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UNCOUPLER-STIMULATED OXIDATION OF CHOLINE BY RAT-LIVER MITOCHONDRIA

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SUMMARY

The main product of uncoupler-stimulated oxidation of choline by rat-liver mitochondria is betaine, which is found almost exclusively extramitochondrially. The uncoupled oxidation of choline is stimulated by intramitochondrial phosphate. The effect of intramitochondrial phosphate is to induce adenine nucleotide efflux, which in its turn allows the efflux of betaine from the mitochondria.

INTRODUCTION

Rat-liver mitochondria oxidize choline either stimulated by uncouplers of oxidative phosphorylation[1–3] or under State-3 conditions[4–8]. Although Jellinek *et al.*[9] and Yue *et al.*[10] found betaine aldehyde as the product of mitochondrial choline oxidation, others showed directly[3,6–8,11] or indirectly[12,13] that rat-liver mitochondria also can oxidize choline to betaine. Recently we demonstrated that under State-3 conditions the main product of mitochondrial choline oxidation is betaine and that this is accumulated in the matrix space[8]. Under these conditions the oxidation rate is relatively low[4,8].

The uncoupler-stimulated choline oxidation is much faster[1,2] and is measured best in phosphate-containing media[14]. We suggested that phosphate influences betaine efflux from the mitochondrial matrix[8] and this could result in enhanced choline oxidation.

In this investigation we studied some parameters of uncoupled choline oxidation including the effect of phosphate.

Abbreviations. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone, FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; Tricine, Tris(hydroxymethyl)methylglycine, S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide, MOPS, morpholinopropane sulphonic acid, TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid.

MATERIALS AND METHODS

Rat-liver mitochondria were isolated according to the method of Hogeboom[15] as described by Myers and Slater[16]

Loading of mitochondria with ^{14}C-labeled adenine nucleotides was achieved according to Out *et al.*[17]

Protein was determined according to Cleland and Slater[18].

Phosphate was determined according to Wahler and Wollenberger[19].

Separation of mitochondria from incubation mixtures for analysing the content of the mitochondrial matrix was achieved by centrifugation–filtration according to the method of Werkheiser and Bartley[20] as described by Harris and Van Dam[21]. The lower layer consisted of 15 % perchloric acid. The volume of the matrix space, when not determined *via* ^{14}C-labeled sucrose and ^3H_2O, was assumed to be 1 μl per mg protein. The sucrose-impermeable space was 20 % of the total volume spun down through the silicone layer as measured by ^3H_2O.

^{14}C-labeled choline, betaine and betaine aldehyde were separated *via* paper chromatography according to Wilken[11] When the supernatant fraction had to be analysed it was acidified immediately after centrifugation. After chromatography the papersheets were scanned with an Actigraph III, Nuclear Chicago and after cutting, the compounds were counted *via* liquid scintillation counting in toluene–ethanol (3 : 1, v/v) containing 50 mg POPOP and 4.0 g PPO per l.

In order to obtain mitochondrial fractions that contain only choline dehydrogenase or betaine aldehyde dehydrogenase activity we followed the procedure of Feinberg *et al.*[12], at pH 7.0. Mitochondria used for these experiments were washed with 0.25 M sucrose in order to avoid contamination of mitochondrial betaine aldehyde dehydrogenase with extramitochondrial enzyme[7]. The particle and supernatant fractions were dialysed overnight to remove phosphate and NAD^+

Oxygen uptake was measured with a Clark oxygen electrode fitted in a thermostatted vessel with a volume of 1.66 ml. When also an H^+ electrode was used the volume of the vessel was 2.5 ml. Stirring was performed with a magnetic stirrer. The vessel was closed with a glas stopper with a narrow inlet to avoid back diffusion of oxygen into the solution. All additions were made through this stopper and were minimal in volume. Unless stated otherwise, the oxygen consumption is corrected for consumption in the absence of added substrate

[U-^{14}C]Choline and [U-^{14}C]betaine were obtained from New England Nuclear. ^3H_2O from Philips Duphar and choline and betaine from B. D H. Oligomycin and antimycin A were obtained from Sigma Chemical Company. Betaine aldehyde was a gift from Drs F. M. Kaspersen.

RESULTS

Localization of choline dehydrogenase and betaine aldehyde dehydrogenase

We found that the reduction of the non-permeant anion ferricyanide by choline, in the presence of intact rat-liver mitochondria is inhibited for more than 90 % by antimycin A. In the presence of sonified mitochondria this inhibition is 60 %. These results are comparable to those obtained with succinate as a substrate, indicating that the active centres of both choline dehydrogenase and succinate de-

hydrogenase are located at the same side of the mitochondrial inner membrane, *i.e.* the inner side[22].

Betaine, produced during choline oxidation by intact rat-liver mitochondria conceivably could be formed by an enzyme attached to the outside of the mitochondrial membrane. This, however, seems very unlikely because only in the mitochondrial matrix NAD^+, necessary for betaine aldehyde dehydrogenation[10], is present. Furthermore, betaine aldehyde is formed in the matrix space and also its oxidation product, betaine, is found there under State-3 conditions. Finally, semicarbazide does not influence choline oxidation by intact rat-liver mitochondria, but inhibits when

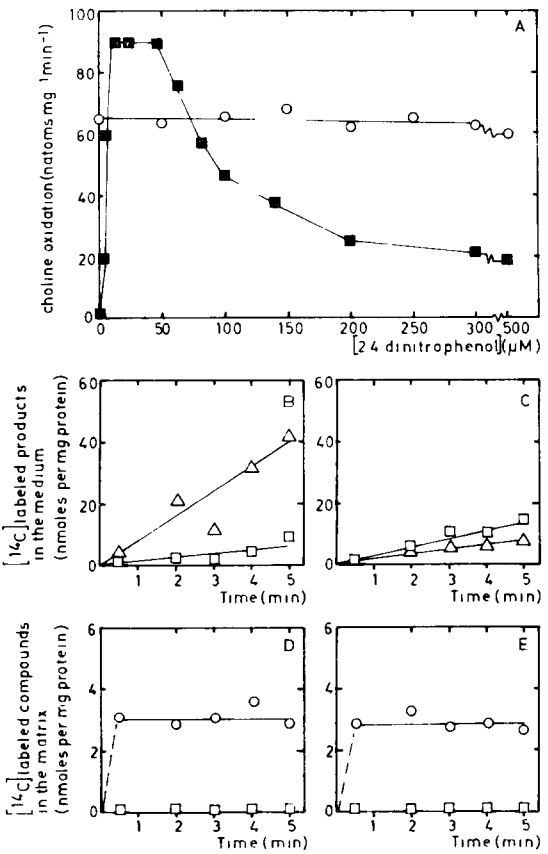

Fig 1 Influence of uncoupler on choline oxidation and its influence on the distribution of the products Rat-liver mitochondria, 1.4 mg protein (A), 4 3 mg protein (B–E), were incubated in a medium containing 185 mM sucrose and 5 mM phosphate, pH 7 0, temp. 25 C. Expt A was carried out in an oxygraph vessel as described in Materials and Methods. Choline chloride concentration was 10 mM and concentrations of 2,4-dinitrophenol were added as indicated. ○–○, sonified mitochondria, ■–■, intact mitochondria. Expts B–E were carried out in a final volume of 5 ml Apart from the reagents mentioned above, 1 mM choline was present *plus* 2 0 μCi ^{14}C-labeled choline. At different times samples of 0 9 ml were taken and analysed for ^{14}C-labeled compounds as described in Materials and Methods In the experiment in B and D 20 μM 2,4-dinitrophenol was present; in the experiment in C and E 200 μM In B and C, products in the medium are shown; in D and E, the metabolites in the matrix space. ○–○, choline; △–△, betaine, □–□, betaine aldehyde

the mitochondria are aged[13] or sonified, indicating again that in intact rat-liver mitochondria betaine is not generated outside the mitochondria.

Products of choline oxidation

Under State-3 conditions the main product of mitochondrial choline oxidation is betaine and this is found in the matrix space[8]. Also under uncoupled conditions the main product is betaine (Fig. 1), but in this case there is formed 5–10 times as much and it is found in the extramitochondrial space. In most experiments small amounts of betaine aldehyde are found under uncoupled conditions. Others[3,5,7] showed that under some conditions mitochondria produce large amounts of betaine aldehyde as a product of choline oxidation. In these experiments, however, the incubation time was at least 30 min, instead of 5. We could confirm the production of larger amounts of betaine aldehyde in long term experiments. This, combined with the fact that sonified mitochondria also produce betaine aldehyde as a product, suggests that prolonged incubation causes the loss of a permeability barrier for betaine aldehyde.

Optimal conditions for uncoupled choline oxidation

All uncouplers of oxidative phosphorylation tested [2,4-dinitrophenol, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), laurylamine, lauric acid, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) and dicumarol] stimulate choline oxidation, although at relatively low concentrations. At higher concentrations choline oxidation by intact mitochondria is severely inhibited, up to 80 % in all cases tested (see Fig. 1A). The inhibition is non-competitive with respect towards substrate. The K_m for choline is 1.2 mM (± 0.4 mM S.E. of mean, 10 measurements). Above 20 mM substrate inhibits markedly.

Potassium inhibits choline oxidation competitively (K_i = 7 mM), supporting the suggestion of Williams[1] that K^+ inhibits choline oxidation *via* inhibition of its uptake. Other monovalent cations (Li^+, NH_4^+, Na^+, ornithine$^+$, Tris$^+$) and nearly all tested divalent cations (Mg^{2+}, Mn^{2+}, Zn^{2+}, Ba^{2+}) also inhibit. More than 90 % inhibition in the latter cases is already brought about by 1 mM of these salts. Calcium, as Williams results already suggested[1], does not inhibit.

Inhibition of choline oxidation by high uncoupler concentration

When the uncoupler concentration exceeds a concentration less than is optimal for oxidation of other substrates, choline oxidation is already inhibited (Fig. 1A) The enzymes choline dehydrogenase and betaine aldehyde dehydrogenase, isolated according to Feinberg *et al* [12] (see Materials and Methods) appeared to be no more sensitive to high uncoupler concentrations than are other mitochondrial enzymes[23]. This fits with the fact that aged or sonified mitochondria are not influenced very much by 10 times the uncoupler concentration that inhibits freshly isolated mitochondria (Fig. 1A). On the other hand, in intact mitochondria in the presence of phenazine methosulphate and catalase (not shown), when the electrons from choline do not follow the electron-transport chain, but can be transferred directly from the dehydrogenase to oxygen *via* phenazine methosulphate, high uncoupler concentrations inhibit severely

In Figs 1B–1E the localization of the products of choline oxidation at optimal

and inhibitory uncoupler concentration is shown. The formation of betaine is inhibited approximately 80 %, but the formation of betaine aldehyde is slightly stimulated by 200 μM 2,4-dinitrophenol (compare Figs 1B and 1C) resulting in 65 % inhibition of oxygen uptake under these conditions, in agreement with Fig. 1A. Under both conditions the amounts of intramitochondrial betaine aldehyde and betaine are very low and also the intramitochondrial concentration of choline is not influenced very much.

Stimulation by phosphate of uncoupled choline oxidation

The stimulation of uncoupled choline oxidation by phosphate[14] is partly duplicated by arsenate, but not by other anions tested, including malonate, up to 50 mM, and acetate, up to 40 mM.

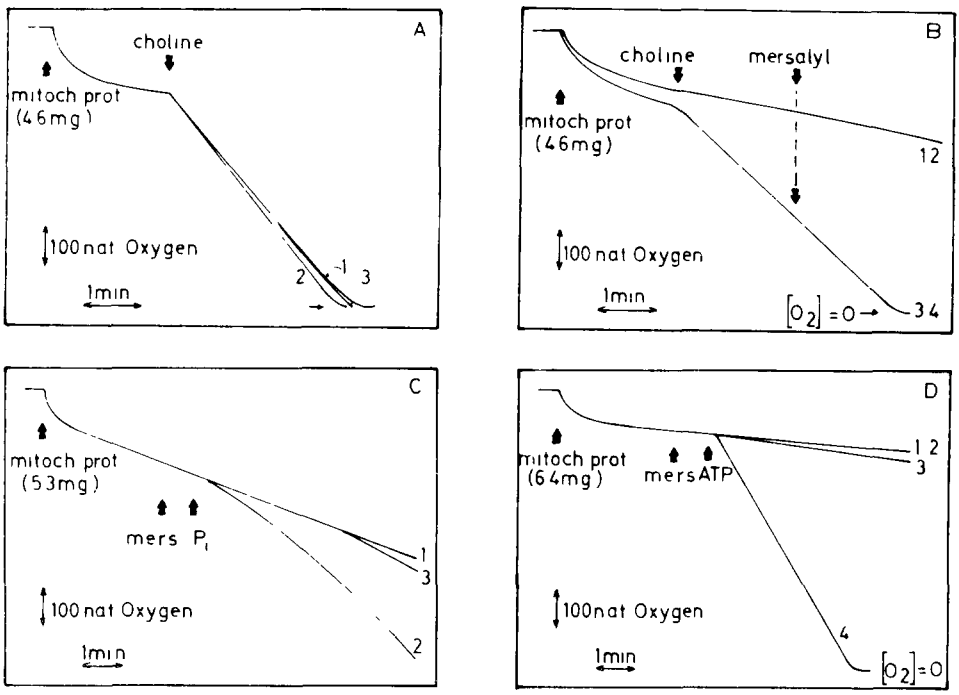

Fig 2 The effect of intra- and extramitochondrial phosphate on choline oxidation. Mitochondria were incubated in an oxygraph vessel at pH 6 9, at 30 C The medium contained 185 mM sucrose, 20 μM 2,4-dinitrophenol and 5 mM MOPS buffer (A) At the time indicated in the figure 10 mM choline is added Trace 1, the incubation contained 5 mM phosphate, Trace 2, the mitochondria are preincubated with 40 nmoles mersalyl per mg protein, Trace 3, phosphate was present, and the mitochondria were preincubated with mersalyl (B) 10 mM choline is added at the time indicated Trace 1, no further addition; Trace 2, 40 nmoles mersalyl per mg protein were added at the time indicated, Trace 3, the medium contained 5 mM phosphate, Trace 4, the medium contained phosphate and mersalyl was added during the incubation (C) 10 mM choline, was present in the medium. Trace 1, 40 nmoles mersalyl per mg protein were added at the time indicated, Trace 2, 5 0 mM phosphate was added at the time indicated, Trace 3, both mersalyl and phosphate were added (D) 10 mM choline was present in the medium. Trace 1, no addition, Trace 2, 200 nmoles ATP were added at the time indicated, Trace 3, 40 nmoles mersalyl per mg protein were added at the time indicated in the figure, Trace 4, both ATP and mersalyl were added

This means that the stimulation is specific for phosphate and structurally related anions and is not due to the non-specific action of a permeant anion.

Mersalyl, an inhibitor of phosphate translocation in mitochondria[24], also stimulates choline oxidation to about the same extent as phosphate does (Fig. 2A) Adding phosphate together with mersalyl does not give further stimulation. For just-maximal stimulation 22 nmoles mersalyl per mg protein are required. When mersalyl is added during choline oxidation it has no influence, neither in the absence nor in the presence of phosphate (Fig. 2B). When phosphate is added during the incubation it stimulates choline oxidation, and mersalyl prevents this, although not completely (Fig. 2C). When, however, after mersalyl a small amount of ATP is added, choline oxidation is again stimulated (Fig. 2D), presumably by the phosphate generated in the matrix *via* the uncoupler-stimulated intramitochondrial breakdown of ATP. That the uncoupler-stimulated ATPase indeed plays a role is shown by the fact that the effect is oligomycin-sensitive and that AMP does not duplicate the effect of ATP. Thus, intramitochondrial phosphate stimulates choline oxidation

We measured the intramitochondrial phosphate concentration under conditions of rapid choline oxidation *i.e.* after preincubation with mersalyl or after addition of mersalyl and subsequent addition of ATP (Fig. 3) The amount of mersalyl stimulating choline oxidation maximally does not completely prevent but only inhibits phosphate efflux. For complete inhibition of phosphate efflux from the mitochondria much more mersalyl is required. Also phosphate, generated in the matrix space *via* uncoupler-stimulated ATPase, leaves the mitochondria rapidly in the presence of an amount of mersalyl that stimulates choline oxidation maximally

Although it does not appear to be necessary that phosphate remains present

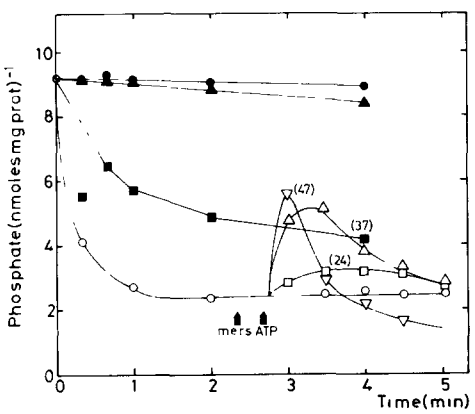

Fig. 3. Intramitochondrial phosphate concentration at low and high choline oxidation rates. Rat-liver mitochondria, 4 mg protein per ml incubation mixture, were incubated in a medium containing 185 mM sucrose, 5 mM Tris–MOPS buffer and 10 mM choline chloride At different times samples were taken and analysed for phosphate as described in Materials and Methods ●–●, no addition, ○–○, 20 μM 2,4-dinitrophenol, ■–■, 20 μM 2,4-dinitrophenol, mitochondria were preincubated with 40 nmoles mersalyl per mg protein during 2 min. ▲–▲, 20 μM 2,4-dinitrophenol, preincubation with 200 nmoles mersalyl. ▽–▽, △–△, □–□, mersalyl added at the time indicated in the figure ▽–▽, 7 5 nmoles ATP; △–△, 2.5 nmoles ATP, □–□, 0 6 nmole ATP. The values in brackets indicate maximal choline oxidation observed, at this amount of ATP.

in the mitochondrial matrix during choline oxidation, the amount of ATP added and therefore the amount of phosphate generated in the matrix space (Fig. 3) is important. Half-maximal stimulation was obtained by addition of 3.3 nmoles ATP per mg protein. This corresponds with a maximal internal phosphate concentration of 4 mM When we compare this with the K_m for stimulation by added phosphate (3 mM) also measured at 10 mM potassium, we see good agreement. Also, the more ATP is added the earlier the maximal internal phosphate concentration is reached and the earlier the choline oxidation starts after addition of ATP and the earlier its maximum rate is reached.

Once this maximum is reached it does not change for several minutes, even though the mitochondrial phosphate concentration has long returned to its initial value. The time of addition of choline has no influence, even if it is added after phosphate activation.

During choline oxidation in the absence of phosphate, after 5 min about 2 nmoles betaine per mg protein are accumulated. Betaine aldehyde is not present in measurable amounts. It is noteworthy that mitochondria do not accumulate as much betaine under these conditions as in State-3. In the medium less than 0.2 μM betaine was found after 5 min, so a gradient of 10 000 was built up, indicating that both phosphate and uncoupler are needed for betaine efflux (see also ref. 8)

TABLE I

LOSS OF ADENINE NUCLEOTIDES FROM THE MITOCHONDRIAL MATRIX UNDER VARIOUS CONDITIONS

^{14}C-labeled adenine nucleotides-loaded rat-liver mitochondria (4 0 mg per ml incubation mixture) were incubated in a medium containing 185 mM sucrose, 5 mM MOPS–Tris, 20 μM 2,4-dinitrophenol and 10 mM choline chloride. Termination of the reaction was achieved by centrifugation-filtration (see Materials and Methods) Mersalyl (60 nmoles per mg protein), when added, was added 3 min after starting the reaction ATP (12 nmoles per mg protein), when added, 3 min 30 s after starting the reaction Oligomycin (2 μg per mg protein) in Expt 5 was present in the medium Final pH 6 9, temp 25 C The amount of adenine nucleotides present in the matrix at 3 min after starting the reaction was taken as 100 %

Expt	*Additions*	*Loss of ^{14}C-labeled adenine nucleotides from the matrix (%) in 90 s*
1	–	28
2	Mersalyl	51
3	ATP	54
4	Mersalyl, ATP	92
5	Oligomycin, mersalyl, ATP	36

We have shown already that in the presence of uncoupler *plus* phosphate, or uncoupler *plus* mersalyl there is a loss of adenine nucleotides from the matrix space[8] as is also suggested by the results of Dresel[25] and Siekevitz and Potter[26]. In Table I it is shown that addition of ATP after mersalyl during an incubation in the presence of choline not only stimulates choline oxidation (see Fig. 2D) but also stimulates adenine nucleotide efflux in an oligomycin-sensitive way. 90 s after addition of a small amount of ATP almost all adenine nucleotides have disappeared from the

matrix, whereas in the control experiments still half of the adenine nucleotides (or more) is retained.

DISCUSSION

The results described in this paper support our earlier conclusion[8] that the rate of choline oxidation by rat-liver mitochondria is regulated by the rate of efflux of its oxidation product, betaine, from the matrix.

The specific stimulation by phosphate of uncoupled choline oxidation can be attributed to a specific stimulation of adenine nucleotide efflux by a combination of intramitochondrial phosphate *plus* an uncoupler. It is remarkable that the adenine nucleotide efflux can even be brought about through intramitochondrial hydrolysis of added ATP, especially if a low concentration of mersalyl is present (see Table 1).

That these conditions do not simply lead to swelling and aspecific leakage of the mitochondria follows from the observations that: (i) the mitochondria retain a low permeability for betaine aldehyde and, (ii) the betaine aldehyde is almost quantitatively further oxidized to betaine, indicating that NAD has not leaked from the mitochondria.

How the loss of adenine nucleotides from the mitochondrial matrix leads to permeability of the membrane for betaine is a matter of further investigation.

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