THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 239, No. 11, November 1964
Printed in U.S.A.

Oxidation of Choline in Rat Liver Mitochondria*

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(Received for publication, April 1, 1964)

The involvement of diphosphopyridine nucleotide in choline oxidation has been the subject of many investigations. Oxidation of choline by liver preparations was first studied by Mann, Woodward, and Quastel (1) and was considered not to involve the intervention of pyridine nucleotides. An opposite conclusion was reached by Strength, Christensen, and Daniel (2), who found that oxidation of choline is stimulated considerably by the addition of DPN. In subsequent studies, Rothschild, Cori, and Barron (3) found that choline oxidation is stimulated by flavin adenine dinucleotide, and that DPN is not reduced after addition of choline. The P:O ratio of 2 found by Rothschild *et al.* (3) agreed well with that observed with other flavoprotein substrates, namely, succinate and glycerophosphate, which are known to utilize only the two phosphorylations of the cytochrome region of the respiratory chain. Rendina and Singer (4) found that the isolated choline dehydrogenase contains flavin adenine dinucleotide and is not able to reduce DPN. In contrast to the oxidation of succinate and glycerophosphate, choline oxidation is completely inhibited by Amytal (5–7).

The oxidation of choline also appears to be limited by a restricted permeability of the mitochondrial membrane. Williams (8) has shown that rat liver mitochondria suspended in 0.15 M KCl do not oxidize choline at an appreciable rate unless the mitochondrial integrity is altered by the addition of Ca^{++}, deoxycholate, or aging at 38°. Recently Kimura, Singer, and Lusty (6, 9) have concluded that choline is oxidized by a separate respiratory chain, which is interlinked with that oxidizing succinate only at the level of cytochrome c_1.

The mechanism of choline oxidation by rat liver mitochondria has been reinvestigated in the present studies with the use of rotenone, a compound which has recently been shown to share all the effects of Amytal on electron transfer but none of the effects of Amytal on energy transfer (10). It will be shown that the low rate of choline oxidation in intact liver mitochondria is largely sensitive to rotenone. In contrast, the high rate of choline oxidation in swollen mitochondria is insensitive to rotenone and is uncoupled from phosphorylation. It will be also shown that choline can induce a reduction of oxaloacetate to malate, and that the oxidation of choline may be stimulated by the addition of DPN. The possible source of hydrogen for the rotenone-sensitive part of the respiration during choline oxidation is discussed.

EXPERIMENTAL PROCEDURE

Rat liver mitochondria were prepared as described previously by Ernster and Löw (11) in 0.25 M sucrose-5 mM Tris buffer, pH 7.4. Deionized sucrose solutions were used throughout. After two washings, the mitochondria were usually resuspended to contain about 30 mg of protein, corresponding to about 1.5 g of liver, wet weight, per ml of suspension.

Assay Procedures—The composition of the medium for measuring respiration and phosphorylation was as follows, in a final volume of 2 ml (unless otherwise specified): 15 mM $^{32}P_i$, pH 7.4, 25 mM Tris-HCl, pH 7.4, 4 mM $MgCl_2$, 25 mM KCl, 162 mM sucrose, 2 mM EDTA, 0.1% albumin, 0.6 mM arsenite, 25 mM choline, 1 mM ATP, 25 mM glucose, 1 mg of hexokinase (type III, Sigma), and mitochondria from 0.5 to 0.75 g of liver, wet weight.

Phosphate-aged mitochondria were obtained by diluting 1 volume of liver mitochondrial suspension, corresponding to 1 g of liver per ml, with 2 volumes of 50 mM phosphate buffer, pH 7.4, and incubating this suspension at 30° in an Erlenmeyer flask for 15 minutes. The aging of the mitochondria was controlled by measurement of the optical density at 520 mμ. Usually the optical density was reduced to one-third in 15 minutes.

Mitochondrial swelling was followed by shaking the mitochondria in open tubes under conditions identical with those used during the incubation in Warburg flasks. At fixed times, samples were withdrawn from the tubes and the optical density was measured at 520 mμ in cuvettes of 1-mm light path.

For measuring the stoichiometry of the reaction, the experiments were carried out in Warburg flasks of 100- to 120-ml volume. The concentrations of all components of the standard incubation medium were unaltered, whereas the final volume of the incubation medium was multiplied by a factor of 10 to 12.

Phosphate uptake was determined by the isotope distribution method recommended by Lindberg and Ernster (12). Reduced intramitochondrial pyridine nucleotides were measured with an Eppendorf fluorometer equipped with a recording apparatus (13).

Determination of Betaine Aldehyde—The amount of betaine aldehyde was determined enzymatically with betaine aldehyde dehydrogenase. The enzyme was isolated from rat liver according to Rothschild and Barron (14), with some modifications. A rat liver supernatant fraction in 0.25 M sucrose after separation of the mitochondria at 4,500 × *g* was made 2 mM with cysteine and recentrifuged at 14,000 × *g* for 15 minutes. The supernatant fluid, kept in an ice bath, was made 50% saturated with ammonium sulfate (31.5 g/100 ml); the suspension was stirred slowly for 30 minutes and then centrifuged at 14,000 × *g* for 15 minutes. The clear supernatant fluid was made 65% saturated with ammonium sulfate (9.8 g/100 ml) and recentrifuged at 14,000 × *g* for 15 minutes. The precipitate, which contained more than 90% of the enzyme activity of the original supernatant fraction, was washed once with a 65% saturated ammonium sulfate solution and then kept in a refrigerator in 65% ammonium

* Aided by a grant from the Muscular Dystrophy Associations of America, Inc.

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sulfate. Enzyme activity and the amount of betaine aldehyde were determined in 3 ml of a medium composed of 33 mM Tris buffer, pH 7.4, 5 mM $MgCl_2$, 5 mM cysteine, 0.3 mM DPN, and 0.1 ml of the enzyme suspension. The betaine aldehyde dehydrogenase was left for 8 minutes in the incubation medium, and the optical density was read at 340 mμ. The sample containing betaine aldehyde was then added, and the optical density was read after 8 and 15 minutes.

Determination of Choline and Betaine—Determinations of choline and betaine were done according to the method of Jel-

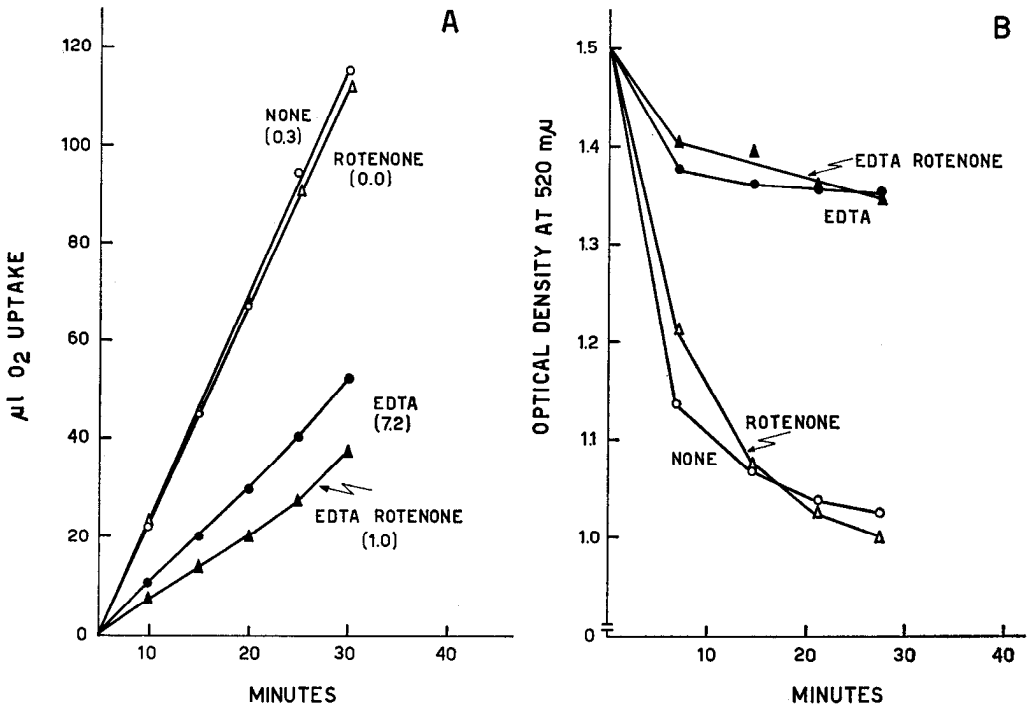


FIG. 1. Effect of rotenone on respiration and optical density of liver mitochondria oxidizing choline in EDTA-deficient and EDTA-supplemented systems. The incubation medium, in a final volume of 2 ml, contained 25 mM P_i, pH 7.4, 25 mM Tris buffer, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 162 mM sucrose, 25 mM choline, 1 mM arsenite (added to the mitochondria 10 minutes before the experiment was begun), 1 mM ATP, 0.015% hexokinase (type IV, Sigma), 25 mM glucose, and mitochondria from 0.75 g of liver, wet weight. Where indicated, 2 mM EDTA and 3.5×10^{-6} M rotenone (in 10 μl of acetone) were added. Mitochondrial swelling was measured in cuvettes of 1-mm light path. *Figures in parentheses* indicate the phosphate uptake.

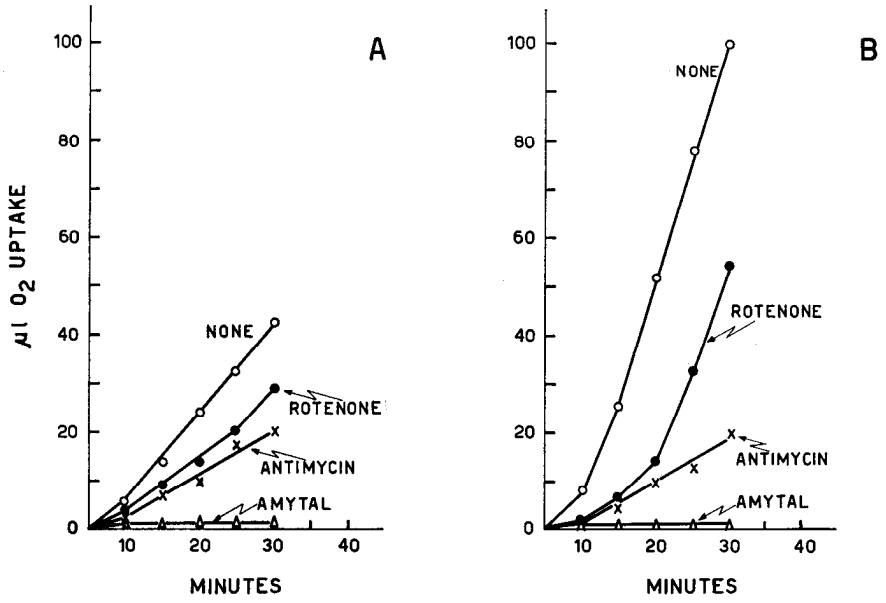


FIG. 2. Loss of the sensitivity of respiration to rotenone during the incubation. The incubation medium in Experiment *A* was the same as in Fig. 1. In Experiment *B* the reaction contained 20 mM P_i, pH 7.4, 12.5 mM Tris buffer, pH 7.4, 12.5 mM KCl, 4 mM $MgCl_2$, 62 mM sucrose, and 1 mM ATP. Arsenite was added as in Fig. 1. Where indicated, 3.5×10^{-6} M rotenone (in 10 μl of acetone) antimycin A, (in 10 μl of acetone) 1 μg per ml, and 2 mM Amytal were also added. Mitochondria from 0.75 g of liver, wet weight, were used.

linek, Strength, and Thayer (15) with slight modifications. The deproteinized incubation mixture was passed through a Seitz G4 filter and then adjusted to pH 8 with N NaOH. A 1% ammonium reineckate solution, which had also been filtered through a Seitz G4 filter, was added to the filtrate. After standing for 10 minutes at room temperature, choline and betaine aldehyde reineckates were separated by filtrations through a weighed Seitz G4 filter. The precipitate was washed with saturated choline reineckate solution. The filtrate was cooled at 0° and acidified with 4 N HCl, and more ammonium reineckate solution was added. After standing for 3 hours at 0°, betaine reineckate was separated by filtration through a weighed Seitz G4 filter. The precipitate was washed with slightly acidic cold distilled water. The Seitz filters were put in desiccators over P_2O_5, kept overnight at 55°, and then weighed on a Mettler H16 balance. In each determination, control samples of standard solutions of choline and betaine were introduced. The error in the recoveries was no greater that 2 to 3%. The amount of choline in the reineckates precipitated at pH 8 was obtained by subtracting the betaine aldehyde determined enzymatically.

Malate was determined according to the procedure described by Hohorst (16). Betaine aldehyde was a commercial product of the Delta Company, New York. Betaine aldehyde solutions were titrated by the bisulfite method as described by Rothschild and Barron (14).

RESULTS

Rate of Choline Oxidation and Mitochondrial Swelling; Conditions for Rotenone Inhibition—As mentioned in the introductory section, Williams (8) has reported that the rate of oxidation of choline by intact liver mitochondria is extremely low and may be considerably increased by the addition of agents that are known to alter mitochondrial integrity and to produce swelling. Kimura *et al.* (9) have also observed an initial lag in the oxidation of choline, which was abolished by the addition of Ca^{++}. Kimura *et al.* (9) obtained high rates of choline oxidation by incubating mitochondria in 0.05 M phosphate at 38°. Williams and

TABLE I

Respiration and phosphorylation of liver mitochondria oxidizing choline in presence of rotenone and antimycin

Concentrations of the substances in a final volume of 2 ml were as follows: 15 mM $^{32}P_i$, pH 7.4, 25 mM Tris buffer, pH 7.4, 25 mM KCl; 4 mM $MgCl_2$, 162 mM sucrose, 0.1% albumin, 25 mM choline, 2 mM EDTA, 0.6 mM arsenite, 1 mM ATP, 0.015% hexokinase (type IV, Sigma), 25 mM glucose, and, when indicated, 3.5×10^{-6} M rotenone, 2×10^{-6} M vitamin K_3, and 4 μg of antimycin A. Mitochondria from 0.75 g of liver, wet weight, were used. Time of incubation, 30 minutes.

Additions	Oxygen uptake	Phosphate uptake
	μatoms/10 mg protein/hr	*μmoles/10 mg protein/hr*
None	2.8	3.2
Choline	9.8	11.6
Choline; rotenone	4.2	1.6
Choline; antimycin A	3.2	0
Choline; rotenone; antimycin A	3.5	0
Choline; rotenone; vitamin K_3	10.2	6.5
Choline; rotenone; vitamin K_3; antimycin A	3.4	0

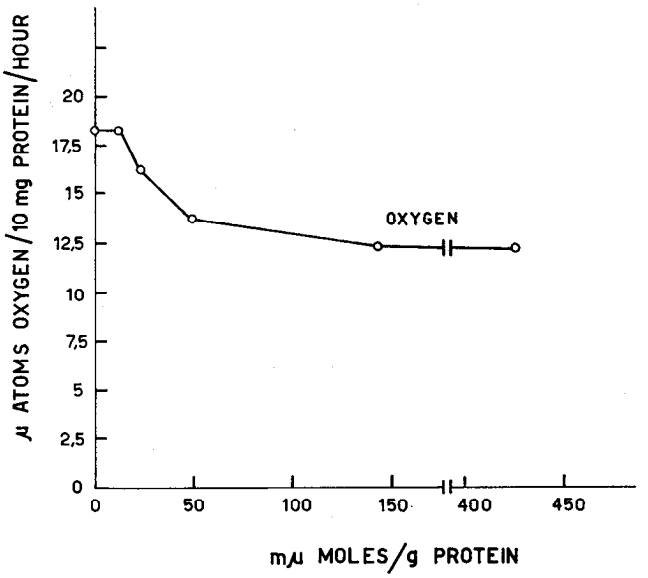


FIG. 3. Titration of the rotenone-sensitive site. The incubation medium, in a final volume of 2 ml, contained 15 mM P_i, pH 7.4, 25 mM Tris, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 162 mM sucrose, 25 mM choline, 1% albumin, 0.6 mM arsenite, 2 mM EDTA, 1 mM ATP, 0.015% hexokinase (type IV, Sigma), 25 mM glucose, and mitochondria from 0.75 g of liver, wet weight. Time of incubation, 30 minutes; temperature, 30°.

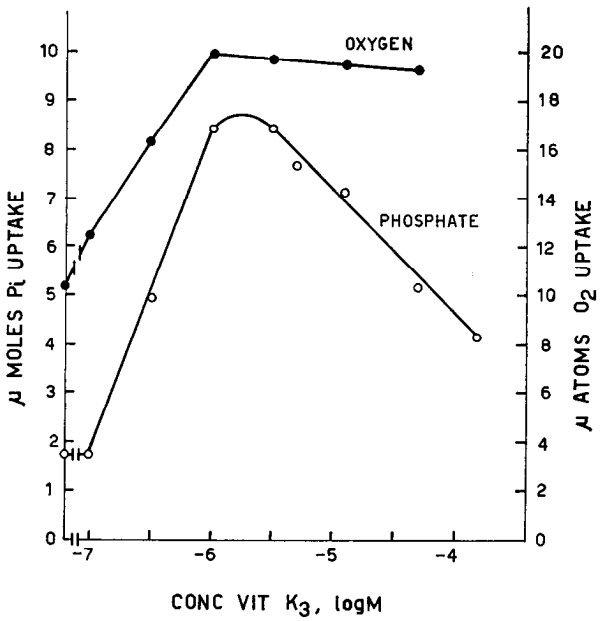


FIG. 4. Bypass of rotenone-sensitive site by vitamin K_3. Experimental conditions were the same as in Fig. 3 except that the concentration of rotenone was 3.5×10^{-6} M (in 10 μl of ethanol) and mitochondria from 0.5 g liver, wet weight, were added per flask. Time of incubation, 35 minutes.

Kimura *et al.* did not measure the phosphate uptake under conditions of maximal rate of choline oxidation. Fig. 1, *A* and *B*, shows that liver mitochondria, when incubated with choline as substrate under the conditions that are usually used to measure oxidative phosphorylation with Krebs cycle metabolites, underwent considerable swelling. The rate of oxidation of choline under these conditions was high, but the phosphorylating capac-

ity was almost completely lost. Complete protection against swelling of the mitochondria and loss of phosphorylating capacity was obtained by the addition of EDTA. In the presence of EDTA, the rate of choline oxidation was kept at a low level, about one-third of that found in the EDTA-deficient system. It is seen also in Fig. 1 that rotenone, which did not interfere with the changes in optical density in either the EDTA-deficient or the EDTA-supplemented system, inhibited the oxygen uptake about 30 to 40% and the phosphate uptake about 90% only in the EDTA-supplemented system. Several parameters were tested with regard to their capacity to affect the rate of choline oxidation; namely, the concentration of phosphate and of ATP, and the addition of arsenate, uncoupling agents, calcium, etc. In general, all conditions which promoted mitochondrial swelling also stimulated the oxidation of choline; furthermore, under conditions of a high rate of choline oxidation, liver mitochondria invariably lost the capacity to phosphorylate and were insensitive to rotenone.

The increase in rate of choline oxidation and loss of sensitivity of the respiration to rotenone may be followed during the incubation. In the experiment shown in Fig. 2*B*, the mitochondria were incubated in a slightly hypotonic, EDTA-deficient medium and with 1 mM ATP. The rate of oxidation of choline increased after about 10 minutes of incubation, and at about this time the sensitivity to rotenone was lost. Contrary to the case with rotenone, the effects of two other respiratory chain inhibitors, Amytal and antimycin A, were independent of the structural state of the mitochondrion and were identical in intact and swollen mitochondria (Fig. 2, *A* and *B*). In phosphorylating mitochondria, rotenone and antimycin A inhibited the oxidation of choline to about the same extent. Thus, in intact liver mitochondria, only about half of choline oxidation proceeds through a rotenone- and antimycin-sensitive pathway. The rotenone-

TABLE II

Lack of bypass of Amytal-sensitive site by addition of vitamin K_3

Experimental conditions are described in Table I.

Additions	Oxygen uptake	Phosphate uptake
	μatoms/10 mg protein/hr	*μmoles/10 mg protein/hr*
None	15.2	17.0
Amytal, 0.6 mM	11.6	7.1
Amytal, 0.6 mM; vitamin K_3	13.3	7.1
Amytal, 0.6 mM; vitamin K_3; antimycin A	4.2	0
Amytal, 2 mM	2.2	0.7
Amytal, 2 mM; vitamin K_3	2.6	0.4
Amytal, 2 mM; vitamin K_3; antimycin A	3.2	0

TABLE III

Oxidation of betaine aldehyde by rat liver mitochondria

Concentrations of the substances, in a final volume of 2 ml, were as in Table I, with 5.5 mM betaine aldehyde and, when indicated, 3.5×10^{-6} M rotenone, 0.1 mM dinitrophenol, and 1.5 mM DPN. Time of incubation, 55 minutes.

Additions	Oxygen uptake	Betaine aldehyde utilized
	μatoms	*μmoles*
Experiment 1		
Mitochondria, 100 mg	0.89	1.1
Mitochondria, 200 mg	2.70	2.2
Mitochondria, 500 mg	5.40*	3.9
Mitochondria, 500 mg, rotenone	4.80	3.8
Mitochondria, 500 mg; dinitrophenol	5.5	3.6
Experiment 2		
Phosphate-aged mitochondria, 500 mg	3.6	3.6
Phosphate-aged mitochondria, 500 mg; DPN	7.0†	3.8
Experiment 3		
Mitochondria, 500 mg	2.54‡	1.7
Mitochondria, 500 mg; rotenone	2.76	2.5

* Endogenous respiration was 0.95 μatom of oxygen.

† Addition of DPN stimulated the endogenous respiration of 3.4 μatoms of oxygen.

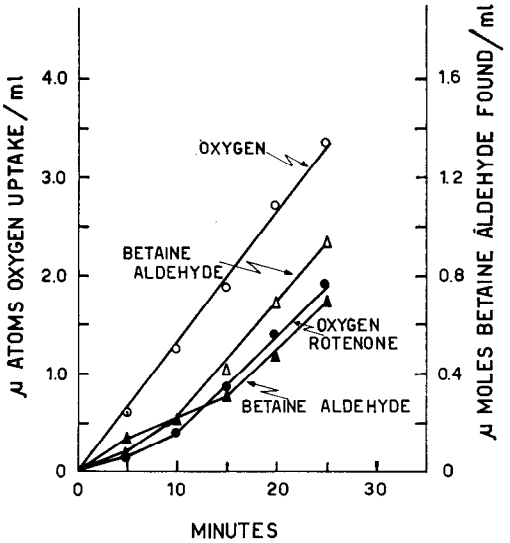
‡ Endogenous respiration was 0.82 μatom of oxygen.

TABLE IV

Stoichiometry of choline oxidation and formation of betaine aldehyde and betaine in intact and aged liver mitochondria

The composition of the medium in Experiments 1 and 2 was as follows: 10 mM P_i, pH 7.4, 25 mM Tris buffer, pH 7.4, 4 mM $MgCl_2$, 25 mM KCl, 162 mM sucrose, 2 mM EDTA, 10 mM choline, 1% albumin, 0.6 mM arsenite, 1 mM ATP, 0.015% hexokinase (type IV, Sigma), and 25 mM glucose. In Experiments 3, 4, and 5 the composition was identical except that Tris buffer was 12.5 mM, KCl 12.5 mM, and sucrose 62 mM; 1 mM 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane ("EGTA") was used instead of EDTA. Final volumes were 24, 24, 16, 20, and 20 ml for Experiments 1 through 5, respectively. Mitochondria from 0.375 g of liver, wet weight, per ml were used. Times of incubation were 55, 35, 50, 35, and 35 minutes for Experiments 1 through 5, respectively. The temperature was 30° for Experiments 1 and 2 and 37° for Experiments 3, 4, and 5. Experiment 5 was carried out with phosphate-aged mitochondria.

Additions	Oxygen uptake	Choline utilized	Betaine aldehyde found	Betaine found	ATP synthesized
	μatoms	*μmoles*	*μmoles*	*μmoles*	*μmoles*
Experiment 1					
None	4.9				11.1
Rotenone	5.7				1.2
Choline	88.1		13.0	35.8	76.1
Choline, rotenone	60.1		27.7	15.0	13.4
Experiment 2					
None	9.2				15.0
Choline	63.0	36.2	5.2	30.5	79.8
Choline, rotenone	34.2	18	6	11.8	14.0
Experiment 3					
None	18.9				8.75
Rotenone	8.2				2.4
Choline	104.0	52.2	8.3	40	45.2
Choline, rotenone	63.7	31.4	11.4	22	11.05
Experiment 4					
None	15.2				16.8
Rotenone	2.0				5.7
Choline	78.0	44	13.3	31	57.3
Choline, rotenone	49.8	37	12.3	24	18.6
Experiment 5					
None	2.5				
Choline	108.0	80	53.7	22	0



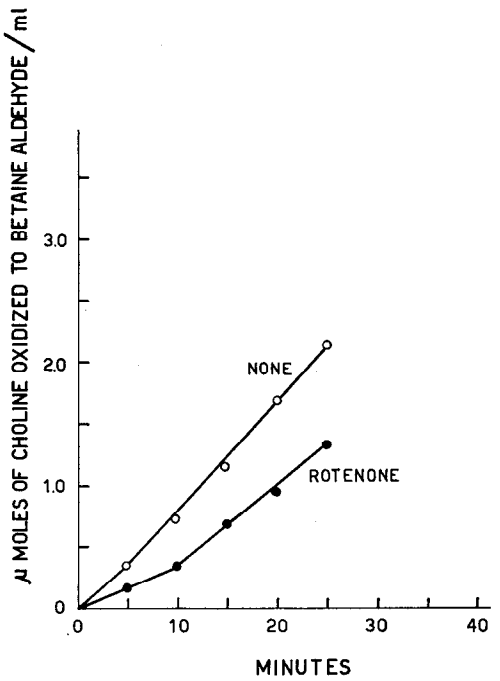


FIG. 5. Kinetics of choline oxidation and betaine aldehyde formation in the presence and absence of rotenone. The incubation medium, in a final volume of 2 ml, contained 15 mM $^{32}P_i$, pH 7.4, 12.5 mM Tris buffer, pH 7.4, 12.5 mM KCl, 4 mM $MgCl_2$, 62 mM sucrose, 2 mM arsenite, 1 mM 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane ("EGTA"), 1% albumin, 10 mM choline, 1 mM ATP, 0.025% hexokinase (type IV, Sigma), 25 mM glucose, and, where indicated, 3.5×10^{-6} M rotenone. Mitochondria from 0.75 g of liver, wet weight, were used. Parallel samples, of identical composition in a final volume of 5 ml, were incubated in open Erlenmeyer flasks from which 0.5-ml aliquots were taken at fixed times and stopped with 2 ml of 12% trichloroacetic acid; temperature, 30°. The figures for oxidation of choline to betaine aldehyde were calculated from the figures for oxygen uptake and betaine aldehyde found.

and antimycin-sensitive pathway is the only one that is coupled to phosphorylation, as shown by the findings that the phosphate uptake was inhibited about 90% by rotenone and almost completely by antimycin (Fig. 1 and Table I).

In agreement with observations of others (7), Amytal was able to abolish the oxygen uptake completely under all conditions.

Titration of Rotenone-sensitive Sites—Fig. 3 shows the effect of rotenone on oxygen consumption. Maximal inhibition of the oxygen uptake was obtained between 25 and 50 mμmoles of rotenone per g of mitochondrial protein. Since the amount of rotenone required to inhibit other DPN-linked oxidations has been reported to be 26 to 28 mμmoles per g of mitochondrial protein (10), it may be concluded that the site of action of rotenone in the case of choline as substrate is probably identical with that of other DPN-linked substrates.

Bypass of Rotenone-sensitive Site by Vitamin K_3—Conover and Ernster (17) have reported that addition of vitamin K_3 to Amytal-inhibited mitochondria resulted in a complete restoration of the oxygen uptake. The respiration was accompanied by 2 instead of 3 μmoles of ATP synthesized per μatom of oxygen uptake. Similar results were reported when rotenone instead of Amytal was used as the inhibitor of DPN-linked oxidations (10). A typical experiment is reported in Table I, and shows the effect of rotenone on endogenous respiration and on choline oxidation, and the bypass of the rotenone inhibition by addition of vitamin K_3. Both respiration and phosphorylation were restored by vitamin K_3, the first completely and the second partially. The vitamin K_3-dependent respiration was sensitive to the addition of antimycin A.

As shown in Fig. 4, maximal stimulation of oxygen and phosphate uptake was reached at about 10^{-6} M vitamin K_3, a concentration slightly lower than that used in previous investigations (17). A severe inhibition of the phosphate uptake, probably caused by an uncoupling effect of vitamin K_3, was observed when higher concentrations of the naphthoquinone were used. In contrast to the case with rotenone, the inhibition by Amytal of choline oxidation was not relieved by the addition of vitamin K_3 (Table II). A slight stimulation of the oxygen uptake was observed at 0.6 mM Amytal, but this was not accompanied by an increase of phosphate uptake.

Stoichiometry of Choline Disappearance and Formation of Betaine Aldehyde and Betaine—Rothschild *et al.* (3) have concluded that almost all the betaine aldehyde dehydrogenase is found in the soluble fraction of liver. Subsequent reports have appeared, however, concerning the capacity of liver mitochondria to catalyze an oxygen uptake of 2 μatoms per mole of added choline (18). A nonspecific DPN-linked aldehyde dehydrogenase has been isolated from liver mitochondria (18).

The isolation of a DPN-linked betaine aldehyde dehydrogenase from rat liver mitochondria has been confirmed in our studies. An amount of betaine aldehyde dehydrogenase activity corresponding to 5% of that of the whole liver homogenate could always be extracted from washed liver mitochondria by treatment with an Ultra Turrax blender.[1] Furthermore, as shown in Table III, addition of betaine aldehyde resulted in a stimulation of the oxygen uptake which was accounted for by the betaine aldehyde utilized. It is to be noted in Table III that rotenone did not inhibit the oxidation of added betaine aldehyde. On the other

[1] Model TP 18/2, Janke and Kunkel KG, Staufen in Breisgau, West Germany.

hand, the oxidation of intramitochondrially generated betaine aldehyde was largely sensitive to rotenone in intact mitochondria (Table IV).

In Table IV are reported the results of some typical experiments on the stoichiometry of the reaction, namely, the measurement of the oxygen uptake, of choline disappearance, and of betaine aldehyde and betaine formation. At the arsenite concentrations used in these experiments, 0.6 mM, the endogenous respiration was rather different among the various mitochondrial preparations, *e.g.* low in Experiments 1 and 2 and high in Experiments 3 and 4. Inasmuch as the formation of 1 mole of betaine aldehyde requires 1 atom of oxygen, and the formation of 1 mole of betaine requires 2 atoms of oxygen, in general a good agreement was found between the oxygen uptake and the formation of betaine aldehyde and betaine. Also, the amounts of betaine aldehyde and of betaine found corresponded to the disappearance of choline. The stoichiometry between oxygen uptake and betaine aldehyde and betaine formation has been recently measured by Jellinek *et al.* (15). These authors found that more than 90% of the oxygen uptake was accounted for by the formation of betaine aldehyde and less than 10% by the formation of betaine. The low levels of betaine found by Jellinek *et al.* (15) are due to the experimental conditions used, which are known to lead to extensive swelling and loss of DPN. When phosphate-aged mitochondria were used in our studies, instead of intact mitochondria (Table IV, Experiment 5), the formation of betaine was also reduced to lower levels. From the data of Table IV it can be concluded that the decrease in oxygen uptake induced by the addition of rotenone is accounted for partly by an inhibition of the oxidation of betaine aldehyde to betaine, and partly by an inhibition of the oxidation of choline to betaine aldehyde.

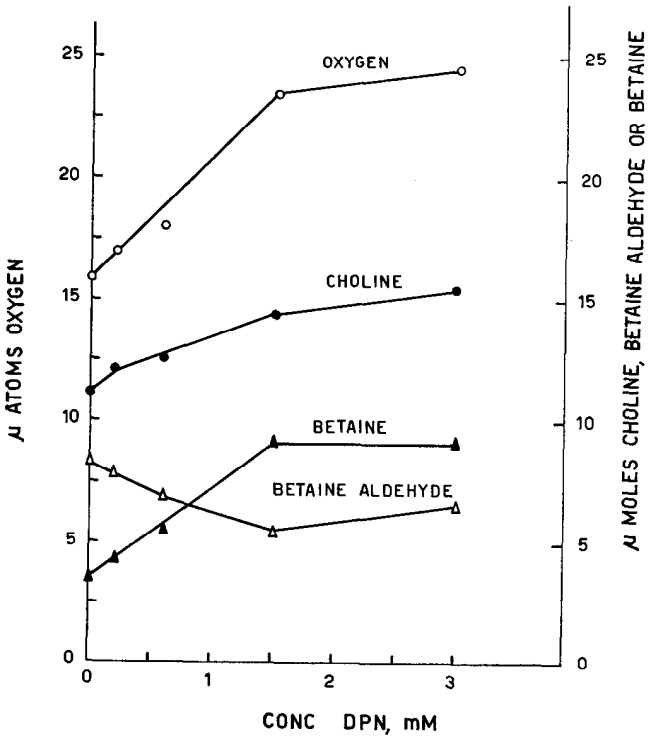
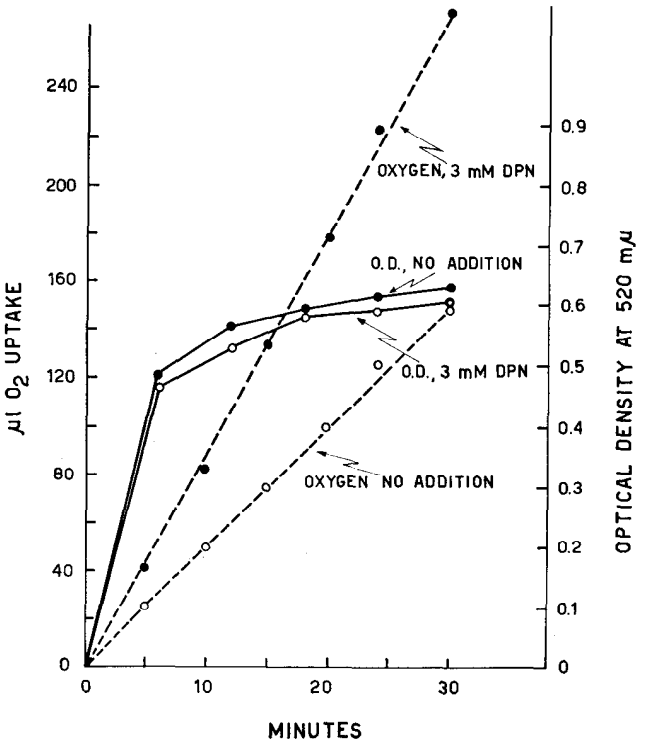


FIG. 6. Effect of various DPN concentrations on choline oxidation in the absence of rotenone. The medium, in a final volume of 2 ml, contained 20 mM P_i, pH 7.4, 12.5 Tris buffer, pH 7.4, 12.5 mM KCl, 4 mM $MgCl_2$, 62 mM sucrose, 2 mM arsenite, 20 mM choline, 1 mM ATP, and mitochondria from 0.75 g of liver, wet weight. Time of incubation, 40 minutes. The figures for oxidation of choline and betaine formation were calculated from the figures for oxygen uptake and betaine aldehyde found.



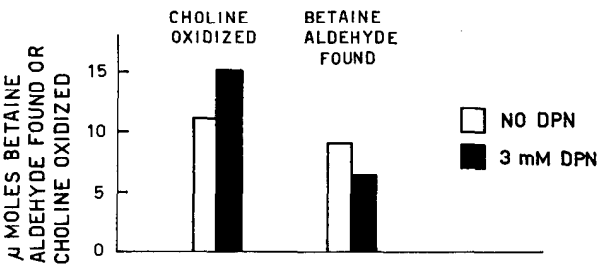


FIG. 7. Effect of DPN on choline oxidation and optical density. Experimental conditions were as follows: 10 mM P_i, pH 7.4, 25 mM Tris buffer, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 162 mM sucrose, 20 mM choline, 1 mM ATP, 2 mM arsenite, 0.025% hexokinase (type IV, Sigma), 25 mM glucose, and mitochondria from 0.75 g of liver, wet weight. Optical density was measured in cuvettes of 1-mm light path as described in "Experimental Procedure."

It is seen in Table IV that the inhibition by rotenone of choline oxidation was much higher in Experiments 2 and 3 than in Experiments 1 and 4. Indeed, the effect of rotenone on the various mitochondrial preparations varied with respect both to the extent of inhibition of the initial rate and to the duration of inhibition as a function of the incubation time. After 20 to 30 minutes of incubation, the inhibition by rotenone was considerably reduced, even in the EDTA-supplemented systems.

The stoichiometry data of Table IV support the procedure, used in the kinetic experiments reported below, of calculating the amount of choline used or of betaine formed from the values for oxygen uptake and for the betaine aldehyde found. An

example of one of these experiments is shown in Fig. 5. It is seen that the inhibition by rotenone on choline oxidation, under the experimental conditions used, was almost linear during the incubation period.

Stimulation of Choline Oxidation by Addition of DPN—The experiments reported in Figs. 6 to 8 show that added DPN can, under suitable conditions, stimulate choline oxidation. The experiments reported in Figs. 6 to 9 were carried out with mitochondria incubated in an EDTA-deficient medium, and with DPN added. It is seen in Fig. 6 that 0.2 to 3 mM DPN considerably stimulated the oxygen uptake of liver mitochondria oxidizing choline.

Stimulation of the oxygen uptake after addition of DPN had been previously reported by Strength *et al.* (2), who attributed it to a stimulation of choline oxidation. This conclusion was questioned by Rendina and Singer (4), who found that the stimulation of the oxygen uptake by DPN was absent at the beginning of the incubation and then increased with time. Rendina and Singer (4) attributed the DPN effect to an activation of betaine aldehyde dehydrogenase and subsequent removal of betaine aldehyde, which is a competitive inhibitor of choline oxidation. The concentration of betaine aldehyde was not measured in the experiments of Rendina and Singer.

In Fig. 6 oxygen uptake, disappearance of choline, and formation of betaine aldehyde and betaine at various DPN concentrations are reported. The stimulation of oxygen uptake was not accounted for by the removal of the betaine aldehyde. Thus, addition of DPN induced a net stimulation of the oxidation of choline to betaine aldehyde and a slight stimulation of the oxidation of betaine aldehyde to betaine.

As shown in Fig. 7, the stimulation of oxygen uptake occurred from the beginning of the incubation, and the respiration was linear throughout the whole incubation period. This finding does not support the interpretation of Rendina and Singer, since the concentration of betaine aldehyde at the beginning of the incubation is presumably too low to account for an inhibition of choline dehydrogenase. Similarly, the changes in the levels of betaine aldehyde induced by the addition of DPN in Figs. 6 and 7 are inadequate to account for a release of the inhibition of choline dehydrogenase by betaine aldehyde after addition of DPN. In Fig. 7 is also reported the optical density of the mitochondrial suspension as measured in a parallel sample. The addition of DPN induced no substantial change of rate and extent of mitochondrial swelling under these conditions. It must be concluded, therefore, that the stimulation of choline oxidation by DPN cannot be attributed to an increased permeability of the mitochondrial membrane to choline.

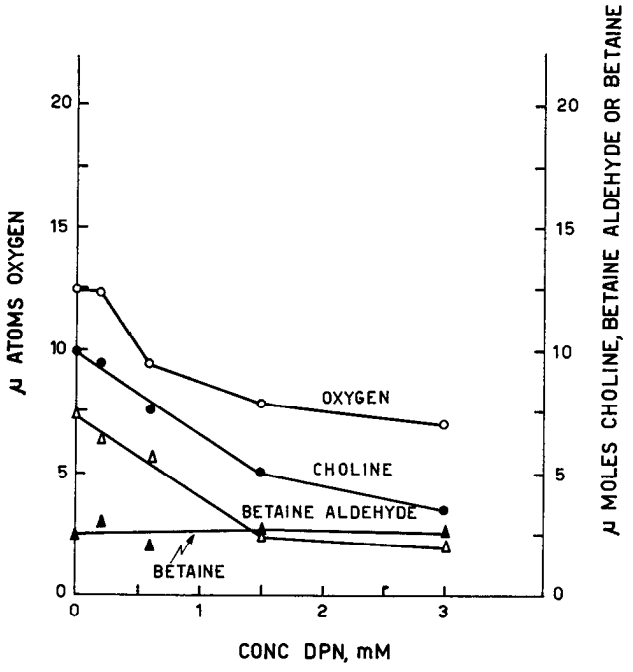


FIG. 8. Effect of various DPN concentrations on choline oxidation in the presence of rotenone. Experimental conditions were the same as in Fig. 7, with rotenone, 3.5×10^{-6} M (in 10 μl of acetone). Mitochondria from 0.75 g of liver, wet weight, were used. Time of incubation, 40 minutes. The figures for choline oxidation and betaine formation were calculated from the figures for oxygen uptake and betaine aldehyde found.

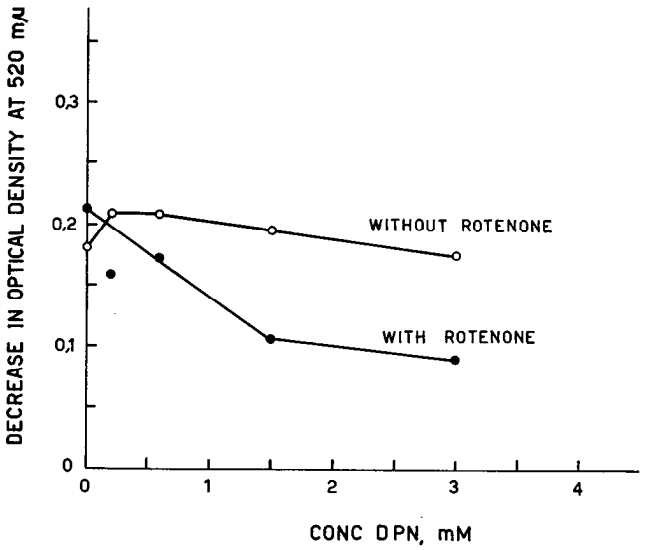


FIG. 9. Effect of various DPN concentrations on mitochondrial optical density in the presence and absence of rotenone. Experimental conditions were the same as in Fig. 7. Time of incubation, 35 minutes. Optical density was measured in cuvettes of 1-mm light path as described in "Experimental Procedure."

In Fig. 8, oxygen uptake, disappearance of choline, and formation of betaine aldehyde and betaine are reported as a function of various DPN concentrations, in the presence of rotenone. It is seen that rotenone, which had no effect on choline oxidation when measured in a hypotonic EDTA-deficient medium, strongly inhibited oxygen uptake and choline oxidation in the same medium when DPN was added. The inhibition was roughly proportional to the concentration of DPN in the range from 0.2 to 1.5 mM. Only the formation of betaine was equal with or without DPN added.

Data on the optical density of the mitochondrial suspensions, as measured in parallel samples, are reported in Fig. 9. In the presence of DPN, rotenone induced a certain inhibition of the decrease in optical density that occurred under the experimental conditions used. Although the changes in optical density induced by the presence of rotenone are too small to account for the severe inhibition of the rate of choline oxidation, it is possible that part of the effect of rotenone in the presence of DPN is due to an interference with the mitochondrial permeability to choline.

The finding reported above, that addition of DPN to swollen

mitochondria causes restoration of the inhibition of the respiration by rotenone, strongly suggests that the loss of the rotenone-sensitive respiration that accompanies mitochondrial swelling is largely related to the release of mitochondrial DPN.

Reduction of Pyridine Nucleotide by Choline—Reduction of DPN by succinate or glycerophosphate has been reported to be an energy-requiring reaction (19–21), in which the energy is supplied either by ATP or by the aerobic oxidation of succinate or glycerophosphate. The formation of reduced pyridine nucleotide after addition of choline was followed either by determining the reduction of oxaloacetate to malate or by measuring the reduced pyridine nucleotide fluorometrically. Addition of choline to liver mitochondria under anaerobic conditions resulted in a net formation of malate, and the reduction of oxaloacetate was not decreased by the addition of dinitrophenol or of rotenone (Table V). The rate of formation of malate was low, about one-half or one-third of the rate of the rotenone-sensitive part of the respiration as measured under similar conditions.

A fluorometric recording of the changes in mitochondrial pyridine nucleotide fluorescence is shown in Fig. 10. Addition of ADP produced oxidation of the pyridine nucleotide. Addition of 15 mM choline resulted in increased fluorescence. The increase in fluorescence caused by glutamate was about 5 times that caused by choline (Fig. 10).

In the presence of 0.03 mM dicoumarol, the extent of reduction of the intramitochondrial pyridine nucleotide induced by the addition of choline was diminished about 50%, as compared to the reduction obtained under the conditions of the experiment of Fig. 10. It should be noted, however, that the reduction of pyridine nucleotide by glutamate was also decreased about 50% by the addition of 0.03 mM dicoumarol. The finding reported in Fig. 10 is not in contrast with the observation of Rothschild *et al.* (3), who reported that external DPN was not reduced after addition of choline.

TABLE V

Anaerobic reduction of oxaloacetate to malate induced by choline

Concentrations of the substances in a final volume of 2 ml were as follows: 12.5 mM Tris, pH 7.4, 12.5 mM KCl, 4 mM $MgCl_2$, 2 mM EDTA, 2 mM DPN, 1 mM ATP, 2 mM malonate, 62 mM sucrose, and, when indicated, 0.6 mM arsenite, and 15 mM P_i, pH 7.4. Phosphate-aged mitochondria from 0.75 g of liver, wet weight, were used. The mixture was incubated at 30° for about 5 minutes, during which air was evacuated with a water pump. The reaction was started by addition from the side arm of 10 mM oxaloacetate and choline. The reaction was stopped after 30 minutes in Experiments 1 and 2, and after 20 minutes in Experiment 3, with 0.2 ml of 100% trichloroacetic acid.

Additions	Malate formed		
	Without choline	With choline	
		10 mM	20 mM
	μmoles	*μmoles*	
Experiment 1			
P_i	1.9	3.5	
Experiment 2			
P_i	0.9		1.72
Arsenite	0.02		0.74
Arsenite; P_i	0.07		0.66
Arsenite; P_i; dinitrophenol, 0.1 mM			0.82
Experiment 3			
None	0.37		1.17
None	0.57		1.41
Arsenite	0.17		0.53
Arsenite; rotenone, 0.0035 mM			0.58

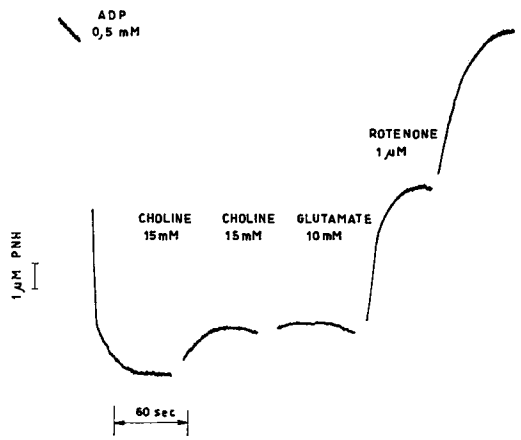


FIG. 10. Changes in fluorescence of pyridine nucleotide in liver mitochondria induced by the addition of choline. The medium contained, in a final volume of 3 ml, 25 mM Tris buffer, pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 2 mM EDTA, 120 mM sucrose, and mitochondria from 0.2 g of liver, wet weight.

DISCUSSION

The conclusion that during choline oxidation part of the electron flux goes through the site of the first respiratory chain phosphorylation is supported by the following findings. (*a*) About 40 to 50% of the initial rate of respiration and a larger part of the accompanying phosphorylation are abolished by rotenone in concentrations that are known to inhibit the oxidation of other DPN-linked substrates. The rotenone block is bypassed by vitamin K_3. (*b*) The respiration of liver mitochondria oxidizing choline is considerably stimulated by the addition of DPN. The effect of DPN is not accompanied by an increased permeability of the mitochondrial membrane to choline. (*c*) Addition of choline induces an increased reduction of the intramitochondrial pyridine nucleotide.

That the source of hydrogen for the reduction of the pyridine nucleotide is the FAD-linked choline dehydrogenase and not the DPN-linked betaine aldehyde dehydrogenase is apparently supported by three kinds of observations. Firstly, the data on the stoichiometry of the reaction reported in Table III indicate that rotenone inhibits not only the oxidation of betaine aldehyde to betaine but also that of choline to betaine aldehyde. Secondly, addition of DPN to swollen liver mitochondria produces a real stimulation of the oxidation of choline to betaine aldehyde. Thirdly, choline induces a reduction of oxaloacetate to malate under anaerobic conditions.

The first two findings may perhaps be also explained by assuming that the oxidation of choline is limited under those conditions in which the betaine aldehyde formed cannot be immediately removed by oxidation to betaine. Addition of rotenone and lack of DPN would be two of these conditions. It should be pointed out, however, that the addition of neither rotenone nor DPN caused significant changes in the level of betaine aldehyde as determined in the experiments of Table IV and Figs. 6 to 8. Furthermore, both the inhibition by rotenone and the stimulation of choline oxidation by DPN were present from

the first few minutes of incubation, when only small amounts of betaine aldehyde were formed. Therefore, the assumption that the rate of choline oxidation is controlled by the rate of removal of betaine aldehyde would obviously require that betaine aldehyde be more efficient as an inhibitor of choline dehydrogenase in particulate than in structure-free preparations, because its "actual" concentration is increased by compartmentation.

That the reduction of the pyridine nucleotide is coupled to the oxidation of choline to betaine aldehyde, on the other hand, is strongly supported by the finding that choline induces a reduction of oxaloacetate to malate. In fact, in anaerobiosis the formation of betaine aldehyde requires the simultaneous reduction of oxaloacetate, which is the only hydrogen acceptor present in the system. The reduction of oxaloacetate occurred, however, at a rather low rate. Equilibrium studies between the oxaloacetate-malate and choline-betaine aldehyde couples are therefore still required before it can be concluded that the FAD-linked choline dehydrogenase can reduce intramitochondrial pyridine nucleotide without a stoichiometric supply of energy.

It has been shown above that the phosphate uptake was inhibited about 80% by the addition of rotenone and completely inhibited by antimycin. Rotenone and antimycin inhibited respiration by 30 and 50%, respectively. It appears, therefore, that in intact, tightly coupled mitochondria a considerable part of the respiration with choline as substrate proceeds through an antimycin-insensitive, nonphosphorylating pathway. The low P:O ratios recorded (5) with choline as substrate are thus due to the simultaneous occurrence of phosphorylating and nonphosphorylating respirations. The large inhibition of the phosphorylation by rotenone also indicates that the phosphate uptake is largely dependent on the operation of the first respiratory chain phosphorylation. If the hydrogen for this phosphorylation is provided by the betaine aldehyde dehydrogenase, it must be concluded that the oxidation of choline is largely uncoupled from phosphorylation.

In previous studies the conclusion was reached that no difference exists between rotenone and Amytal with respect to their effects on mitochondrial electron transfer (10). From the present data it appears, however, that rotenone and Amytal differ in two respects: (*a*) in contrast to the rotenone block of choline oxidation, the Amytal block is not bypassed by the addition of vitamin K_3, and (*b*) choline oxidation is inhibited by Amytal completely in intact as well as in aged mitochondria, whereas choline oxidation is inhibited only partially by rotenone, and only in intact mitochondria. In the case of choline oxidation, therefore, the sites of action of the two inhibitors are clearly different. We suggest that whereas the action of rotenone is specifically in the flavin region of the respiratory chain, that of Amytal is less specific and involves also the electron flux originating from choline dehydrogenase. Indeed, Amytal has been reported to inhibit several flavoprotein enzymes involved in both electron and energy transfer (10, 22).

SUMMARY

The oxygen uptake of intact liver mitochondria oxidizing choline as substrate is partly inhibited by rotenone and antimycin. The respiration is increased by all conditions that favor mitochondrial swelling. In swollen mitochondria, the respiration is inhibited by antimycin and not by rotenone. The respiration of liver mitochondria oxidizing choline is inhibited by rotenone at concentrations equal to those required to inhibit the oxidation of other diphosphopyridine nucleotide-linked substrates. The rotenone block is bypassed by the addition of vitamin K_3, with subsequent stimulation of oxidation and phosphorylation. The stoichiometry of choline oxidation in intact and swollen liver mitochondria has been determined. It is found that rotenone inhibits the oxidation both of choline to betaine aldehyde and of betaine aldehyde to betaine.

Addition of diphosphopyridine nucleotide results in a considerable stimulation of the oxidation of choline to betaine aldehyde. The oxidation of choline by swollen mitochondria supplemented with diphosphopyridine nucleotide becomes highly sensitive to the addition of rotenone. Addition of choline to liver mitochondria under anaerobic conditions results in an energy-independent reduction of oxaloacetate to malate. Addition of choline to liver mitochondria in the presence of adenosine diphosphate results in a reduction of the intramitochondrial pyridine nucleotides.

From the data presented, it is concluded that in intact liver mitochondria part of the electron flow during choline oxidation proceeds through the site of the first respiratory chain phosphorylation. The possible source of hydrogen for this part of the respiration is discussed.

Acknowledgments—The excellent technical assistance of Mr. Leonardo Agosti is gratefully acknowledged. The authors are also indebted to Dr. G. Penso for collaborating in part of the experiments described in this paper.

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