TYLER

Department of Physiology, Royal Veterinary College, University of London, London, N.W. 1

(Received 13 December 1967)

The energy conserved during oxidoreduction in the mitochondrial respiratory chain may be used either for ATP synthesis or in other energy-linked reactions, including the uptake of univalent and bivalent cations (Ernster & Lee, 1964). A sustained phase of cation uptake by isolated mitochondria, and a concurrent increase in the respiration rate, usually requires the addition of a permeant anion, e.g. phosphate or acetate. Univalent cation uptake is stimulated strongly by the antibiotics valinomycin and gramicidin (Moore & Pressman, 1964; Pressman, 1965; Chappell & Crofts, 1965). A reaction mechanism for Ca^{2+} uptake by mitochondria (Chance, 1965), in terms of the chemical coupling hypothesis of respiratory-chain phosphorylation (Slater, 1953), postulates that the cation reacts with a high-energy intermediate ($\text{X} \sim \text{I}$) formed in an energy-transfer pathway between the respiratory chain and the locus of ATP synthesis. According to the chemiosmotic theory of respiratory-chain phosphorylation, the respiration-dependent uptake of cations by mitochondria is a consequence of some combination of an electric-potential gradient and a proton gradient across the mitochondrial membrane and does not involve the interaction between cations and high-energy intermediates (Mitchell, 1966a).

Fonyo & Bessman (1966) found that, when liver mitochondria were suspended in a phosphate-buffered mixture, the stimulation of substrate oxidation by either ADP or Ca^{2+} was suppressed by the organic mercurial *p*-hydroxymercuribenzoate, whereas the oxidation rate observed in the presence of 2,4-dinitrophenol was only partially inhibited. This result could be interpreted in terms of the chemical coupling hypothesis by postulating that the mercurial inhibited the reaction: $\text{A} \sim \text{I} + \text{X} \rightarrow \text{A} + \text{X} \sim \text{I}$. If this were so, respiratory stimulation due to the reaction of Ca^{2+} with $\text{X} \sim \text{I}$ would be sensitive to mercurial, whereas respiratory stimulation by 2,4-dinitrophenol, caused by $\text{A} \sim \text{I}$ hydrolysis, would be insensitive to mercurial. It is not easy to explain these effects in terms of the chemiosmotic coupling theory.

The present communication describes the in-

hibition of cation-stimulated respiration by the organic mercurial mersalyl [sodium *o*-(3-hydroxymercuri - 2 - methoxypropyl)carbamoylphenoxy - acetate] and by formaldehyde. It is shown that their effects resemble those of *p*-hydroxymercuribenzoate, but that further experimental studies support an alternative site for their action that is consistent with the chemiosmotic theory.

Rat liver mitochondria were isolated in 0.25M-sucrose and 5mM-tris-HCl buffer, pH 7.4, by a standard method (Schneider, 1953). Respiration rates were measured by the polarographic technique (Chappell, 1964a). Mitochondrial swelling was assayed in suspending media similar to those described by Chappell & Haarhoff (1967). A decrease in the extinction of mitochondrial suspensions at $750\text{m}\mu$ was assumed to be due to mitochondrial swelling. Sodium mersalyl was obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.) and formaldehyde (A.R. grade) from British Drug Houses Ltd. (Poole, Dorset).

The influence of mersalyl and formaldehyde on succinate oxidation stimulated by the addition of either ADP, Ca^{2+} , K^{+} in the presence of valinomycin, or the uncoupling agent 2,4-dinitrophenol, is shown in Table 1 (Expt. 1). Both mersalyl and formaldehyde inhibited respiration stimulated by ADP and phosphate, without having much effect on the rate observed in the presence of 2,4-dinitrophenol, in agreement with previous results (Fonyo & Bessman, 1966; Van Buskirk & Frisell, 1967). It was also found that both reagents strongly inhibited respiration stimulated by Ca^{2+} or K^{+} with valinomycin when phosphate was added as the permeant anion, but had only slight effects when phosphate was replaced by acetate (Table 1, Expt. 1). Similar results were obtained when *p*-chloromercuribenzoate was used instead of mersalyl, when Ca^{2+} was replaced by Sr^{2+} , or when valinomycin was replaced by gramicidin. Mersalyl had comparable effects when NAD-linked substrates were added instead of succinate. The concentration of formaldehyde used strongly inhibited the oxidation of NADH by mitochondria (and by submitochondrial particles), and the addition of 2,4-dinitrophenol was therefore

Table 1. *Inhibition of the respiration and swelling of liver mitochondria by mersalyl and formaldehyde*

In Expt. 1, rat liver mitochondria (4.0 mg. of protein) were added to an oxygen-electrode vessel (volume 3.2 ml.) containing sucrose (0.2 M), tris-HCl buffer (20 mM), succinate (tris salt) (5 mM), KCl (15 mM), rotenone (5 μ g.) and cytochrome *c* (10 μ M), at pH 7.4. The mitochondria were incubated for 3 min. with or without mersalyl (10 μ moles/g. of protein) or formaldehyde (2.5 μ moles/mg. of protein) before the addition of ADP (1 μ mole), CaCl_2 (1 μ mole), valinomycin (0.2 μ g.), phosphate (tris salt) (2.5 mM), acetate (tris salt) (25 mM) and 2,4-dinitrophenol (0.1 mM) as indicated. The rate of oxygen uptake is expressed as μ g.atoms of O/min./g. of protein. In Expt. 2, rat liver mitochondria (2 mg. of protein in 25 μ l. of cold sucrose-tris medium) were mixed for 1 min. with 5 μ l. of cold water with or without mersalyl (10 μ moles/g. of protein) or formaldehyde (2.5 μ moles/mg. of protein) before addition to a 3 ml. cuvette (light-path 1 cm.) in a Beckman model DB spectrophotometer fitted with a recorder and set at 750 m μ . The cuvette contained rotenone (5 μ g.), antimycin (5 μ g.), ethylene glycol bis(aminoethyl)tetra-acetate (0.1 mM), tris-HCl buffer (5 mM) and ammonium salt, at pH 7.4. The ammonium salt present was ammonium phosphate (0.1 M), ammonium acetate (0.15 M) or ammonium succinate (0.1 M) plus ammonium phosphate (5 mM), as indicated. The rate of swelling is expressed as the rate of decrease, in *E* units/min., of the extinction recorded 15 sec. after the addition of the mitochondria. Respiration and swelling were both measured at 22°.

Expt. no.	Additions	Rate of oxygen uptake (Expt. 1) or rate of swelling (Expt. 2)		
		No inhibitor	With mersalyl	With formaldehyde
1	None	8.0	6.5	6.5
	ADP + phosphate	45	9.5	10
	Dinitrophenol	105	81	75
	ADP + phosphate + dinitrophenol	105	87	78
	Ca^{2+} + phosphate	91	13	14
	Ca^{2+} + phosphate + dinitrophenol	100	75	73
	Ca^{2+} + acetate	80	73	70
	Valinomycin	17	15	15
	Valinomycin + phosphate	98	19	18
	Valinomycin + acetate	82	73	70
	Valinomycin + phosphate + dinitrophenol	100	70	68
	Valinomycin + phosphate + acetate	94	77	75
2	Ammonium phosphate	0.43	0.02	0.03
	Ammonium acetate	1.10	1.20	0.91
	Ammonium succinate + ammonium phosphate	0.14	0.01	0.01

unable to restore a rapid rate of NAD-linked substrate oxidation in mitochondria treated with formaldehyde. The results in Table 1 (Expt. 1) suggested that both mersalyl and formaldehyde can inhibit only those reactions that require phosphate, and that they might act by preventing the entry of phosphate into the mitochondria.

Chappell & Crofts (1966) and Chappell & Haarhoff (1967) have shown that the swelling of liver mitochondria in solutions of ammonium salts is dependent on the ability of the anion present to enter the mitochondria. A study was therefore made of the influence of mersalyl and formaldehyde on mitochondrial swelling. In agreement with previous results (Chappell & Haarhoff, 1967), liver mitochondria were found to swell in solutions of ammonium phosphate or ammonium acetate, whereas swelling in ammonium succinate required the addition of a low concentration of ammonium phosphate. Mersalyl and formaldehyde strongly inhibited the swelling in ammonium phosphate or

ammonium succinate with ammonium phosphate, but had relatively slight effects on swelling in ammonium acetate (Table 1, Expt. 2). The results of these and other swelling studies show that concentrations of mersalyl and formaldehyde sufficient to decrease strongly the phosphate-dependent respiration of mitochondria also inhibit specifically those types of swelling that require the entry of phosphate into the mitochondria. They may be simply interpreted by postulating that mersalyl and formaldehyde prevent the entry of phosphate into mitochondria. It seems likely that *p*-hydroxymercuribenzoate has a similar action, and that the inhibition of phosphate-dependent respiration described by Fonyo & Bessman (1966), by Van Buskirk & Frisell (1967) and in the present work (Table 1, Expt. 1) are all due to an inhibition of phosphate entry.

Chappell & Crofts (1966) and Chappell & Haarhoff (1967) have concluded that the mitochondrial membrane contains a phosphate exchange-diffusion

carrier, which permits the entry of phosphate or arsenate into the mitochondria. It is postulated that this carrier is inactivated by mercurials and by formaldehyde and thus becomes unable to mediate the transfer of phosphate across the mitochondrial membrane. The fact that both mercurials and formaldehyde form complexes with proteins suggests that the carrier may be a protein or lipoprotein. The inhibited state of respiration and swelling induced by mersalyl was abolished by the further addition of cysteine, indicating that the mercurial did not act by promoting an irreversible protein denaturation or swelling of the mitochondria.

The hypothesis that mercurials and formaldehyde decrease respiration by inhibiting the phosphate exchange-diffusion carrier is consistent with previous observations (Fonyo & Bessman, 1966; Van Buskirk & Frisell, 1967) that the reagents inhibit succinate oxidation uncoupled by arsenate without having much effect on that uncoupled by 2,4-dinitrophenol. In the former case the uncoupling action of arsenate is presumably dependent on the entry of arsenate into the mitochondria via the phosphate exchange-diffusion carrier. When 2,4-dinitrophenol is present a rapid rate of oxidation can occur because added phosphate is not required for the oxidation of most substrates, including succinate, by liver mitochondria treated with this uncoupling agent (Chappell, 1964b).

At the present time the influence of compounds that inhibit energy-linked reactions appears to be confined to the inactivation of either the reversible coupling adenosine triphosphatase [e.g. oligomycin (Kagawa & Racker, 1966), dicyclohexylcarbodiimide (Beechey, Holloway, Knight & Robertson, 1966) and organotin compounds (Sone & Hagihara, 1964)] or the phosphate exchange-diffusion carrier (mercurials and formaldehyde). It appears to be unnecessary to postulate, as Fonyo & Bessman (1966) and Van Buskirk & Frisell (1967) have done, that mercurials and formaldehyde suppress mitochondrial respiration by inhibiting an energy-transfer pathway between the respiratory chain

and the site of ATP synthesis, or more specifically that they inhibit the reaction: $A \sim I + X \rightarrow A + X \sim I$. The results of studies with inhibitors of energy-linked reactions are consistent with the view (Mitchell, 1966b) that both the reversible adenosine triphosphatase and the phosphate exchange-diffusion carrier are essential components of the mechanism of respiratory-chain phosphorylation, and also with his suggestion that the mechanism does not involve a reaction of the type: $A \sim I + X \rightarrow A + X \sim I$.

The author is grateful to Dr E. J. Harris for a generous gift of valinomycin and to Mr N. Pettet for excellent assistance.

- Beechey, R. B., Holloway, C. T., Knight, I. G. & Robertson, A. M. (1966). *Biochem. biophys. Res. Commun.* **23**, 75.
 Chance, B. (1965). *J. biol. Chem.* **240**, 2729.
 Chappell, J. B. (1964a). *Biochem. J.* **90**, 225.
 Chappell, J. B. (1964b). *Biochem. J.* **90**, 237.
 Chappell, J. B. & Crofts, A. R. (1965). *Biochem. J.* **95**, 393.
 Chappell, J. B. & Crofts, A. R. (1966). In *Regulation of Metabolic Processes in Mitochondria*, p. 293. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
 Chappell, J. B. & Haarrhoff, K. N. (1967). In *Biochemistry of Mitochondria*, p. 75. Ed. by Slater, E. C., Kaniuga, Z. & Wojtczak, L. London: Academic Press (Inc.) Ltd.
 Ernster, L. & Lee, C. P. (1964). *Annu. Rev. Biochem.* **33**, 729.
 Fonyo, A. & Bessman, S. P. (1966). *Biochem. biophys. Res. Commun.* **24**, 61.
 Kagawa, Y. & Racker, E. (1966). *J. biol. Chem.* **241**, 2467.
 Mitchell, P. (1966a). In *Regulation of Metabolic Processes in Mitochondria*, p. 65. Ed. by Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. Amsterdam: Elsevier Publishing Co.
 Mitchell, P. (1966b). *Biol. Rev.* **41**, 445.
 Moore, C. & Pressman, B. C. (1964). *Biochem. biophys. Res. Commun.* **15**, 562.
 Pressman, B. C. (1965). *Proc. nat. Acad. Sci., Wash.*, **53**, 1076.
 Schneider, W. C. (1953). *J. Histochem. Cytochem.* **1**, 212.
 Slater, E. C. (1953). *Nature, Lond.*, **172**, 975.
 Sone, N. & Hagihara, B. (1964). *J. Biochem., Tokyo*, **56**, 151.
 Van Buskirk, J. J. & Frisell, W. R. (1967). *Biochim. biophys. Acta*, **143**, 292.