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CONTROL OF CHOLINE OXIDATION BY RAT-LIVER MITOCHONDRIA

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SUMMARY

The steady-state concentrations of choline and its reaction products in intact rat-liver mitochondria were determined under different conditions. From these measurements, it is concluded that in a sucrose medium choline dehydrogenation and betaine aldehyde dehydrogenation are the rate-limiting steps in overall choline oxidation under "State-3" or uncoupled conditions, respectively.

Ageing of the mitochondria leads to changes in the mitochondrial membrane, resulting in a markedly different pattern of oxidation products. This finding explains why rotenone inhibits oxygen uptake with choline as substrate in fresh but not in aged mitochondria.

INTRODUCTION

The oxidation of choline by freshly prepared rat-liver mitochondria results mainly in the formation of betaine [1–3]. Only after prolonged incubation or ageing of the mitochondria is betaine aldehyde also found [4–7].

The localization of the product betaine depends on the incubation conditions. Under phosphorylating conditions it is mainly found in the matrix space, whereas in the presence of an uncoupler of oxidative phosphorylation virtually all betaine appears in the surrounding medium [1–3].

If the rate of oxygen uptake under the two conditions is compared, one finds that "State-3" oxidation is relatively slow [2, 3, 5, 8]. However, after prolonged incubation the rate of "State-3" choline oxidation appears to be enhanced [4, 5]. Williams [9] showed under comparable conditions a diminished integrity of the mitochondrial membrane. This was further substantiated by the demonstration of swelling [4, 5], loss of adenine nucleotides from the matrix space [5] and loss of coupling between oxidation and phosphorylation [4, 5].

The effect of rotenone on overall choline oxidation is variable. Whereas Tyler et al. [10] find no or nearly no inhibition, Azzone and coworkers report inhibition between 10 % and 75 % depending on the time of (pre)incubation [4, 11].

In this paper we report the results of a systematic investigation of the oxidation of choline by rat-liver mitochondria and the effect of the degree of ageing on this

process. In this way we hoped to be able to establish which factors determine the overall rate of choline oxidation under various conditions.

MATERIALS AND METHODS

Rat-liver mitochondria were isolated according to the method of Hogeboom [12], as described by Myers and Slater [13]. Protein was determined according to Cleland and Slater [14]. Phosphorylation was determined by measuring the incorporation of $^{32}P_i$ into ATP after extraction of inorganic phosphate according to Nielsen and Lehninger [15].

Incubations were carried out in a medium containing 185 mM sucrose, 5 mM potassium phosphate, 1 mM choline plus additions as indicated in the figure or table. Final pH 7.0, and temperature 25 °C, unless stated otherwise.

Separation of mitochondria from incubation mixtures was achieved according to the method of Werkheiser and Bartley [16] as described by Harris and Van Dam [17].

Products of choline oxidation were measured by a tracer method. In these experiments 2 μCi [^{14}C]-labelled choline was added and the products were separated according to Wilken [18].

If accumulation of choline or its products in the mitochondrial matrix was measured 0.1 μCi [^{14}C]choline was added, plus 0.2 μCi ^3H_2O. For further details of sampling, counting of radioactive samples, measuring oxygen and calculations, see our earlier paper [3].

Submitochondrial particles containing choline dehydrogenase activity separated from betaine aldehyde dehydrogenase activity were prepared according to Feinberg et al. [19], at pH 7.0, using mitochondria washed three times to avoid contamination with intramitochondrial aldehyde dehydrogenase [18]. The preparation was dialysed overnight to remove phosphate and adenine nucleotides. Careful analysis of the products of choline oxidation by this preparation showed that less than 5 % of the product was betaine.

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

RESULTS

Products of choline oxidation and their localization

The rate of "State-3" choline oxidation by freshly prepared rat-liver mitochondria and the localization of the products is a function of the time of incubation (Fig. 1A, B; see also ref. 5).

During the first minutes, the oxidation rate is relatively slow ($\approx$ 1 nmol oxygen consumed per mg protein per min) [1–5, 7, 8]. Betaine, the almost exclusive product [1–3] under these conditions is found mainly in the matrix space (Fig. 1B) and its concentration can reach a value 1000 times higher than that in the surrounding medium.

Oxidation of choline to betaine is coupled to phosphorylation (Fig. 1C); a

State-3' choline oxidation, a three step process

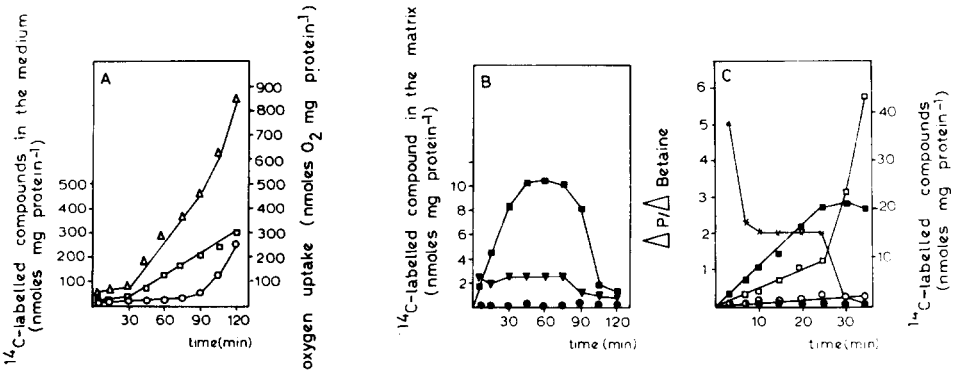

Fig. 1. Rat-liver mitochondria (1.1 mg protein per ml, Fig. 1A, 1B or 3.0 mg protein per ml Fig. 1C) were incubated under conditions as described under Materials and Methods. In the medium for the experiments A and B also 2 mM ADP was present. The experiment C consists of three parallel incubations. The first one contained [^{14}C]choline, the two others $^{32}P_i$, one in the presence of choline, the other in its absence. The difference in phosphate incorporation of these incubations is ascribed to oxidation of choline. At times indicated in the figures, samples of 0.9 ml were taken and analysed according to Materials and Methods. Symbols: □—□, betaine in the medium; ■—■, betaine in the matrix; ○—○, betaine aldehyde in the medium; ●—●, betaine aldehyde in the matrix; ▼—▼, choline in the matrix; △—△, oxygen consumption; ×—×, $\Delta P/\Delta$ betaine.

$\Delta P/\Delta$betaine ratio of 2 is calculated, comparable to a $\Delta P/\Delta O$ ratio of 1 [4, 5, 20], because the oxidation of one molecule choline to betaine requires two oxygen atoms.

After about 30 min the situation changes markedly. The rate of oxygen uptake increases 5–10 times and the betaine formed now is found mainly in the medium. No or nearly no further increase of betaine concentration in the matrix space is observed (Fig. 1B). Furthermore, a dramatic decrease in $\Delta P/\Delta$betaine can be seen (Fig. 1C). In fact, the oxidation of choline is no longer coupled to phosphorylation.

It may be noted, however, that no significant decrease of the betaine or choline concentration in the matrix occurs and that betaine remains the main product.

About 75 min after starting the experiment, a further increase in rate of oxygen uptake can be seen (Fig. 1A). This enhancement can be completely accounted for by formation of betaine aldehyde. It is accompanied by a decrease of the mitochondrial concentrations of choline and betaine to values comparable to those in the surrounding medium (Fig. 1B).

Under uncoupled conditions (Fig. 2A) a period with a low oxidation rate is also found. However, it lasts only a few minutes and there is no betaine accumulated in the matrix space (not shown).

After this lag period uncoupler-stimulated choline oxidation shows a picture that resembles "State-3" oxidation after 30 min in some respects. The rate of oxygen uptake is about the same under both circumstances and so is the localization of the main product betaine produced during this period. In the matrix space choline is present in a concentration of 2–4 mM; betaine aldehyde is present only in trace amounts. During this incubation mitochondria swell.

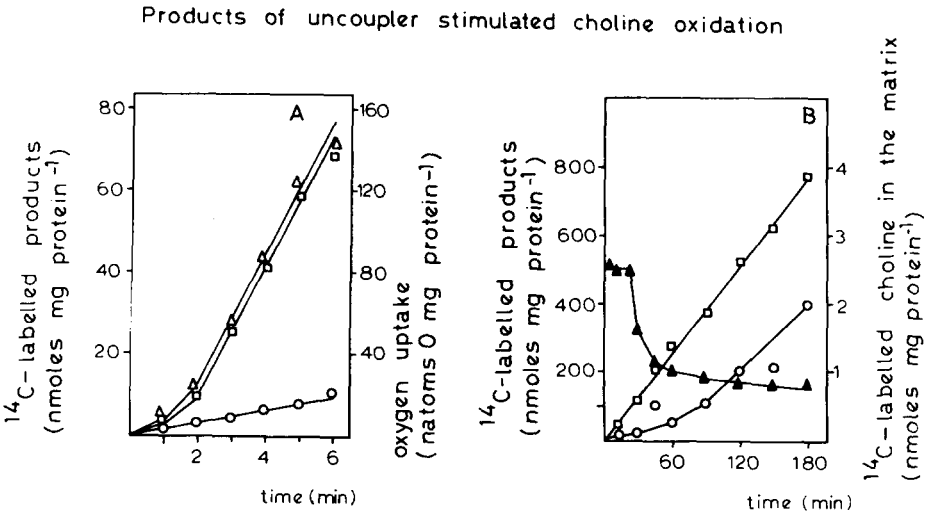

Fig. 2. Rat-liver mitochondria were incubated in a medium as described under Materials and Methods, plus 20 μM 2,4-dinitrophenol. At times indicated in the figure samples were taken and analysed according to Wilken [18]. See Materials and Methods for further details. A. 4.1 mg protein per ml was incubated in an oxygraph vessel. When the oxygen concentration was nearly zero, catalase plus H_2O_2 was added. B. Two parallel experiments were performed. For measuring products of choline oxidation 2 μCi [^{14}C]choline was added and 3.7 mg protein per ml. For measuring uptake of [^{14}C]choline by mitochondria, 0.12 mg protein, 0.2 μCi [^{14}C]choline and 0.2 μCi ^3H_2O were added. Symbols: ○—○, betaine aldehyde in the medium; □—□, betaine in the medium; ▲—▲, choline in the matrix, △—△, oxygen uptake.

When the incubation time is prolonged also in the presence of uncoupler betaine aldehyde appears in the medium (Fig. 2B). The choline concentration at the same time drops to the value found in the medium. Again the situation is comparable to that under "State-3" conditions after an incubation period of 90 min. When betaine aldehyde appears in the medium the intramitochondrial choline concentration drops. Extensively aged [4, 5], sonicated or calcium-treated [21] mitochondria do not show a lag time in choline oxidation, betaine or betaine aldehyde production. Immediately, choline is oxidized at the maximal rate and no stimulation is found by ADP or 2,4-dinitrophenol. The rate of betaine aldehyde formation is twice the rate of betaine formation. We confirmed the higher capacity of choline dehydrogenase when compared to (betaine) aldehyde dehydrogenase with the separate enzymes, isolated according to Feinberg [19] under various conditions.

Careful analysis of the contents of the matrix space of mitochondria, oxidizing choline in the presence of uncoupler and under "State-3" conditions shows that in the first case 0.16 mM betaine aldehyde is present, and this is increased markedly by rotenone, whereas under "State-3" conditions the concentration of betaine aldehyde is only 0.06 mM and this value is hardly influenced by rotenone (Table I).

The influence of acetoacetate and β-hydroxybutyrate on the amount of betaine aldehyde in the matrix space can be seen in lines 5 and 6. In the presence of β-hydroxybutyrate there is a slight increase, whereas in the presence of acetoacetate there is no change in concentration of betaine aldehyde in the matrix space, when compared to

TABLE I

CHOLINE AND ITS OXIDATION PRODUCTS IN THE MATRIX SPACE UNDER VARIOUS CONDITIONS

6 mg mitochondrial protein per ml were incubated and analysed as described under Materials and Methods. The medium contained 5 μCi [^{14}C]choline. Time of incubation 2 min.

Expt	Additions	Choline (nmol/mg protein)	Betaine aldehyde (nmol/mg protein)	Betaine (nmol/mg protein)
1	2,4-dinitrophenol (20 μM)	2.2	0.16	0.2
2	2,4-dinitrophenol (20 μM), rotenone (2 μg/mg protein)	1.9	0.26	0.1
3	ADP (2 mM)	1.7	0.05	2.1
4	ADP (2 mM), rotenone (2 μg/mg protein)	1.6	0.06	1.7
5	2,4-dinitrophenol (20 μM), acetoacetate (2 mM)	2.1	0.17	0.2
6	2,4-dinitrophenol (20 μM), β-hydroxybutyrate (10 mM)	2.2	0.20	0.2

line 1. The amount of choline and betaine in the matrix space is in agreement with our earlier publications [1–3].

The "steady state" concentration of choline in the matrix space is reached within the dead time of the measurements, about 8 s. Therefore, at a concentration of 1 mM choline in the medium the rate of uptake of choline by rat-liver mitochondria is at least 15 nmol per mg protein per min, probably higher. At an outside concentration of 1 mM the rate of choline oxidation is about 5 nmol per mg protein per min.

Influence of rotenone on uncoupler-stimulated choline oxidation

Rotenone is an efficient inhibitor of uncoupler-stimulated choline oxidation when added to fresh mitochondria or after a short preincubation (Fig. 3A, traces 1 and 2). After a longer period of preincubation the effect of rotenone diminishes (Fig. 3A, trace 3) and, when added after 10 min the influence of rotenone on uncoupler-stimulated choline oxidation is negligible (not shown).

When aged mitochondria are used for the same experiment instead of freshly prepared ones we obtain results as depicted in Fig. 3B. It is clear that aged mitochondria are less sensitive to rotenone added after a short preincubation period than fresh mitochondria. When mitochondria are aged overnight or sonicated no inhibition by rotenone is found at all.

It is noteworthy that the moment of addition of choline does not affect its oxidation rate after a given preincubation time to any degree.

The amount of rotenone needed for maximal inhibition was found to be about 20 pmol per mg protein, in good agreement with the value reported by Ernster and coworkers [22] for complete inhibition of NADH dehydrogenase. Furthermore, we checked the influence of rotenone on the uptake of choline, the efflux of betaine from the matrix space and on the dehydrogenases prepared from mitochondrial protein according to Feinberg [19]. Under the conditions used in Fig. 3 the influence of rotenone on these processes was negligible.

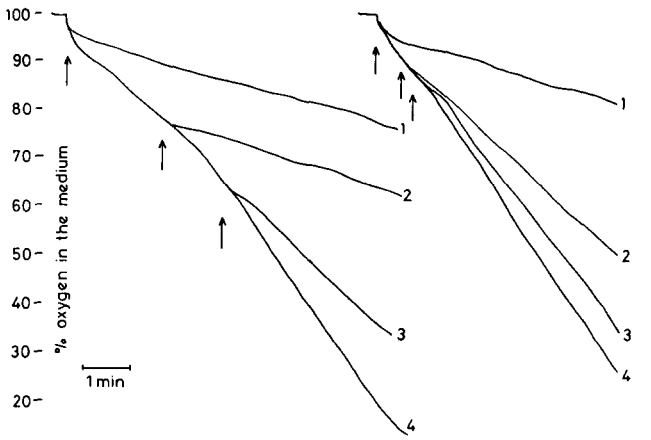

Fig. 3. Influence of rotenone on uncoupler-stimulated choline oxidation. Mitochondrial protein (3.2 mg per ml) was incubated in an oxygraph vessel. Medium and description of methods see Materials and Methods. The choline chloride concentration used was 10 mM instead of 1 mM; 20 μM 2,4-dinitrophenol was present. At times, indicated in the figure with arrows, rotenone was added (1 μg per mg protein). Both figures: Trace 1, rotenone added at the first arrow; Trace 2, rotenone added at the second arrow; Trace 3, rotenone added at the third; Trace 4, no rotenone added. A. Freshly prepared mitochondria. B. Mitochondria aged for 1 h at 25 °C before use.

Substrates that influence the degree of reduction of NAD also have a marked influence on uncoupler-stimulated choline oxidation (Table II). Acetoacetate stimulates choline oxidation, both in the presence and absence of rotenone, and β-hydroxybutyrate inhibits. It may be noted that acetoacetate not only stimulates betaine forma-

TABLE II

INFLUENCE OF THE DEGREE OR REDUCTION OF NAD ON UNCOUPLER-STIMULATED CHOLINE OXIDATION

Rat-liver mitochondria (5.1 mg per ml) were incubated and analysed according to Materials and Methods. 20 μM 2,4-dinitrophenol was added to the medium. Incubation time 5 min.

Expt	Additions	Products in the medium (nmol/mg protein)	
		Betaine aldehyde	Betaine
1	None	5.7	26.0
2	Rotenone (1 μg per mg protein)	1.6	3.2
3	β-hydroxybutyrate (10 mM)	9.0	3.9
4	Acetoacetate (2 mM)	20.2	24.5
5	Rotenone (1 μg per mg protein), β-hydroxybutyrate (10 mM)	1.7	3.2
6	Rotenone (1 μg per mg protein), acetoacetate (2 mM)	19.6	25.0
7	Rotenone (1 μg per mg protein) added after 5 min	6.4	24.2
8	Rotenone (1 μg per mg protein) plus β-hydroxybutyrate (10 mM) added after 5 min	5.7	13.7

tion, it also stimulates betaine aldehyde production.

When the effectors are added after 5 min preincubation, rotenone has only a minor effect (Fig. 3A). However, added together with β-hydroxybutyrate, rotenone can inhibit choline oxidation 50 %.

Acetoacetate and β-hydroxybutyrate do not have a very marked effect on the maximal rate of uncoupler-stimulated choline oxidation, but both have an influence on the time it takes to reach the maximal oxidation rate. In the presence of acetoacetate the maximal rate of oxidation is reached within the dead time of the measurement, whereas in the presence of β-hydroxybutyrate the lag-time is enlarged and now lasts between 5 and 8 min. The lagtime in the absence of acetoacetate or β-hydroxybutyrate lasts about 2 min. (see Fig. 2A, Fig. 3).

"State-3" choline oxidation is influenced by rotenone to an extent of maximally 40 %, usually much less (Compare Table I, Lines 3 and 4).

Properties of choline and (betaine) aldehyde dehydrogenase

Dehydrogenase reactions involved in overall choline oxidation by rat-liver mitochondria are choline dehydrogenase and (betaine) aldehyde dehydrogenase, both intramitochondrial enzymes [3, 7].

The aldehyde dehydrogenase, probably the enzyme reported by Smith and Packer [23] does not show much activity without NAD^+. The K_m for betaine aldehyde of the isolated enzyme is 0.6 mM, in good agreement with the results reported by Smith and Packer [23]. Chemicals that influence overall choline oxidation by intact mitochondria such as adenine nucleotides [5], phosphate [3] or choline do not significantly influence the activity.

Isolated choline dehydrogenase shows a K_m for substrate of 0.7 mM (see Table III). This value is strongly influenced by adenine nucleotides. Not only the K_m changes, but also the V. AMP in general favours a high oxidation velocity, ATP a low one and ADP an oxidation rate somewhere in between. However, when both ATP and AMP

TABLE III

INFLUENCE OF ADENINE NUCLEOTIDES ON CHOLINE DEHYDROGENASE

Particles from rat-liver mitochondria (1.4 mg protein per ml) prepared as described under Materials and Methods, were incubated in a medium containing sucrose and phosphate buffer in an oxygraph vessel, as described under Materials and Methods. Each incubation contained 1.7 μg oligomycin per mg protein. Each experiment consists of a titration with the following choline concentrations: 0.2, 0.5, 1.0, 2.5 and 10 mM. The rate of choline dehydrogenation was determined as the rate of oxygen uptake. K_m and V were calculated from Lineweaver-Burk plots.

Expt	Additions	K_m (mM)	V (nmoles · mg protein^{-1} · min^{-1})
1	None	0.70	35
2	AMP	0.47	44
3	ADP	0.77	24
4	ATP	1.00	20
5	AMP+ADP	0.80	23
6	AMP+ATP	1.00	19
7	ADP+ATP	1.02	18
8	AMP+ADP+ATP	1.00	18

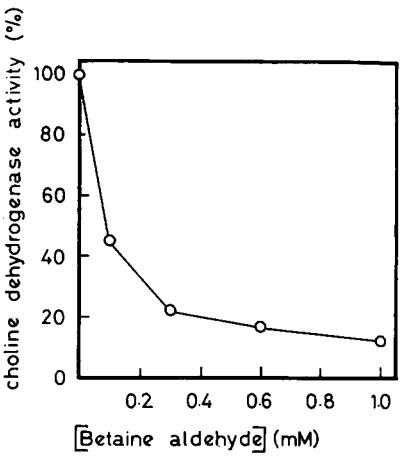

Fig. 4. Influence of betaine aldehyde on choline dehydrogenase activity. A particle preparation [19] (0.4 mg protein per ml) was incubated in a medium as described under Materials and Methods. The products of choline oxidation were determined as described under Materials and Methods. Betaine in all cases was less than 10 % of total products. Temp. 20 °C, time of incubation 10 min.

are present the influence of ATP is dominant.

Another effector of choline dehydrogenase is betaine aldehyde [24]. At a concentration of only 0.1 mM the activity of choline dehydrogenase diminishes 55 % (Fig. 4). At a concentration of 0.16 mM betaine aldehyde, as was measured in the presence of uncoupler (Table I), the oxidation rate is lowered by about 62 %. Rendina and Singer [24] reported a K_i of 2 mM, but they measured in whole liver homogenate.

Betaine has much less influence on choline dehydrogenase. About 10 mM is needed for 40 % inhibition. NAD^+ has no influence at all. Phosphate and rotenone, added in concentrations that inhibit choline oxidation by intact mitochondria strongly do not influence choline dehydrogenase significantly.

DISCUSSION

When "State-3" and uncoupler-stimulated choline oxidation by fresh rat-liver mitochondria are compared, large differences can be seen only in the first minutes of incubation. In the presence of ADP choline oxidation is relatively slow [2, 5, 8], coupled to phosphorylation [4, 5] and accompanied by accumulation of betaine [2]. In the presence of uncoupler the rate of oxidation is about 5 times as high [3, 10] and there is no intramitochondrial accumulation of betaine [3].

When, however, prolonged "State-3" choline oxidation (30–90 min) is compared with uncoupler-stimulated oxidation (0–60 min) there are strong similarities (compare Figs 1 and 2) .There is about the same oxidation rate, no coupling of oxidation to phosphorylation or to product accumulation and mitochondria under both conditions appear to be swollen [4] and have lost their adenine nucleotides [2, 5]. Betaine remains the main product up to this point.

When incubation is prolonged even further betaine aldehyde is also found as a product. Furthermore, mitochondria lose their ability to accumulate choline from the

medium after prolonged incubation (as when sonicated or calcium-treated mitochondria [24] are used). In the presence of uncoupler these changes in mitochondrial permeability appear more rapidly than under "State-3" conditions.

From the above-mentioned observations, it can be deduced that either prolonged incubation [9] or the presence of uncoupler [2] cause diminished integrity of the mitochondrial inner membrane. The longer the period of incubation, the more semipermeability-dependent functions of the mitochondrial inner membrane are lost.

The mitochondrial choline oxidase system, apart from the electron carrier chain, consists of the following steps: (i) the uptake of choline; (ii) oxidation of choline to betaine aldehyde by choline dehydrogenase; (iii) oxidation of betaine aldehyde to betaine by betaine aldehyde dehydrogenase and (iv) efflux of betaine from the matrix space. Extensively aged mitochondria also allow betaine aldehyde to leave the matrix space; we doubt, however, if this is a natural function of the mitochondrial inner membrane.

The rate of the first reaction, the uptake of choline from the medium, is at least three times higher than the rate of choline oxidation at the same choline concentration in the medium, probably more. Also the fact that, both in the presence of uncoupler and under "State-3" conditions, the amount of choline in the matrix is about the same, 1.5–4 nmol per mg protein, whereas the oxidation rates differ about five times, makes the suggestion of Kagawa and coworkers [5, 25] that the uptake of choline under "State-3" conditions would be the rate-limiting step, improbable. In fact, if choline uptake were rate-limiting one should expect no or nearly no choline in the matrix space.

As the rate-controlling step of "State-3" choline oxidation in our opinion betaine efflux does not come into consideration because of the constant rate of choline oxidation under these conditions at increasing intramitochondrial betaine concentrations [3]. Therefore, although we cannot rule out completely an indirect inhibition by betaine, we think that inhibition of the efflux of betaine and the low oxidation rate of choline have the same cause.

Under "State-3" conditions ATP and ADP are present in the matrix space. These adenine nucleotides are known to inhibit betaine efflux [2] but also favour a low rate of choline dehydrogenation (Table III). On the other hand, during uncoupled oxidation mainly AMP is present in the matrix.

That indeed choline dehydrogenase is the most strongly controlled step in overall "State-3" choline oxidation also follows from the low amount of betaine aldehyde present in the matrix under these conditions. If the rate-limiting step were after choline dehydrogenase one should expect accumulation of betaine aldehyde, especially in the presence of rotenone, and this is not found (Table I).

On the basis of these arguments we conclude that choline dehydrogenase is the rate-determining factor in overall "State-3" choline oxidation.

Uncoupler-stimulated choline oxidation shows a different pattern. The adenine nucleotide concentration in the mitochondrial matrix is low, betaine aldehyde is three times higher as compared to "State-3" conditions, the oxidation rate is enhanced about five times, and the betaine formed is found in the medium.

Because ATP and ADP are converted to AMP in the presence of uncoupler, choline dehydrogenase is activated.

At the same time betaine becomes permeant as a consequence of the decreased

concentration of adenine nucleotides in the matrix space [2]. The enhanced choline dehydrogenase activity causes accumulation of betaine aldehyde indicating that now betaine aldehyde dehydrogenation is rate-determining for oxygen uptake. As expected, rotenone in this case has a pronounced inhibitory effect on overall choline oxidation.

Thus, under uncoupled conditions betaine aldehyde dehydrogenation is the rate-determining reaction.

Inhibition of choline dehydrogenase by NADH would also explain the influence of rotenone on uncoupler-stimulated oxidation. However, it would not explain why in the absence of rotenone, under "State-3" conditions, when the degree of reduction is low, choline oxidation is only slow. Also it would not explain why in the presence of acetoacetate (low degree of reduction of NAD) the betaine aldehyde produced is found in the medium. The amount of betaine aldehyde in the matrix space does not depend in itself on the degree of reduction of NAD (see Table I, lines 5 and 6), suggesting that NADH does not regulate a step in overall choline oxidation before betaine aldehyde production. To explain the high amounts of betaine aldehyde in the medium in the presence of acetoacetate, we suggest that betaine aldehyde efflux is inhibited by a high degree of reduction of NAD.

As Bianchi and Azzone [4] already showed, rotenone inhibits choline oxidation by inhibition of NADH oxidation. The influence of β-hydroxybutyrate and acetoacetate on choline oxidation supports this idea strongly. If mitochondrial NAD is reduced (in the presence of rotenone plus substrate or in the presence of β-hydroxybutyrate) and at the same time betaine aldehyde dehydrogenase is the rate-determining step, rotenone will inhibit overall choline oxidation (Fig. 3A). If, however, no substrate is present [10] or choline dehydrogenase is rate-determining [4], rotenone will not inhibit overall choline oxidation (Fig. 3B). It may be noted here that the rate of choline oxidation is only 10–20 % of that of succinate oxidation; 5 % leakage through the rotenone block will account for nearly maximal choline oxidation.

When mitochondria are aged extensively both choline dehydrogenase and betaine aldehyde dehydrogenase work on their maximal capacity and the permeability of the membrane to all metabolites is high.

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