The Effect of Nicotinamide Adenine Dinucleotide and Rotenone on the Oxidation of Choline by Rat Liver Mitochondria*

(Received for publication, June 14, 1967)

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SUMMARY

The effect of semicarbazide on the oxidation of choline by rat liver mitochondria was studied. Semicarbazide was used to prevent both further oxidation of betainealdehyde and product inhibition of choline dehydrogenase by the aldehyde. NAD did not stimulate choline-based respiration when semicarbazide was present to prevent the oxidation of enzymatically formed betainealdehyde. In contrast, NAD did stimulate respiration when the semicarbazide was omitted. NAD stimulation of choline-supported respiration appears to be entirely due to its effect on the oxidation of betainealdehyde. In both the presence and the absence of semicarbazide, rotenone inhibited the oxidation of choline.

It was concluded that the rate of choline oxidation to betainealdehyde is decreased by rotenone and that this inhibition is not due to product inhibition by betainealdehyde. Choline oxidation by submitochondrial particles was insensitive to rotenone. Inhibition of respiration by rotenone was found to be competitive with respect to choline. Acetate ion eliminated the rotenone sensitivity of choline oxidation. These results suggest that rotenone may inhibit choline oxidation by reducing the rate of entry of choline into intact mitochondria.

Stimulation of choline oxidation by NAD was reported by Strength, Christensen, and Daniel (2). As a result of this report the role of NAD in choline oxidation has been the subject of several investigations. Bianchi and Azzone (3) reported that choline oxidation by rat liver mitochondria is inhibited partially by rotenone. Rotenone was used as a specific inhibitor of the NADH dehydrogenase complex (4). Inhibition by rotenone of choline oxidation was unexpected since isolated choline dehydrogenase contains FAD and does not reduce NAD (5, 6). Bianchi and Azzone (3) proposed that part of the electron flow from choline oxidation proceeds through reduction and reoxidation of NAD.

The product of choline dehydrogenase action is betainealdehyde, which subsequently is oxidized either by a cytoplasmic NAD-linked betainealdehyde dehydrogenase or by a mitochondrial NAD-linked nonspecific aldehyde dehydrogenase (3, 7, 8). Since rotenone prevents the reoxidation of NADH, the oxidation of enzymatically formed betainealdehyde would be inhibited, thus causing accumulation of the aldehyde. Product inhibition of choline dehydrogenase has been demonstrated (5). Thus, inhibition of choline dehydrogenase by accumulated betainealdehyde offered a hypothesis for the mechanism of rotenone action in choline oxidation. However, Bianchi and Azzone (3) found that inhibition by rotenone was high initially, which is not consistent with product inhibition.

We report here our studies on the effect of rotenone on choline-induced respiration in the presence of semicarbazide. Semicarbazide was chosen with the aim of preventing product inhibition by trapping the betainealdehyde as its semicarbazone (9).

METHODS

Respiration was measured with a Gilson differential manometer at 30°. Air was used as the gas phase and 0.2 ml of 10% KOH was placed in the center well. The incubation was started by the addition of substrate after an 8-min preliminary incubation. Data are reported after correction for endogenous respiration.

Rat liver mitochondria were prepared in 0.25 M sucrose, essentially by the method of Weinbach (10). In our hands, inhibitory effects of rotenone on choline oxidation were not seen when homogenization was carried out with more than a minimum number of vertical passes of the Teflon pestle in the tissue homogenizer. The procedure adopted was to make two additional passes of the pestle after fragments of the liver were no longer visible. The mitochondria were routinely washed once with 0.25 M sucrose, and were used within 20 min of preparation. Phosphate aging was according to the procedure of Bianchi and Azzone (3), except that the concentration of mitochondria was 0.5 g of tissue equivalent per ml. Rotenone, obtained from Columbia Organic Chemicals Company, Columbia, South Carolina, was recrystallized from ethanol water. Rotenone additions were in 10 μl of 95% ethanol. The same volume of ethanol was added to control flasks.
Semicarbazide was prepared salt-free by passing recrystallized semicarbazide hydrochloride solution over a column of Dowex 1 anion exchange resin in the hydroxide form. The semicarbazide solution was stored frozen. Submitochondrial particles were prepared by sonic oscillation of liver mitochondria suspended in 0.01 M, pH 7.4, Tris-chloride buffer (1 g of tissue equivalent per ml) with a Branson S-110 sonic oscillator (11). Sonic treatment was at 0° for 1 min at a setting of 3 (current of 3.5 amp), with a ¾-inch probe. After removal of the remaining mitochondria by centrifugation at 8,700 x g for 10 min, the submitochondrial particles were removed by centrifugation in a Spinco model 50 ultracentrifuge rotor for 30 min at 50,000 rpm. Particles were resuspended in 0.01 M Tris-chloride, pH 7.4, buffer. Submitochondrial particles from 3 g of tissue were used in each manometer flask.

RESULTS

Fig. 1 shows the effect of semicarbazide on the stoichiometry of choline oxidation by rat liver mitochondria. The stoichiometry was determined by adding a limiting amount of choline and measuring respiration until completion of the reaction was virtually reached. In the presence of semicarbazide, total oxygen consumption was 1.0 μatm per μmole of choline. In the absence of semicarbazide the total oxygen consumption at 6 hours was 1.7 μatm per μmole of choline. It is apparent that semicarbazide prevents the oxidation of internally generated betainealdehyde. Under the conditions used, the oxidation of betainealdehyde was not complete in 6 hours. During the first 10 to 20 min of the incubation, flasks having no semicarbazide did not respire more rapidly than those which contained semicarbazide.

Fig. 2 shows the course of respiration with intact mitochondria and 25 mM choline as substrate. Under these conditions the addition of semicarbazide caused an almost immediate inhibition. The time course of the inhibition was not consistent with significant inhibition of choline oxidation by either betainealdehyde or its semicarbazone.

If one assumes that semicarbazide has no effect on the dehydrogenation of choline, then the amount, in micromoles of betainealdehyde oxidized, would equal the number of microatoms of oxygen consumed in the absence of semicarbazide minus the number of microatoms of oxygen consumed in the presence of semicarbazide. It was observed that increasing semicarbazide resulted in increasing inhibition until a maximum inhibition was
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reached which was not further increased by higher semicarbazide concentrations. The mean maximal inhibition was 36%, with a range of 25 to 50% in seven determinations. Rapid oxidation of the enzymatically formed betainealdehyde in the control and complete prevention of its oxidation in the presence of semicarbazide would result in a 50% maximal inhibition of respiration.

With the above method of calculation, the data of Figs. 1 and 2 indicate that there is a lag phase in the oxidation of enzymatically generated betainealdehyde, which diminishes with increasing choline concentration. With 25 mm choline the lag was much decreased. The reasons for the lag are not as yet apparent.

Rotenone inhibited respiration in both the presence and the absence of semicarbazide. Thus, there is a semicarbazide-sensitive component of respiration with choline as substrate. Rotenone inhibition is not limited to this component.

Fig. 3 shows the effect of semicarbazide upon respiration with choline as substrate with phosphate-aged mitochondria. The addition of NAD caused a stimulation of respiration if semicarbazide was not added. When semicarbazide was added, NAD produced essentially no stimulation. Rotenone caused an inhibition of respiration in the presence of semicarbazide and added NAD with choline as substrate. In other experiments (not shown), more extensive aging of mitochondria eliminated the rotenone sensitivity.

Because phosphate aging experiments had indicated a structural dependence of rotenone sensitivity (3), we examined the rotenone sensitivity of submitochondrial particles prepared from rat liver. Respiration was measured in the presence of 25 mM choline and 0.1% cytochrome c. The medium was otherwise as in Fig. 2, except that KCl and sucrose were omitted. Rotenone (3.5 μM) had no effect on choline oxidation under these conditions.

Variation of respiration as a function of rotenone concentration is depicted in Fig. 4. With choline concentration at 25 mM, rotenone inhibited respiration 50% at a concentration of 160 μmole/g of protein. When β-hydroxybutyrate (25 mM) was used as a substrate, 50% inhibition was obtained at 19 μmole/g of protein. Bianchi and Azzone (3) achieved maximal inhibition at 50 μmole/g of protein with choline in the absence of semicarbazide. Fig. 5 shows the dependence of respiration rate on choline concentration by means of a double reciprocal plot according to the method of Lineweaver and Burk (12). The data indicate that rotenone is a competitive inhibitor with respect to choline.

Table I shows the effect of substituting 5 mM potassium acetate for part of the potassium chloride present in the incubation mixture. Phosphate was not added, and its presence was not necessary for respiration to be shown. Acetate itself had little effect on the control rate of choline oxidation. In the presence of acetate, no inhibition of choline oxidation by rotenone was observed.

**DISCUSSION**

It can be seen from the effect of semicarbazide on the stoichiometry of the reaction that semicarbazide was effective in prevent-

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**Table I**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Respiration</th>
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<tr>
<td>Control + rotenone</td>
<td></td>
</tr>
<tr>
<td>25 mM KCl</td>
<td>85</td>
</tr>
<tr>
<td>20 mM KCl-5 mM potassium acetate</td>
<td>76</td>
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ing further oxidation of betainealdehyde produced in the oxidation of choline by intact mitochondria.

Rotenone inhibited choline oxidation in the presence of semicarbazide; therefore, rotenone inhibits the oxidation of choline to betainealdehyde quite apart from the expected effect of rotenone on NAD-linked betainealdehyde oxidation. The action of semicarbazide as a trapping agent eliminates the possibility that rotenone inhibition is due to the accumulation of betainealdehyde, causing the consequent inhibition of choline dehydrogenase.

With phosphate aged mitochondria, added NAD stimulated respiration following choline addition. Since semicarbazide eliminated this increase in respiration, it is apparent that the stimulating effect of NAD is limited to its effect on betainealdehyde oxidation by mitochondria. Strength et al. (2) found that choline-supported respiration was stimulated by NAD. Rothschild, Cori, and Barron (13) found that added NAD was not reduced after choline addition. However, Bianchi and Azzone (3) reported small increases in mitochondrial NADH after choline addition. They also reported that NAD stimulated the oxidation of choline to betainealdehyde.

Bianchi and Azzone (3) found that, with mitochondria in hypotonic solution, the addition of NAD was necessary for rotenone inhibition. This is consistent with the hypothesis that the inhibition of choline oxidation is related to the interaction of rotenone with the NADH dehydrogenase complex. On the other hand, since NAD did not stimulate choline oxidation in the presence of the semicarbazide, the evidence presented here is not consistent with the suggestion of Bianchi and Azzone (3) that part of the electron flow during choline oxidation to betainealdehyde proceeds through NADH reduction and resorption. It may be that the involvement of the rotenone-sensitive step is an indirect one.

Submitochondrial particles were not sensitive to inhibition of choline oxidation by rotenone plus NAD, suggesting that the rotenone inhibition depends upon structural features which are lost in the preparative procedure.

One possible interpretation of the structural dependence of the inhibition is that rotenone interferes with the entry of choline into the mitochondrial compartment containing choline dehydrogenase. This hypothesis is made less attractive by the observation that mildly swollen mitochondria are still subject to rotenone inhibition, even though they are permeable enough to require NAD supplementation. However, further swelling causes elimination of rotenone plus NAD sensitivity.

Other laboratories have reported (13, 14) that, in the absence of trapping agent, betainealdehyde is oxidized more slowly than choline, so that some accumulation of the aldehyde occurs when choline is added. The partial inhibition of choline-based respiration by semicarbazide reported here is not consistent with the recent report of Yue, Russel, and Mulford (15) that rat liver mitochondria are unable to oxidize added betainealdehyde. Differences may perhaps be ascribed to differences in experimental conditions or to the fact that betainealdehyde in the present experiments was freshly generated inside the mitochondrion. The lag phase in betainealdehyde oxidation herein described may indicate that under certain conditions intact mitochondria are unable to oxidize internally generated betainealdehyde. A knowledge of the mechanism involved in the lag phase of betainealdehyde oxidation would help in assessing the quantitative importance of intramitochondrial oxidation of the aldehyde under physiological conditions.

At a substrate level of 25 mM, the amount of rotenone required to achieve 50% inhibition of choline oxidation was 11-fold greater than that required for β-hydroxybutyrate oxidation. However, the respiratory rate with β-hydroxybutyrate was much greater than when choline was used as substrate. When the absolute respiratory rates are compared in the presence of various rotenone concentrations, it is seen that the concentrations of rotenone required to reduce choline-based respiration allowed a greater residual respiration when β-hydroxybutyrate was the substrate.

The finding that rotenone inhibition of respiration is competitive with respect to choline is also not consistent with a direct effect of rotenone on electron flow from choline through NADH to oxygen. Since there is little structural analogy between choline and rotenone, a competition between these two compounds on the site of an enzyme seems unlikely. On the other hand, if rotenone acted to interfere with the entry of choline in some way, then high concentrations of choline might be expected to overcome inhibition by rotenone.

Wilken, Kagawa, and Lardy (16) reported that with mitochondria in a "controlled state" choline entry was rate limiting. Inhibition of choline-dependent respiration by uncoupling agents has been reported by Feinberg (17). These findings were interpreted by us as indicating that the entry of choline into intact mitochondria is energy dependent. The inhibition of choline oxidation by rotenone may be related to the effect of uncoupling agents, in that oxidative phosphorylation with endogenous NAD-linked substrates would be prevented, thus limiting the energy supply for choline transport.

The rotenone sensitivity of choline-dependent respiration was eliminated by the addition of acetate. Acetate has been reported to facilitate the entry of K+ and Ca++, and di- or tricarboxylic acid salts of potassium into mitochondria (18-21). Acetate may also facilitate the entry of choline, thus preventing the entry step from becoming rate limiting for respiration in the presence of rotenone. Apparently acetate facilitates the entry of both cations, owing to its action as a permeant counter ion. An alternative possibility is that the movement of undissociated acetic acid through the membrane barrier, followed by its dissociation, reduces the alkalinity of the internal medium (matrix) of the mitochondria. The reduced pH of the internal medium may aid the cation entry in some way. If choline transport is an ion exchange process in which choline moves in with the concurrent outward movement of protons, then reducing the internal pH of the mitochondrion could facilitate the exchange.

Tyler, Gonze, and Estabrook (22) reported recently that rotenone did not inhibit choline oxidation in rat liver mitochondria. Significant differences in their experiments were lower rotenone concentration, the absence of uncoupling agents, and the lack of EDTA, Mg++, and arsenite in the medium.

REFERENCES

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