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THE UPTAKE OF CHOLINE BY RAT LIVER MITOCHONDRIA

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SUMMARY

1. Rat liver mitochondria can accumulate choline against a concentration gradient. Maximally about 30 nmol choline per mg mitochondrial protein are found in the matrix space.

2. The process of choline uptake is biphasic. After a rapid uptake of 1.5–15 nmol per mg protein, a slower uptake occurs if an energy supply is present. In the absence of energy, only the rapid uptake is found.

3. The inhibition of uncoupler-stimulated choline oxidation by cations is the result of an inhibition of choline uptake.

INTRODUCTION

As a substrate the dehydrogenase of which is located at the inner side of the mitochondrial inner membrane [1], choline must be able to pass this membrane. Williams [2, 3] and later Kagawa and coworkers [4, 5] ascribed a regulatory role in overall choline oxidation to the uptake process.

The presence of (labelled) choline in the mitochondrial matrix space was demonstrated by several authors [1, 5, 6] and concentrations of choline in the matrix space higher than those in the surrounding medium have been reported [1, 6].

On the basis of swelling experiments, Mitchell and Moyle [7] concluded that choline did not enter the matrix space via a proton-coupled antiport.

In the experiments we performed, up to 30 nmol choline per mg protein have been found in the matrix space. For reasons of electroneutrality, either an anion has to move together with the choline into the matrix space, or a cation has to move in the opposite direction. The present report deals with the uptake of choline as a regulatory process in overall choline oxidation and the mechanism of this uptake.

MATERIALS AND METHODS

Rat liver mitochondria were isolated according to the method of Hogeboom [8], as described by Myers and Slater [9]. Protein was determined according to

Abbreviations: EGTA, ethyleneglycol-bis(aminoethyl)tetraacetic acid; DMO, dimethyloxazolidinedione-2,4.

Cleland and Slater [10]; separation of mitochondria from incubation mixtures was achieved according to the method of Werkheiser and Bartley [11] as described by Harris and Van Dam [12]. Products of choline oxidation were measured by a tracer method. 2 μCi ^{14}C-labelled choline were added to the incubation mixture and the products were separated according to Wilken [13].

If the accumulation of choline in the matrix was to be measured, 0.1 μCi ^{14}C-labelled choline were added, plus 0.2 μCi $^{3}H_2O$. For further details of the sampling, counting of radioactive samples, measurements of oxygen, H^+, and calculations, see our earlier paper [1].

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

RESULTS

Rat liver mitochondria, in the presence or absence of a source of energy, are able to accumulate choline. At a choline concentration of 1 mM in the medium, usually 2–4 nmol choline per mg protein are taken up within the dead time of the measurements (minimally 4 s; Fig. 1A). The amount of choline taken up in this short time is nearly independent of the temperature between 0 and 37 °C. The K_m of this process for choline is 1.4 mM. In separate experiments, it was verified (using Millipore filtration) that there was no binding of choline to frozen or sonicated mitochondria.

In the presence of a source of energy, i.e. ATP, a slower phase is observed after the rapid uptake. At a choline concentration of 1 mM in the medium, the rate of this slow uptake is 0.5–1.5 nmol/min per mg protein, at pH 7.0 and 25 °C. This process is strongly temperature-dependent. A Q_{10} value of 3.1 was calculated. The K_m of this slow choline uptake is 0.3 mM.

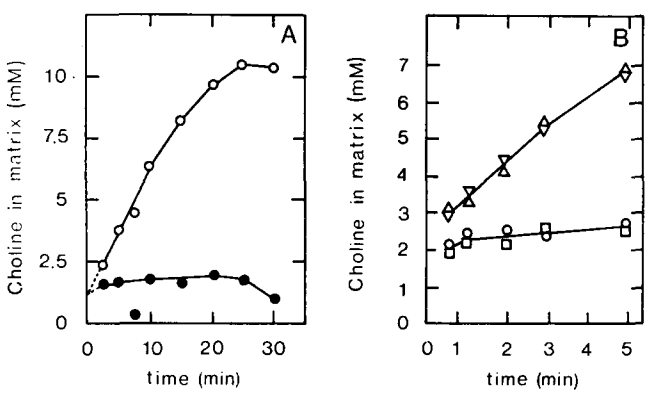

Fig. 1. Uptake of choline by rat liver mitochondria. Rat liver mitochondria (4.1 or 2.6 mg per ml in Expts. A and B, respectively) were incubated at pH 7.0 and 25 °C. The media contained 185 mM sucrose, 5 mM phosphate buffer and 1 mM choline chloride. In Expt. A 0.9 μg antimycin per mg protein were also present. Expt. A: ○—○, plus 2 mM ATP; ●—●, plus 20 μM, 2,4-dinitrophenol. Expt. B: ○—○, plus 0.6 μg antimycin per mg protein; □—□, plus 20 μM 2,4-dinitrophenol; △—△, plus 2 mM ATP; ▽—▽, plus 0.6 μg antimycin per mg protein plus 2 mM ATP.

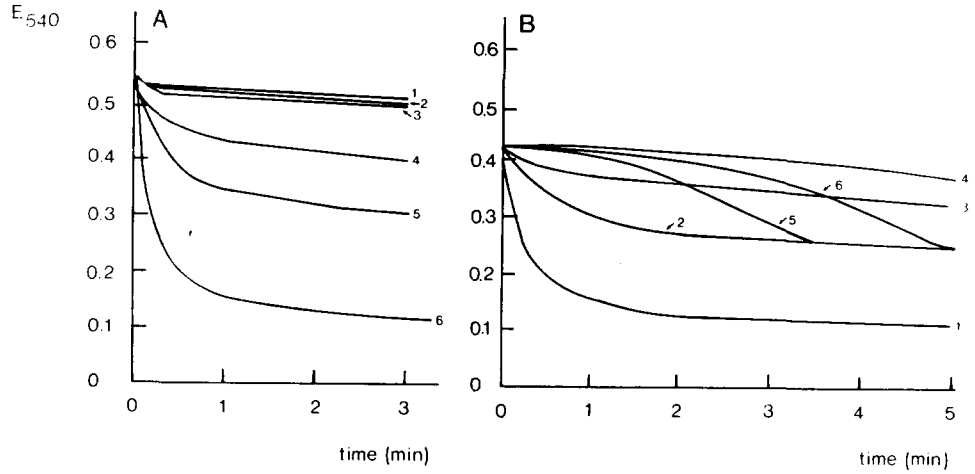

Fig. 2. Swelling of rat liver mitochondria in choline salts. Rat liver mitochondria (0.1 mg protein per ml) were incubated at pH 7.0 and 25 °C. The media contained 5 mM Tris · HCl buffer, 0.05 mM EGTA and 1.5 μg rotenone per mg protein. Salt solutions were isotonic. Expt. A: 1, choline chloride or ammonium chloride; 2, choline nitrate; 3, choline acetate; 4, potassium phosphate; 5, choline phosphate; 6, ammonium phosphate. Expt. B: 1, ammonium phosphate; 2, choline phosphate; 3, choline phosphate plus 20 μM 2,4-dinitrophenol; 4, choline phosphate plus 100 μM 2,4-dinitrophenol; 5, choline phosphate plus 1 mM ATP; 6, choline phosphate plus 3 mM ATP.

The maximal choline concentration measured in the matrix space was 30–50 mM. In the absence of energy, the maximal amount is found after 5 min, at a concentration of 15 mM in the medium. Above this concentration, choline strongly inhibits its own uptake. In the presence of a source of energy, the maximal amount of choline in the matrix is found after 25 min. All high-energy conditions tested stimulate choline uptake after the first rapid phase, whereas under all low energy conditions the slow uptake phase of choline is absent. In Fig. 1B, some examples are shown.

The energy-dependent choline uptake process can be observed both under State-3 and State-4 conditions. Under State-4 conditions, half-maximal stimulation is given by 0.02 mM ATP. When energy for choline uptake is provided by succinate oxidation AMP does not influence choline uptake. State-3 choline oxidation itself, however, does not stimulate choline uptake. Calcium ions or uncouplers of oxidative phosphorylation completely prevent the slow choline uptake by rat liver mitochondria.

We also tested the uptake of choline by measuring swelling of mitochondria in isotonic salt solutions in comparison with some reference salts. Rotenone was added in all swelling experiments; it was verified that this addition completely inhibited respiration under these conditions [1]. As can be seen in Fig. 2A, mitochondria swell extensively in choline phosphate (contrast Mitchell and Moyle, ref. 7), but not in choline nitrate or choline acetate. However, when either ATP or uncoupler is added, the swelling of the mitochondria decreases.

In the case of ATP, a lag time is observed. This lasts the longer the more ATP is added. In the case of uncoupler, swelling is inhibited during the whole period.

In a medium containing a high concentration of cations, both the fast and the

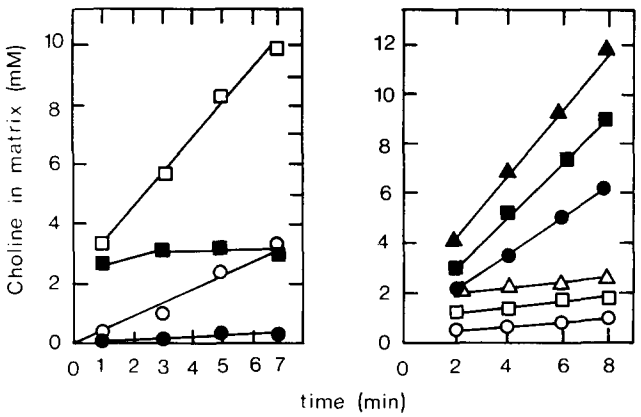

Fig. 3. Choline uptake as a function of the cations present. Mitochondria were incubated in media of 185 mosM at 25 °C. The osmolarity was maintained at 185 mosM by addition of sucrose. 5 mM phosphate buffer, pH 7.0, unless otherwise stated, and 1 mM choline was present. For further details, see Materials and Methods. Expt. A: 3.4 mg protein plus 1.1 μg antimycin per ml were present. □—□, ■—■, sucrose media; ○—○, ●—●, 80 mM KCl present; open symbols, plus 2 mM ATP; closed symbols, plus 20 μM 2,4-dinitrophenol. Expt. B: 3.7 mg protein, plus 0.7 μg antimycin per ml were present. ○—○, ●—●, pH 6.6; □—□, ■—■, pH 7.1; △—△, ▲—▲, pH 7.7; open symbols, plus 2 mM ATP; closed symbols, plus 20 μM 2,4-dinitrophenol.

slow uptake are diminished (Fig. 3A). In the presence of 80 mM KCl, the fast uptake is nearly abolished and the slow uptake is more than halved. The inhibition of choline uptake by K^+ is competitive; a K_i of 15 mM was found for K^+. Lowering the pH has the same effect. Other cations tested (laurylamine, ornithine, Na^+, NH_4^+, Mg^{2+} and La^{3+}) also inhibit choline uptake. Laurylamine is the most effective: at a concentration of 40 μM, the choline uptake is halved (competitively), whereas the structurally related lauric acid at 150 μM does not inhibit to a significant extent. The other monovalent cations are approximately as effective as potassium. Mg^{2+} is about 10 times and La^{3+} about 50 times as effective as K^+ in inhibiting choline uptake by rat liver mitochondria.

We also tested some zwitterions and anions in their ability to inhibit choline uptake. Citrulline, betaine, acetate, nitrate and thiocyanate had no effect. Carnitine severely inhibited (90 % at 10 mM) and phosphate slightly stimulated choline uptake by rat liver mitochondria.

The influence of K^+ on uncoupler-stimulated choline oxidation [1] is plotted in Fig. 4 as a function of the amount of choline and its oxidation products in the matrix space. At increasing concentrations of K^+ in the medium, the concentration of choline in the matrix space decreases sharply, whereas the concentration of betaine increases [6]. The amount of betaine aldehyde remains low and constant.

Under State-3 conditions the influence of K^+ is much less marked [14]. The rate of choline oxidation is lowered from 2.8 nmol oxygen per mg protein to 1.6 nmol when 160 mM sucrose is replaced by 80 mM KCl. By this replacement, the concentration of choline in the matrix space is lowered from 3.0 to 0.7 mM. Mg^{2+} and La^{3+} show similar effects.

When, in the presence of uncoupler, extramitochondrial products of choline

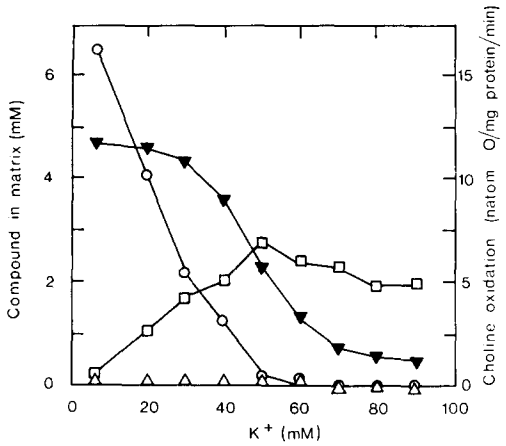

Fig. 4. Effect of K^+ on the concentration of intermediates of choline oxidation. Rat liver mitochondria, 4.7 mg protein per ml when oxygen consumption was measured, and 3.2 mg protein when product analysis was carried out, were incubated in a medium containing KCl as indicated in the figure. Samples have been taken after 2 min. Further details are described under Materials and Methods. ▼ —▼, Oxygen consumption. ○ —○, Choline in the matrix. □ —□, Betaine in the matrix. △ — △, Betaine aldehyde in the matrix.

oxidation are determined, the amount of betaine aldehyde is low [1]. In some cases, however, betaine aldehyde constitutes 20–30 % of the products found. In these cases, both betaine and betaine aldehyde production are inhibited by cations.

The inhibition by K^+ of choline uptake by rat liver mitochondria can be influenced by valinomycin (Fig. 5) when a source of energy is present. At low K^+ con-

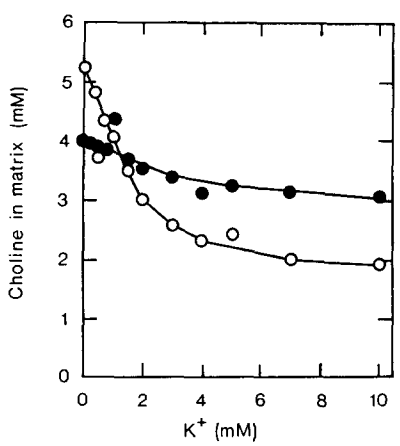

Fig. 5. Influence of valinomycin and K^+ on choline uptake. Mitochondrial protein (1.4 mg protein/ml) was incubated in a medium containing 2 mM Tris · HCl, 1.1 μg antimycin per mg protein, 1 mM ATP, Tris salt. Sucrose was added to maintain the osmolarity at 185 mosM. Final pH, 6.9; temperature 25 °C; incubation time, 3 min. For further details of analysing and taking samples, see Materials and Methods. ● —●, no addition; ○ —○, plus valinomycin (0.01 μg per mg protein).

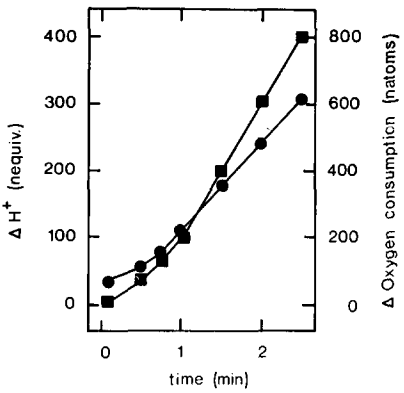

Fig. 6. Production of H^+ and oxygen consumption during uncoupler-stimulated choline oxidation. Rat liver mitochondria (8.5 mg protein) were incubated in an oxygraph vessel containing a pH electrode. The medium contained 185 mM sucrose, 1.5 mM phosphate buffer and 20 μM 2,4-dinitrophenol. Plotted is the difference between an experiment plus and minus choline. Final pH, 7.0; temperature, 25 °C; final volume, 1.7 ml. Calibration was achieved by 10 mM oxalic acid. ■—■, oxygen consumption, ●—●, H^+ production.

centrations in the medium (less than 1.25 mM) valinomycin stimulates choline uptake. At higher concentrations of K^+, valinomycin inhibits strongly. At 10 mM K^+, the amount of choline in the matrix space is the same as in the presence of uncoupler. Under various conditions, we studied the influence of choline on K^+ movement using a K^+-sensitive electrode. No influence could be found.

Whereas no influence of choline on K^+ movement could be found, an effect

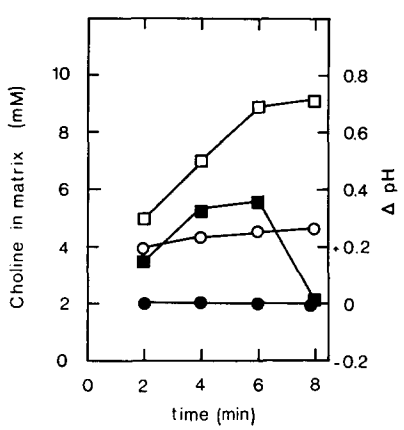

Fig. 7. Intramitochondrial pH and choline uptake. Rat liver mitochondria (4.4 mg protein per ml) were incubated in a medium containing 185 mM sucrose, 1.5 mM phosphate buffer (pH 7.0), 1 mM choline, 1.1 μg antimycin per mg protein. When choline uptake was measured, label was added according to Materials and Methods. The difference between intramitochondrial and extramitochondrial pH was measured by the addition of 0.4 μCi [^{14}C]DMO, plus 1.8 μCi ^3H_2O. The difference of an experiment plus and minus choline is plotted. □—□, ■—■, plus 2 mM ATP. ○—○, ●—●, plus 20 μM 2,4-dinitrophenol. Open symbols, choline uptake was measured. Closed symbols, ΔpH was measured.

could be seen if H^+ production was studied. Fig. 6 shows H^+ production and oxygen consumption during uncoupler-stimulated choline oxidation. Product analysis showed that twice the amount of betaine plus the amount of betaine aldehyde produced, equals the amount of oxygen consumed. In the same experiment it could be shown that the amount of H^+ produced equaled the betaine production if sonified mitochondria were used. The same is true for intact rat liver mitochondria after the first minutes. However, during the first seconds about 25 nmol H^+ are produced which cannot be accounted for by the production of betaine (Fig. 6), because the rate of choline oxidation is very low during the first minutes [15].

Further proof that the intramitochondrial pH can be influenced by choline uptake can be seen in Fig. 7. In the presence of uncoupler, after 2 min, no difference can be seen, the same situation as in Fig. 6 after 2 min. However, in the presence of ATP, the matrix space becomes more alkaline by about 0.4 pH units. When choline uptake stops (in this experiment after 6 min) ΔpH drops to zero. In a KCl medium, no or hardly any choline uptake or pH difference could be found under State-4 conditions.

DISCUSSION

Rat liver mitochondria are able to take up choline from a sucrose medium against a concentration gradient. Two different uptake processes can be distinguished: (1) a rapid energy- and temperature-independent process. Its minimal rate is approx. 40 nmol choline taken up per mg protein per min at a concentration of 1 mM in the medium. The K_m for choline is 1.4 mM. Since there is no binding of choline to mitochondrial proteins, this must represent a rapid entry of choline into the matrix space. (2) a slow energy-dependent process with a Q_{10} of 3.1. At 1 mM choline in the medium, the rate of choline uptake is about 1 nmol per mg protein per min. The K_m for the substrate is 0.3 mM. Both processes are inhibited by cations. All cations tested inhibit both the slow and the rapid uptake process.

It has been suggested that the uptake of choline from the medium is a regulatory step in overall choline oxidation by rat liver mitochondria [3–5, 16]. Under the usual oxidizing conditions only the rapid uptake process takes place. This is obvious in the presence of uncoupler and is demonstrated in Fig. 1 of ref. 6, under State-3 conditions. No accumulation of choline takes place when oxidation of choline itself is the only source of energy.

In a sucrose medium the amount of choline in the matrix space is the same, whether mitochondria oxidize choline or whether choline oxidation is inhibited. Therefore, the rate of choline uptake under these conditions is at least higher than the maximal rate of choline oxidation. This finding does not seem to favour the hypothesis that choline uptake is the regulatory step in overall oxidation, as suggested by Wilken and coworkers [4, 5].

However, another picture emerges in media containing high concentrations of cations. In that case, inhibition of overall oxidation, both in the presence of uncoupler and under State-3 conditions, is accompanied by a decreased choline concentration in the matrix. Also the concentration of betaine in the matrix space is increased and we suggest that this is the reason why choline oxidation is inhibited under these conditions [6].

The concentration range of KCl that inhibits choline oxidation is from 30 to 60 mM. In this range, the intramitochondrial choline concentration drops from 2.1 to 0 mM, while the betaine concentration is increased from 1.6 to 2.5 mM. The K_m of the enzyme choline dehydrogenase, for choline, is 0.7 mM under these conditions [15]. So, the intramitochondrial choline concentration is lowered from 3 times the K_m value to zero, accounting for a change from nearly maximal oxidation to nearly complete inhibition. At 60 mM KCl the rate of choline uptake is so low that all choline entering the matrix space is oxidized immediately, resulting in a steady-state concentration of choline in the matrix of zero. Because KCl also inhibits betaine efflux [11], increasing the KCl concentration in the medium also causes an increase in the betaine concentration in the matrix space. When the rate of formation of betaine becomes low at high concentrations of K^+, no further accumulation of betaine is observed.

Thus, we believe that choline uptake under these particular conditions is rate-limiting, as suggested by Williams [2, 3], even though the betaine concentration in the matrix is enhanced from 1.7 to 2.4 mM.

This is in good agreement with the finding that the rate of choline oxidation is lowered to a lesser degree by 80 mM KCl under State-3 conditions, than under uncoupled conditions. Because State-3 choline oxidation is much slower than uncoupler-stimulated oxidation, inhibition of the much faster process of choline uptake has, relatively, less influence on the overall rate of oxidation. Again, the decrease can be accounted for completely by a decrease of the intramitochondrial choline concentration.

Furthermore, in the presence of 80 mM KCl not only is the formation of betaine inhibited, but also the (small) [1] formation of betaine aldehyde. Again, inhibition of choline uptake is favoured over inhibition of betaine efflux as the mechanism of inhibition of overall choline oxidation [3].

On the basis of swelling experiments, it was concluded that choline did not participate significantly in proton-coupled antiport [7]. Although exactly the same conditions have been used in our laboratory, except that we omitted 0.2 mM cyanide from the medium, we found extensive swelling of mitochondria suspended in isotonic choline phosphate (Fig. 2). Choline has been used as an impermeable cation [18–21], although it became clearer and clearer that choline, to be oxidized, has to pass the mitochondrial inner membrane [1, 3–5, 13–15, 17, 22]. Also, the life-time of a pH gradient across the mitochondrial inner membrane upon addition of oxygen to mitochondria suspended in a choline medium is almost double the life-time of the gradient when mitochondria are suspended in sucrose (ref. 19, Figs. 8 and 10). For reasons of electroneutrality either a cation must leave the matrix space in exchange for choline, or an anion has to accompany choline entering the matrix space.

Choline is taken up by the mitochondria, up to 30 nmol per mg protein under appropriate conditions, in the absence of permeable anions. During the initial stages of choline uptake, an acidification of the external medium (Fig. 6, see also Fig. 10 of ref. 18) and an alkalinization of the matrix space (Fig. 7) is observed. Together with the finding that swelling occurs in choline phosphate, but not in choline nitrate, one is tempted to conclude that a choline-H^+ antiport system is present in the mitochondrial inner membrane.

On the other hand, the finding that choline entry is stimulated under energized conditions (Figs. 1 and 3) as well as under other conditions where a membrane po-

tential, with the extra-mitochondrial space positive, might exist (Fig. 4), suggests that choline enters the matrix electrophoretically. This latter view would be strengthened by the absence of swelling in choline acetate.

Clearly, further experimental work will be needed before we fully understand the movement of choline across the mitochondrial inner membrane.

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