

Transport and Oxidation of Choline by Liver Mitochondria

By D. D. TYLER

Department of Physiology, Royal Veterinary College, University of London, London NW1 0TU, U.K.

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1. Rapid choline oxidation and the onset of P_i -induced swelling by liver mitochondria, incubated in a sucrose medium at or above pH 7.0, required the addition of both P_i and an uncoupling agent. Below pH 7.0, P_i alone was required for rapid choline oxidation and swelling. 2. Choline oxidation was inhibited by each of several reagents that also inhibited P_i -induced swelling under similar conditions of incubation, including EGTA, mersalyl, Mg^{2+} , the Ca^{2+} -ionophore A23187, rotenone and nupercaine. None of these reagents had any significant effect on the rate of choline oxidation by sonicated mitochondria. There was therefore a close correlation between the conditions required for rapid choline oxidation and for P_i -induced swelling to occur, suggesting that in the absence of mitochondrial swelling the rate of choline oxidation is regulated by the rate of choline transport across the mitochondrial membrane. 3. Respiratory-chain inhibitors, uncoupling agents (at pH 6.5) and ionophore A23187 caused a loss of endogenous Ca^{2+} from mitochondria, whereas nupercaine and Mg^{2+} had no significant effect on the Ca^{2+} content. Inhibition of choline oxidation and mitochondrial swelling by ionophore A23187 was reversed by adding Ca^{2+} , but not by Mg^{2+} . It is concluded that added P_i promotes the Ca^{2+} -dependent activation of mitochondrial membrane phospholipase activity in respiring mitochondria, causing an increase in the permeability of the mitochondrial inner membrane to choline and therefore enabling rapid choline oxidation to occur. Nupercaine and Mg^{2+} appear to block choline oxidation and swelling by inhibiting phospholipase activity. 4. Choline was oxidized slowly by tightly coupled mitochondria largely depleted of their endogenous adenine nucleotides, suggesting that these compounds are not directly concerned in the regulation of choline oxidation. 5. The results are discussed in relation to the possible mechanism of choline transport across the mitochondrial membrane *in vivo* and the influence of this process on the pathways of choline metabolism in the liver.

The rate of oxidation of the flavin-linked substrates succinate and α -glycerophosphate and most of the NAD^+ -linked substrates by the respiratory chain is regulated by the respiratory-control mechanism in isolated mitochondria and uncoupled oxidation of these substrates proceeds rapidly even in the absence of added phosphate (Lardy & Wellman, 1953; Chappell, 1964*b*). Choline oxidation by the respiratory chain is also flavin-linked (Singer, 1963) and under optimal conditions the choline-oxidation rate is relatively high, about one-fifth the rate of uncoupled succinate oxidation (Tyler *et al.*, 1966). Studies of the interaction between the choline-oxidation system and the non-penetrant anion ferricyanide indicate that choline dehydrogenase is situated at the inner surface of the inner mitochondrial membrane, showing that external choline must cross the membrane before oxidation can occur (De Ridder *et al.*, 1973).

Williams (1960) found that choline oxidation was unusual, since ADP was unable to stimulate coupled choline oxidation and uncoupler-stimulated oxidation required the addition of P_i . Williams (1960) and Wilken *et al.* (1965) concluded that coupled choline

oxidation was limited by the rate of choline transport into mitochondria. De Ridder & Van Dam (1975) concluded that choline transport is not a rate-limiting step during choline oxidation and that the oxidation is regulated by several factors, including the adenine nucleotides present in the matrix space. The results of the present study provide an explanation for the phosphate requirement during uncoupled choline oxidation and enable a choice to be made between the two hypotheses concerning the control of choline oxidation. A preliminary account of this work has been presented (Tyler, 1976).

Materials and Methods

Reagents

The following compounds were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.: adenine nucleotides, antimycin, choline bicarbonate, rotenone, Ruthenium Red and Triton X-100. Solutions of choline phosphate were prepared by neutralizing choline bicarbonate with H_3PO_4 . Ruthenium Red

was recrystallized and standardized by the method of Reed & Bygrave (1974). Nicotinamide nucleotides and enzymes required for the assay of adenine nucleotides were from Boehringer Corp., London W5 2TZ, U.K. Crystallized bovine plasma albumin was obtained from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. [$U\text{-}^{14}\text{C}$]ADP (559 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other reagents, including choline chloride, were from BDH, Poole, Dorset, U.K.; AnalaR-grade reagents were used whenever possible. Nupercaine (cinchocaine hydrochloride BP) and ionophore A23187 were gifts from Ciba Laboratories, Horsham, Sussex, U.K., and Eli Lilly and Co, Indianapolis, IN, U.S.A., respectively. Aqueous solutions were freshly prepared in glass-distilled water.

Enzyme preparations

Rat liver mitochondria were isolated in 0.25 M-sucrose by the method of Schneider (1953). The mitochondrial pellets were washed twice and again homogenized in sucrose solution to obtain a stock suspension of mitochondria containing 60–100 mg of protein/ml, which was stored at 1°C until used. Some mitochondrial preparations were depleted of lysosomal enzymes by treatment of the preparation with 2 mg of digitonin/100 mg of protein (Loewenstein *et al.*, 1970). Mitochondria were depleted of endogenous adenine nucleotides by preincubation in 0.25 M-sucrose/20 mM-triethanolamine buffer/1 mM-EDTA/5 mM-MgCl₂/10 mM-potassium phosphate buffer, pH 7.2, as described by Meisner & Klingenberg (1968). Mitochondrial adenine nucleotides were labelled by incubation with [$U\text{-}^{14}\text{C}$]ADP as described in Method II of Pfaff & Klingenberg (1968). Sonicated mitochondria were prepared by diluting a stock mitochondrial preparation 10-fold in ice-cold water and sonicating the suspension for 30 s in an MSE 100 W sonicator tuned to maximum power output.

Enzyme assays

Enzymic activities and mitochondrial swelling were assayed at 30°C. The reaction mixture (3 ml) contained 0.185 M-sucrose, bovine plasma albumin (1 mg/ml) and 10 mM-Mops* buffer, adjusted with Tris to the required pH, and mitochondria (0.9–1.4 mg of protein/ml). Bovine plasma albumin was added to eliminate swelling caused by the release of endogenous free fatty acids (Lehninger & Remmert, 1959). It delayed the onset of phosphate-induced swelling at pH values above 7.0. Other additions were made as indicated in the legends to the Figures and Tables. Stock solutions of rotenone and ionophore A23187 were prepared in ethanol. Inhibitors were added in

* Abbreviation: Mops, 4-morpholinepropanesulphonic acid.

small volumes (usually 10 μl) of stock solutions. The amount of ethanol added with the inhibitor had no significant effect on the activities assayed. Respiration rates were recorded by the polarographic technique with a Clark oxygen electrode (Chappell, 1964a), and are expressed as μg -atoms of O/min per g of protein. Rates of mitochondrial swelling were measured at 750 nm in a Beckman model 25 Spectrophotometer and are expressed as A_{750} units/min. The content of mitochondrial adenine nucleotides was measured by enzymic methods (Jaworek *et al.*, 1974; Lamprecht & Trautschold, 1974). Calcium was measured by a colorimetric method (Gindler & King, 1972). When the Ca²⁺ content of incubated mitochondria was measured, the mitochondria were first sedimented at 8000g for 2 min in an Eppendorf Microcentrifuge 3200. The mitochondrial pellets were stored overnight in the small plastic centrifuge tubes at -20°C. The pellet in each tube was then thawed and dissolved in 0.1 ml of 1% (v/v) Triton X-100 and mixed with 2.9 ml of the working reagent used for the Ca²⁺ assay. The average Ca²⁺ content of six stock preparations of mitochondria was 16.7 nmol/mg of protein (range 13.8–21.4). The reaction mixture contained about 11 nmol of Ca²⁺/ml, because of Ca²⁺ present in the bovine plasma albumin (2 nmol/mg of protein) and in the sucrose solution (9 nmol/ml). Protein was measured by the biuret method (Gornall *et al.*, 1949). The numerical data presented are the mean values obtained, and the polarographic and swelling traces are representative of the results obtained, with at least three different mitochondrial preparations.

Results

Effect of pH on choline oxidation

Rapid O₂ uptake by rat liver mitochondria incubated with choline was dependent on the addition of P_i to the reaction mixture within the pH range 6.5–8.0. The additional requirement for the presence of an uncoupling agent, such as 2,4-dinitrophenol or carbonyl cyanide *m*-chlorophenylhydrazone (Williams, 1960; De Ridder *et al.*, 1973), was necessary only at alkaline pH values. At acid pH, 2,4-dinitrophenol inhibited choline oxidation in the presence of P_i (Fig. 1). When endogenous substrates were depleted by either the preincubation of mitochondria with 2,4-dinitrophenol and P_i or sonication, the choline-oxidation rate was decreased to about 70% of that in fresh mitochondria, suggesting that rapid O₂ uptake in the presence of choline (Fig. 1) was mostly due to choline oxidation but partially to the simultaneous oxidation of endogenous substrate.

Effect of pH on mitochondrial swelling

The P_i-dependent swelling of mitochondria (Raaflaub, 1953; Hunter & Ford, 1955), which oc-

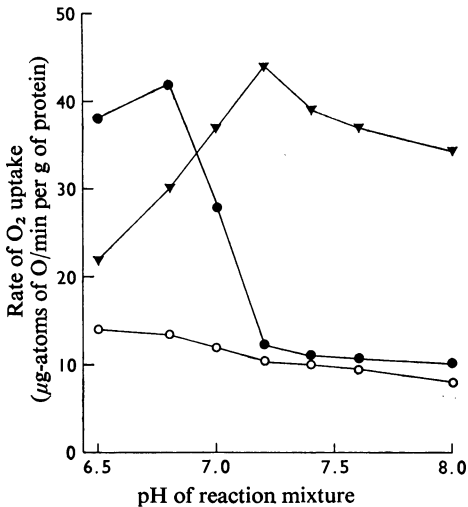


Fig. 1. Effect of pH on choline oxidation

The reaction mixtures contained 5mM-potassium phosphate buffer at the pH indicated. Mitochondria were incubated for 2 min to measure the rate of endogenous substrate oxidation. Choline chloride was then added to measure the choline-oxidation rate, followed 1 min later by the addition of 2,4-dinitrophenol to measure the choline-oxidation rate in the presence of the uncoupler. ○, Endogenous substrate oxidation; ●, with 5mM-choline chloride; ▼, with 5mM-choline chloride and 40 µM-2,4-dinitrophenol.

currred under the same conditions as those of Fig. 1, was also pH-dependent. Above pH7.2, a long incubation period was required before the onset of swelling, unless an uncoupling agent was also added (Fig. 2). At pH6.5, uncoupling agents blocked P_i-dependent swelling. When the reaction mixture contained 5mM-choline chloride, no significant change occurred in the incubation time required for the onset of swelling, but choline increased the extent of swelling considerably both at pH6.5 without uncoupler and at pH7.4 in the presence of uncoupler. Swelling and choline oxidation were blocked by the organic mercurial mersalyl [sodium *O*-(3-hydroxymercuri-2-methoxypropyl)carbamoylphenoxyacetate; 50 nmol/mg of protein], an inhibitor of P_i transport across the mitochondrial membrane (Tyler, 1969). The mersalyl concentration required was higher than that previously used, owing to the presence of bovine plasma albumin, which binds to mersalyl. These observations showed that a close correlation exists between the conditions necessary for rapid choline oxidation and rapid mitochondrial swelling to occur. The P_i requirement for swelling was also satisfied by arsenate, but not by malonate or acetate. The effect of these anions on swelling was therefore similar to their

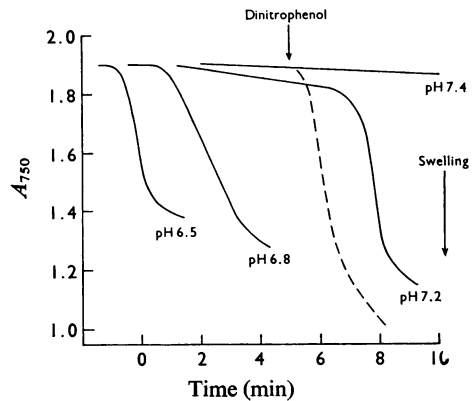


Fig. 2. Effect of pH on mitochondrial swelling

The reaction mixtures contained 5mM-potassium phosphate buffer at the pH indicated. The solid lines show the kinetics of phosphate-dependent swelling. In a separate experiment at pH7.4, 40 µM-2,4-dinitrophenol was added at the time indicated by the arrow and the swelling response to the uncoupling agent is shown by the broken line.

effect on choline oxidation observed previously (De Ridder *et al.*, 1973). The P_i-dependent swelling of mitochondrial preparations depleted of lysosomal enzymes by treatment with a small amount of digitonin showed similar properties to the swelling of untreated preparations. Lysosomal stimulation of mitochondrial swelling (Mellors *et al.*, 1967) was therefore not involved.

Role of endogenous Ca²⁺ ions

The inhibition of P_i-dependent swelling by EDTA (Raaffaub, 1953; Tapley, 1956) and the inhibition of choline oxidation by EDTA (Bianchi & Azzone, 1964) suggested that endogenous Ca²⁺ was involved in both processes. In support of this conclusion, it was observed that at alkaline pH values the incubation time required for the onset of P_i-dependent swelling was decreased when 20 µM-Ca²⁺ was added to the suspending medium, and under these conditions no uncoupling agent was required to stimulate either swelling or choline oxidation. Further evidence was obtained from studies with EGTA, a more specific Ca²⁺ chelator than EDTA, and with the Ca²⁺ ionophore A23187, which causes a depletion of endogenous Ca²⁺ from mitochondria (Reed & Lardy, 1972; and Table 3 below). Each of these reagents strongly inhibited uncoupled choline oxidation and P_i-dependent swelling at pH7.4 (Fig. 3). Similar results were obtained by using pH6.5 buffer without 2,4-dinitrophenol, except that some swelling usually occurred even when the mitochondria were preincubated with

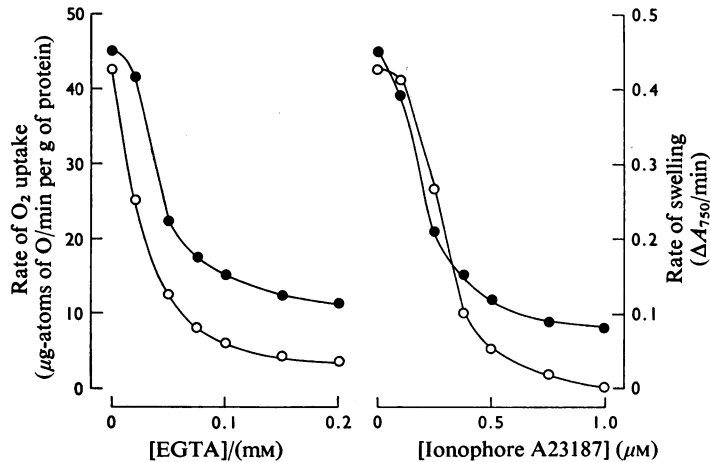


Fig. 3. Effect of Ca^{2+} -complexing agents on swelling and oxidation

Rates of swelling and choline oxidation were measured at pH 7.4 in the presence of 5 mM-potassium phosphate buffer, 5 mM-choline chloride and 40 μM-2,4-dinitrophenol. Mitochondria were incubated for 1 min with the concentration of EGTA or ionophore A23187 indicated, before the addition of P_i , choline and uncoupling agent. ○, Rate of swelling; ●, rate of O₂ uptake.

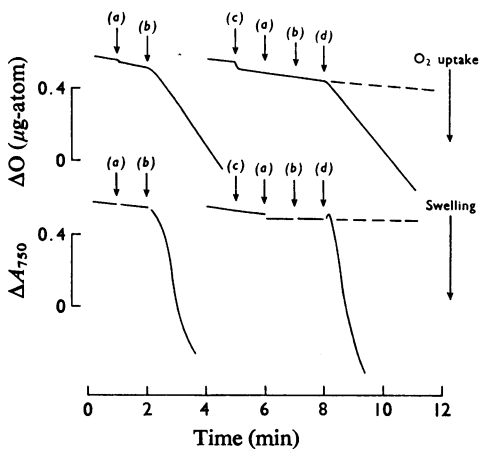


Fig. 4. Effect of added Ca^{2+} ions on mitochondria incubated with ionophore A23187

The left-hand upper and lower traces show the effect of choline and 2,4-dinitrophenol on O₂ uptake and swelling. In the right-hand upper and lower traces the experiments were repeated with mitochondria preincubated with ionophore A23187. The following additions were made to the reaction mixtures as indicated: (a) 15 μl of 1 M-choline chloride; (b) 20 μl of 6 mM-2,4-dinitrophenol; (c) 2 μl of 1 mM-ionophore A23187; (d) 3 μl of 0.1 M-CaCl₂. In the right-hand traces, the broken lines show the effect of adding 3 μl of 1 M-MgCl₂ instead of CaCl₂.

EGTA before the addition of P_i . At pH 7.4, the inhibition of swelling and uncoupled choline oxidation by ionophore A23187 was reversed by the addition of Ca^{2+} (Fig. 4), but not by Mg^{2+} , which is also lost from mitochondria treated with ionophore A23187 (Reed & Lardy, 1972).

Effect of nupercaine

The local anaesthetic nupercaine is a potent inhibitor of pancreatic phospholipase A₂ activity (Scherphof *et al.*, 1972). The protective effect of nupercaine (0.2–0.5 mM) on mitochondrial structure, respiratory control and phosphorylation activities (Scarpa & Lindsay, 1972; Aleksandrowicz *et al.*, 1973) is believed to be due to the inhibition of a phospholipase A₂ enzyme known to be present in the mitochondrial membrane (Waite & Sisson, 1971; Nachbaur *et al.*, 1972). As shown in Table 1, 0.1 mM-nupercaine was a potent inhibitor of choline oxidation and P_i -dependent mitochondrial swelling. In oxygen-electrode experiments, this nupercaine concentration had no significant effect on the stimulation of succinate oxidation by added Ca^{2+} , but it increased the amount of added Ca^{2+} required to cause a loss of respiratory control (Fig. 5). In the absence of nupercaine, a very low concentration of Ca^{2+} was sufficient to abolish respiratory control during succinate oxidation at pH 6.5, compared with pH 7.4. In all three experiments, the loss of respiratory control after the

Table 1. *Effect of nupercaine on swelling and choline oxidation*

At pH6.5, mitochondria were incubated for 1 min in reaction mixtures containing the concentration of nupercaine indicated, before addition of 5mM-potassium phosphate buffer (pH6.5) and, 2min later, 5mM-choline chloride. At pH7.4, mitochondria were incubated for 1 min in a reaction mixture containing 5mM-potassium phosphate buffer and the concentration of nupercaine indicated, before the addition of 5mM-choline chloride and 40µM-2,4-dinitrophenol. Nupercaine had no effect on the endogenous substrate oxidation rate (9.8 at pH6.5 and pH7.4). Rates of mitochondrial swelling are expressed as A_{750} unit/min and rates of O_2 uptake as µg-atoms of O /min per g of protein

Nupercaine concn. (µM)	Rates			
	At pH6.5		At pH7.4, with 2,4-dinitrophenol	
	Swelling	O_2 uptake	Swelling	O_2 uptake
0	0.380	37.5	0.550	40.6
20	0.175	22.3	0.300	28.1
50	0.030	14.7	0.110	19.6
100	0.010	12.1	0.035	12.1

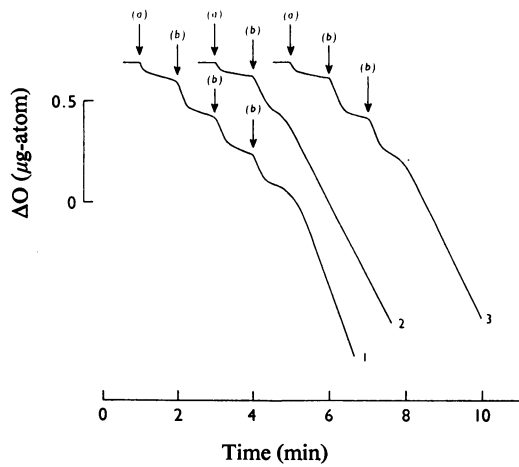


Fig. 5. *Effect of pH and nupercaine on Ca^{2+} -stimulated respiration*

The reaction mixtures contained 5mM-potassium succinate, 5mM-potassium phosphate buffer, 3.3mM- $MgCl_2$ and rotenone (1µg/mg of protein) at either pH7.4 (trace 1) or pH6.5 (traces 2 and 3). In the experiment shown by trace 3, 0.1mM-nupercaine was also present. The following additions were made as indicated: (a) 50µl of stock mitochondrial suspension (4mg of protein); (b) 5µl of 0.1M- $CaCl_2$.

addition of Ca^{2+} to respiring mitochondria coincided with the onset of mitochondrial swelling, as observed previously (Chappell & Crofts, 1965). Swelling induced by added Ca^{2+} and P_i was prevented when the mitochondria were pretreated with Ruthenium Red at a concentration just sufficient to block the activity of the Ca^{2+} transporter (0.1nmol/mg of protein; Reed & Bygrave, 1974) even when 2mM- Ca^{2+} was added to the reaction mixture.

These effects of nupercaine and Ruthenium Red suggest that P_i -dependent swelling is due to the activation of a phospholipase enzyme by Ca^{2+} present in the matrix space. This enzyme activity then causes changes in membrane permeability both to sucrose, resulting in swelling, and to choline, which enables rapid choline oxidation to occur. This explanation is consistent with the high temperature coefficient of P_i -induced swelling (Lehninger, 1962), which is characteristic of an enzyme-catalysed process. It is also supported by studies of the effect of hypo-osmotic conditions on swelling and choline oxidation. When the osmoticity of the reaction mixture at pH7.4 was varied by decreasing the sucrose concentration, the mitochondria swelled very rapidly under hypo-osmotic conditions. At sucrose concentrations below 30mM, the absorption of the mitochondrial suspension was lower than that observed when mitochondria were incubated with P_i and 2,4-dinitrophenol. Despite the severe swelling induced by hypo-osmoticity, the choline-oxidation rate was low, but could be

Table 2. *Effect of osmoticity on swelling and choline oxidation*

Mitochondria were incubated for 2min at pH7.4 in reaction mixtures containing 5mM-potassium phosphate buffer, 0.1mM-EGTA and the concentration of sucrose indicated, before the addition of 5mM-choline chloride and, 1min later, 0.16mM- $CaCl_2$. The A_{750} of the mitochondrial suspension shown was measured after the addition of choline. In each experiment, the addition of Ca^{2+} induced a rapid swelling.

Sucrose concn. (mM)	A_{750}	Rate of O_2 uptake (µg-atoms of O /min per g of protein)		
		Endogenous substrate	With choline	With choline and $CaCl_2$
185	1.80	7.4	9.2	42.0
100	1.71	6.9	6.9	45.2
40	1.18	9.2	9.2	44.8
20	0.91	9.7	10.1	43.8
0	0.60	10.1	18.5	40.2

stimulated by adding Ca^{2+} (Table 2). These experiments indicate that P_i -induced swelling promotes specific changes in mitochondrial-membrane permeability that cannot be induced by physical stresses applied to the membrane by osmotic forces.

Uncoupled choline oxidation by mitochondria incubated with P_i is blocked by a variety of ions, including K^+ (Williams, 1960) and Mg^{2+} (Kagawa *et al.*, 1965). Mg^{2+} is known to inhibit P_i -induced swelling of mitochondria (Raafaub, 1953; Tapley, 1956) and mitochondrial phospholipase activity (Waite *et al.*, 1969a). In the present study, the addition of either KCl (40mM) or $MgCl_2$ (0.5mM), sufficient to inhibit strongly uncoupled choline oxidation at pH7.4, also strongly inhibited P_i -dependent swelling, suggesting that these cations block choline oxidation by inhibiting Ca^{2+} -activated changes in membrane permeability and not by competing with choline for a choline-carrier system as suggested elsewhere (De Ridder, 1976). At pH6.5, in the absence of uncoupler, K^+ had no significant effect on either choline oxidation or P_i -dependent swelling, whereas Mg^{2+} strongly inhibited both processes.

Swelling and choline oxidation were also strongly inhibited when rotenone was added to the reaction mixture before mitochondria, in agreement with previous observations (Bianchi & Azzone, 1964; De Ridder & Van Dam, 1975). P_i -dependent swelling of liver mitochondria is supported by the oxidation of endogenous substrates (Chappell & Greville, 1958). The inhibition of choline oxidation by rotenone therefore suggested that in the absence of rotenone swelling and the consequent uptake and oxidation of choline was supported initially by the oxidation of endogenous substrate. In support of this hypothesis, when mitochondria were depleted of endogenous

substrate by preincubation with 2,4-dinitrophenol before P_i addition, very little choline oxidation occurred, despite the presence of uncoupler and P_i , until swelling was initiated by increasing the Ca^{2+} concentration (Fig. 6).

Choline oxidation by sonicated mitochondria was not significantly influenced by any of the following substances, used at concentrations which either strongly stimulated or strongly inhibited choline oxidation in intact mitochondria: P_i , with or without 2,4-dinitrophenol; EGTA; ionophore A23187; Mg^{2+} ; K^+ ; Ca^{2+} ; or rotenone. Nupercaine (0.1mM) inhibited choline oxidation by sonicated mitochondria by about 15%. Even with higher concentrations of nupercaine (0.5mM) the inhibition was only about 55%.

Effect of Ruthenium Red

Ruthenium Red (1.0nmol/mg of protein) strongly inhibited both P_i -dependent swelling and choline oxidation at pH6.5, and partially inhibited these processes at pH7.4, in the presence of 2,4-dinitrophenol. The amount of Ruthenium Red required to inhibit swelling and oxidation was considerably in excess of the amount required to block the Ca^{2+} -transporter system, indicating that the inhibition was due to an action distinct from the known effect of Ruthenium Red on Ca^{2+} uptake by mitochondria.

Ca^{2+} content of mitochondria

Since the observations described above indicated that endogenous Ca^{2+} was involved in swelling and choline oxidation, the effect of various inhibitors of these processes on the Ca^{2+} content of mitochondria was investigated. In some experiments, the efflux of adenine nucleotides from the mitochondria (Meisner & Klingenberg, 1968; De Ridder & Van Dam, 1973) was also measured. The results are presented in Table 3. At pH6.5, about 83% of the mitochondrial Ca^{2+} was lost to the medium after a 3 min incubation with P_i . Uncoupling agents, respiratory inhibitors and ionophore A23187 stimulated Ca^{2+} efflux from mitochondria even in the absence of added P_i , suggesting that these reagents inhibit swelling by decreasing the Ca^{2+} concentration in the mitochondrial matrix. In contrast, nupercaine and Mg^{2+} had only a slight effect on mitochondrial Ca^{2+} incubated in the absence of added P_i and largely prevented P_i -induced Ca^{2+} efflux, suggesting that they inhibit the Ca^{2+} -activated step involved in mitochondrial swelling. Inhibitors of swelling, including ionophore A23187, nupercaine, Mg^{2+} and rotenone, also strongly inhibited the efflux of adenine nucleotides induced by added P_i (Table 3). De Ridder & Van Dam (1973) found that uncoupling agents stimulated the efflux of betaine from mitochondria and concluded that the

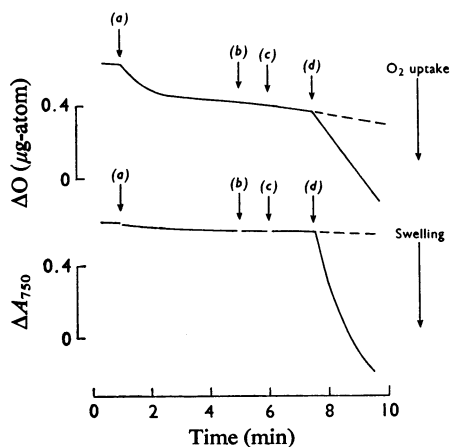


Fig. 6. Effect of preincubation with 2,4-dinitrophenol on choline oxidation

The following additions were made to the reaction mixture (pH7.4) as indicated: (a) 20 μ l of 6mM-2,4-dinitrophenol; (b) 15 μ l of 1M-potassium phosphate buffer, pH7.4; (c) 15 μ l of 1M-choline chloride; (d) 3 μ l of 0.1M- $CaCl_2$. The broken lines show the effect of adding 3 μ l of 1M- $MgCl_2$ instead of $CaCl_2$.

Table 3. Contents of Ca^{2+} and labelled adenine nucleotides in mitochondria

Mitochondria (3.5 mg of protein) labelled with [$U-^{14}C$]ADP were incubated in sucrose/Mops buffer, pH 6.5, for 1 min with the concentrations of reagents indicated below before the addition of either 15 μ l of 1 M-potassium phosphate buffer, pH 6.5, or 15 μ l of water. The incubation was then continued for 3 min before the mitochondria were sedimented by centrifugation at 8000g for 2 min, and their contents of Ca^{2+} and labelled adenine nucleotide determined. The adenine nucleotide content of the stock labelled mitochondria was taken as 100%.

Addition	No added P_i		With 5 mM-phosphate, pH 6.5	
	Calcium (nmol/mg)	Adenine nucleotide (% of control)	Calcium (nmol/mg)	Adenine nucleotide (% of control)
None	25.8	92.3	4.4	36.0
Nupercaine (0.1 mM)	23.1	94.1	21.7	90.7
$MgCl_2$ (3 mM)	21.1	94.0	20.5	83.2
Ionophore A23187 (0.6 μ M)	5.3	94.0	5.3	93.6
Rotenone (1 μ g/mg of protein)	9.7	94.7	4.9	90.3
Antimycin (1 μ g/mg of protein)	5.3		5.4	
KCN (1 mM)	11.2		6.2	
Dinitrophenol (50 μ M)	5.8		7.7	

Table 4. Swelling and choline oxidation by mitochondria depleted of endogenous adenine nucleotides

The following additions were made to the reaction mixture as indicated: EGTA, 0.1 mM; choline chloride, 5 mM; 2,4-dinitrophenol, 40 μ M; $CaCl_2$, 0.16 mM. The mitochondria used, containing 4.3 nmol of adenine nucleotide/mg of protein, were prepared as described by Meisner & Klingenberg (1968) from stock mitochondria containing 13.2 nmol/mg of protein. Rates of mitochondrial swelling are expressed as A_{750} unit/min and rates of O_2 uptake as μ g-atoms of O/min per g of protein.

Addition	Rate of swelling		Rate of oxygen uptake	
	No EGTA	+EGTA	No EGTA	+EGTA
None	0.114	0.012	2.2	2.2
Choline	0.121	0.010	39.1	8.7
Choline+2,4-dinitrophenol	0.115	0.012	39.1	11.2
Choline+ $CaCl_2$	0.160	0.148	39.4	37.7

mitochondrial membrane becomes permeable to betaine under conditions when adenine nucleotides also leak out. The present results indicate that under the experimental conditions that they used, adenine nucleotide efflux and probably also betaine efflux is a consequence of a Ca^{2+} -activated increase in membrane permeability.

Role of endogenous adenine nucleotides

In agreement with the observations of Meisner & Klingenberg (1968), preincubation of mitochondria with P_i , EDTA and Mg^{2+} promotes the loss of 60–70% of the endogenous adenine nucleotides without significantly changing the respiratory-control or P/O ratios of the mitochondria. When these mitochondria were diluted into P_i buffer at pH 7.4, an immediate and rapid swelling occurred even in the absence of uncoupler, and they catalysed rapid choline oxidation (Table 4). Both these processes were blocked by EGTA and could be restored by adding an excess of Ca^{2+} ions.

Swelling in choline phosphate

Mitochondria swelled rapidly when suspended in iso-osmotic choline phosphate and the swelling was partially resistant to the addition of rotenone and EGTA, in agreement with the results of De Ridder (1976). Swelling was abolished when rotenone was replaced by KCN, as in the experiments of Mitchell & Moyle (1969), by antimycin, or by ionophore A23187 (Fig. 7). Under these conditions, O_2 uptake was inhibited almost completely by antimycin or KCN, but only partially inhibited by rotenone. The effects of respiratory inhibitors and ionophore A23187 suggest that swelling in choline phosphate (De Ridder, 1976) is due to a respiration-dependent Ca^{2+} -activated process similar to that taking place in a sucrose/ P_i reaction mixture as described above.

Discussion

Rapid choline oxidation and mitochondrial swelling

These studies favour the conclusion that choline oxidation by coupled mitochondria is limited by the

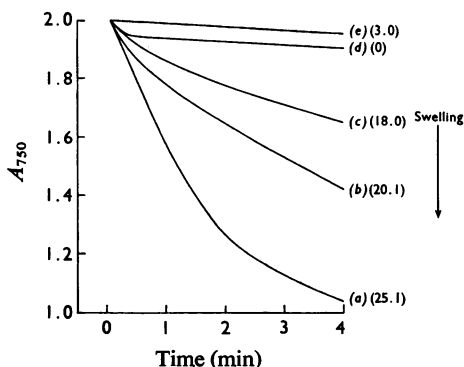


Fig. 7. *Mitochondrial swelling in choline phosphate*
The reaction mixture contained 0.15M-choline phosphate, pH7.0, and the following additions: (a) no addition; (b) rotenone (1 $\mu\text{g}/\text{mg}$ of protein); (c) rotenone+0.1mM-EGTA; (d) 1mM-KCN; (e) either antimycin (1 $\mu\text{g}/\text{mg}$ of protein) or 0.6 μM -ionophore A23187. Mitochondria (4mg of protein) were added at zero time. Numbers in parentheses are the rates of O_2 uptake (μg -atom of O/min per g of protein) observed under the experimental conditions indicated.

rate of choline transport and that added P_i alone, below pH7.0, or together with an uncoupling agent above pH7.0, stimulates choline entry and oxidation by promoting a Ca^{2+} -activated increase in the mitochondrial-membrane permeability to choline. This conclusion is supported by the effects of a number of reagents, including EGTA, ionophore A23187, Mg^{2+} and K^+ , each of which inhibit both swelling and choline oxidation by mitochondria, but have no effect on choline oxidation by broken mitochondria. Rotenone inhibits choline oxidation when added to fresh mitochondria, but not when added after rapid choline oxidation has been established (Bianchi & Azzone, 1964; De Ridder & Van Dam, 1975), suggesting that rotenone also inhibits choline oxidation by inhibiting respiration-dependent swelling and choline entry. The stimulation of swelling and choline oxidation by Ca^{2+} and the inhibitory effect of Mg^{2+} and nupercaine indicate that swelling and changes in permeability to choline are probably due to the hydrolysis of membrane phospholipid by membrane-bound phospholipase. Waite *et al.* (1969b) found that only a very small amount of endogenous phospholipid hydrolysis is required for extensive swelling to occur, suggesting that hydrolysis is limited to a key locus in the mitochondrial structure, perhaps at sites of fusion of the outer and inner mitochondrial membranes (Hackenbrock, 1968; Nachbaur *et al.*, 1972). The inhibition of swelling by high concentrations of Ruthenium Red may also be due to an inhibition of phospholipase, since this compound has been found to inhibit other

Ca^{2+} -activated enzymes (Watson *et al.*, 1971; Vale & Carvalho, 1973).

P_i - and respiration-dependent swelling occurs under conditions favouring the retention of a high concentration of free endogenous Ca^{2+} in the matrix space. One important factor regulating the free Ca^{2+} concentration is the intramitochondrial adenine nucleotide pool, especially ADP, which appears to stabilize calcium phosphate deposits in the matrix (Carafoli *et al.*, 1965). The inhibition of P_i -dependent swelling by oligomycin (Chappell & Greville, 1959; Azzi & Azzone, 1965), rotenone, high uncoupler concentrations and by mersalyl suggests that swelling is dependent on ATP synthesis, which may increase the free Ca^{2+} concentration by removing ADP. Low concentrations of uncoupling agents may stimulate ATP synthesis, and therefore swelling, through substrate-level phosphorylation during endogenous substrate oxidation (Azzone & Azzi, 1966). Calcium phosphate in the matrix should be more soluble at acid pH values, and this factor would explain the more rapid onset of P_i -dependent swelling and loss of respiratory control during calcium uptake at low pH. Meisner & Klingenberg (1968) showed that about 75% of the total intramitochondrial adenine nucleotides could be removed without significantly altering either the rate or the efficiency of oxidative phosphorylation. The structure and biochemical functions of these preparations were found to be very sensitive to Ca^{2+} , suggesting that *in vivo* one function of the apparent excess of adenine nucleotides is to regulate the availability and activity of Ca^{2+} stored in the mitochondria.

De Ridder & Van Dam (1975) concluded that coupled choline oxidation is rate-limited by the activity of choline dehydrogenase, an enzyme thought to be regulated by the ratio of adenine nucleotide forms in the matrix space. However, the 2-fold changes in the K_m and V_{max} values for choline dehydrogenase activity induced by AMP compared with ADP and ATP (De Ridder & Van Dam, 1975) may not be sufficiently large to account for the increase in choline-oxidation rate when phosphate and an uncoupling agent are added. Furthermore when mitochondria were either depleted of endogenous substrate by preincubation with 2,4-dinitrophenol before P_i addition or depleted of endogenous adenine nucleotides, choline was oxidized slowly, whereas the hypothesis of De Ridder & Van Dam (1975) predicts that rapid choline oxidation would occur under these conditions, as a consequence of the low adenine nucleotide concentrations in the matrix space.

Mechanism of choline transport by coupled mitochondria

Liver mitochondria do not swell in iso-osmotic solutions of choline acetate (Mitchell & Moyle, 1969;

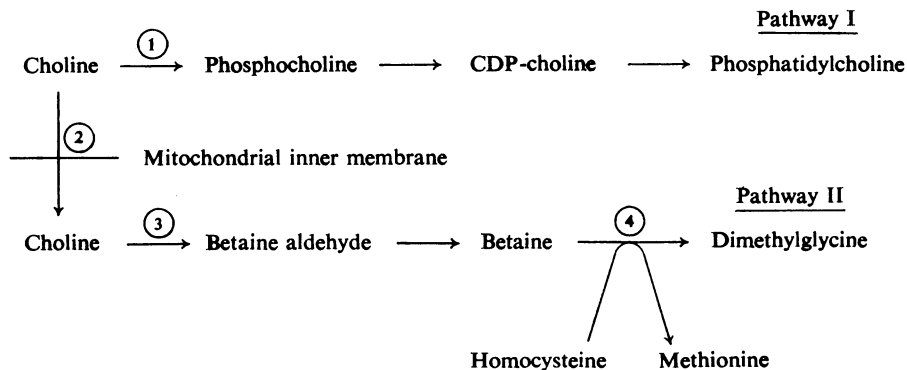
De Ridder, 1976) or choline phosphate (Mitchell & Moyle, 1969). The swelling in choline phosphate observed by De Ridder (1976) was found to be a consequence of respiration-dependent P_i -induced swelling. The failure of mitochondria to swell in solutions of choline salts indicates that the choline cation is unable to penetrate the membrane of non-respiring mitochondria. In coupled respiring mitochondria, choline⁺ transport presumably occurs in response to the electrophoretic gradient set up across the inner membrane during the oxidation of endogenous substrates. The uptake of choline⁺ by liver mitochondria therefore appears to be similar to the mechanism of L-ornithine⁺ uptake described by Gamble & Lehninger (1973). It is noteworthy that the rate of choline oxidation under coupled conditions (1 nmol/min per mg of protein; De Ridder & Van Dam, 1973) is very similar to the rate of energy-dependent choline uptake (0.5–1.5 nmol/min per mg of protein; De Ridder, 1976), as would be expected if choline transport is the rate-limiting step. De Ridder (1976) also observed a rapid uptake of labelled choline (2–4 nmol/mg of protein) by mitochondria, which was energy-independent, nearly temperature-independent and associated with the ejection of 2.9 nmol of H⁺/mg of protein. These properties suggest that the latter uptake of choline is a binding phenomenon, analogous to the respiration-independent displacement of protons from external anionic groups of mitochondria by alkali-metal cations, in which up to 23 nmol of H⁺/mg of protein may be ejected (Gear & Lehninger, 1967).

It is not known whether the liver inner mitochondrial membrane contains a specific choline transporter or whether the energized membrane possesses a low inherent permeability to choline. Gamble & Lehninger (1973) concluded that a specific carrier system

transported L-ornithine⁺ across the membrane at a rate of about 5 nmol/min per mg of protein, or 5 times the choline-transport rate observed by De Ridder (1976) under energized conditions. Thus, despite the low choline-transport rate, it is possible that a specific electrogenic choline transporter is functional in the process of external choline oxidation by tightly coupled liver mitochondria.

Choline oxidation in vivo

In the liver, two metabolic pathways compete for the available choline (see Scheme 1). Choline acetyltransferase activity is present only in nerve tissue (Nachmansohn & Berman, 1946). Pathway I, located mainly in the endoplasmic reticulum (McMurraray & Magee, 1972), utilizes choline for the synthesis of phosphatidylcholine, a phospholipid essential for the formation and maintenance of intracellular membranes. Pathway II oxidizes choline to the methyl donor betaine, which can undergo transmethylation with homocysteine to form methionine, a reaction catalysed by mitochondrial betaine-homocysteine transmethylase (Klee *et al.*, 1961). Pathway II is usually less important than Pathway I, because dietary methionine is the major source of methyl groups in most animal species. In some species, including man, monkey and guinea pig, very little choline dehydrogenase activity appears to be present (Sidransky & Farber, 1960). The free choline content of rat liver is about 0.24 μmol/g of liver (Sundler *et al.*, 1972), which corresponds to an average choline concentration in the cytosol water of 0.34 mM. The K_m value for choline does not appear to have been determined for either liver choline kinase or for choline transport into coupled mitochondria (steps 1 and 2 of Scheme 1). If the K_m value for liver choline kinase



Scheme 1. *Metabolism of choline in rat liver*

Key: 1, choline kinase; 2, choline transport across the mitochondrial membrane; 3, choline dehydrogenase; 4, betaine-homocysteine methyltransferase.

is similar to that for the yeast enzyme (20 μM ; Wittenberg & Kornberg, 1953), the first step of the phospholipid-synthesis pathway would be nearly saturated with substrate under physiological conditions. In the rat fed on a methionine-deficient diet, the methylation of dietary homocysteine provides sufficient methionine for normal growth if choline or another methyl-group donor is supplied in the diet (du Vigneaud *et al.*, 1939). The rate of betaine synthesis in coupled liver mitochondria oxidizing choline is about 1 nmol/min per mg of protein (De Ridder & Van Dam, 1973). Under similar conditions, an adult rat liver weighing 15 g and containing 50 mg of mitochondrial protein/g of liver would synthesize 160 mg of betaine/day. After conversion into methionine, this rate of synthesis is about 8 times the estimated daily methionine requirement in the adult rat (Albanese, 1950). Thus the rate of choline oxidation by coupled mitochondria appears to be more than sufficient to support the rate of methionine synthesis indicated by the dietary experiments. Liver mitochondria were found to swell in an iso-osmotic solution of methionine (D. D. Tyler, unpublished work) at about 5 times the rate observed by Halling *et al.* (1973), suggesting that mitochondria are permeable to methionine. Methionine synthesized in mitochondria would therefore be expected to move freely into the cytosol.

The rate of choline transport and oxidation therefore seems to be well adjusted to the principal functions of choline metabolism in the liver. The oxidation is sufficiently rapid to satisfy the methyl-group requirement when there is a dietary shortage of methionine, but slow enough to ensure that adequate choline is available for phospholipid synthesis. The major pathway of methionine synthesis is usually by transfer of a methyl group, not from betaine, but from methyltetrahydrofolate to homocysteine. The tetrahydrofolate derivative arises in the pool of C_1 compounds formed mainly during the catabolism of glycine and serine (Bender, 1975). When methyl-group synthesis is satisfied by the metabolism of dietary glycine and serine, the enzymes of the choline-oxidation pathway present in liver mitochondria provide a route for methyl-group oxidation coupled to the synthesis of 9 mol of ATP per mol of choline oxidized to glycine and serine (Frisell *et al.*, 1966).

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