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Differential Effects of Rotenone and Amytal on Mitochondrial Electron and Energy Transfer*

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Rotenone, a plant product with wide commercial use as a fish poison, has been shown by Fukami and Tomizawa (1, 2) and by Lindahl and Öberg (3, 4) to act on isolated mitochondria as powerful inhibitor of the aerobic oxidation of substrates linked to diphosphopyridine nucleotide without affecting that of succinate. The inhibition, which was independent of the presence of coupled phosphorylation (3), could be localized to the electron transfer step between diphosphopyridine nucleotide and flavin (3, 4). These effects of rotenone resembled those previously described with Amytal (5–7). By undertaking a further comparison of the effects of the two compounds, in the present study, we found that rotenone duplicates all of the known effects of Amytal on electron transport. In striking contrast, however, is its lack of effect on a number of Amytal-sensitive energy transfer reactions, such as the dinitrophenol-induced adenosine triphosphatase (8, 9) and the inorganic orthophosphate-adenosine triphosphate exchange (10) reactions. Data relating to these observations are presented in this paper, and some of their implications are discussed. It is also shown that rotenone, unlike Amytal, is firmly bound to the electron transfer system, thus rendering possible a "titration" of the rotenone-sensitive catalyst. Calculations derived from such titrations indicate that the rotenone-sensitive catalyst is present at the lowest molar ratio among known components of the liver-mitochondrial electron transport system, and probably constitutes the rate-limiting catalyst during the aerobic oxidation of mitochondrial DPNH in the presence of phosphate and phosphate acceptor.

EXPERIMENTAL PROCEDURE

Preparative Procedures—Rat liver mitochondria were prepared as described previously by Ernster and Löw (11) and washed twice with 0.25 M sucrose. Mitochondrial protein constituted approximately 20 mg per g of liver, wet weight, as determined by the biuret method (12).

Submitochondrial particles with DPNH oxidase activity (13) were prepared by adopting the procedure described by Kielley and Kielley (14) for preparation of ATPase. Mitochondria from 40 g of liver were suspended in 55 ml of 0.003 M phosphate buffer, pH 7.5, and treated for 2 minutes in the cold with an Ultra Turrax blender (24,000 r.p.m.). Large particles were removed by centrifugation in a model L Spinco ultracentrifuge, No. 40 rotor, at 20,000 × *g* for 10 minutes. The supernatant solution was recentrifuged at 105,000 × *g* for 30 minutes, and the sediment was resuspended in 0.003 M phosphate buffer, pH 7.5. The suspension was used in the DPNH oxidase assay.

Pretreatment of mitochondria with rotenone or Amytal was made by adding 4×10^{-7} M rotenone or 0.002 M Amytal (final concentrations) to suspensions of mitochondria in 0.25 M sucrose (the suspension contained mitochondria from 100 mg of liver, wet weight, per ml). The additions of rotenone and Amytal were made in 0.2 ml of ethanol per 10 ml of suspension, and a control containing ethanol only was run. The samples were allowed to stand for 10 minutes at 0°, and then centrifuged at 8000 × *g* for 15 minutes. The mitochondria were resuspended in 0.25 M sucrose and recentrifuged. Submitochondrial particles were pretreated by adding 10^{-6} M rotenone or 3×10^{-6} M antimycin A (final concentrations), both in 0.2 ml of ethanol, to 10-ml aliquots of particle suspension in 0.003 M phosphate buffer, pH 7.5 (the suspension contained particles from 1 g of liver per ml). A control was again run with ethanol. Recentrifugation was carried out at 105,000 × *g* for 30 minutes, and the surface of the pellet was rinsed carefully with phosphate buffer.

Materials—Rotenone, $C_{23}H_{22}O_6$ (for structural formula, see *The Merck Index*), manufactured by S. B. Penick and Company, New York, was a kind gift of Dr. K. E. Öberg, Uppsala. Stock solutions of rotenone, 0.5 to 1×10^{-3} M in absolute ethanol, were prepared and used for further dilutions. Rotenone was added to the samples in 0.02 ml of ethanol. The same amount of ethanol was added to the samples incubated without rotenone. This amount of ethanol had no effect on the reactions studied (*cf.* Table I). Solutions of sodium Amytal were prepared in water.

Oligomycin was generously supplied by Professor Henry A. Lardy, and coenzyme Q_0, by Professor D. E. Green, Madison, Wisconsin. Coenzyme Q_9 (Merck), vitamins K_1, K_2, and K_3 (Hoffmann-La Roche), and all other reagents were commercial products.

Assay Procedures—The composition of the medium for measuring respiration and phosphorylation was as follows (unless otherwise specified): 0.025 M P_i^{32}, pH 7.5, 0.008 M $MgCl_2$, 0.01 or 0.02 M substrate, 0.001 M ATP, 0.03 M glucose and hexokinase in excess, 0.05 M KCl, 0.05 M sucrose, and mitochondria were added to each vessel as described in the legends for the figures and tables. Respiration was measured by the conventional Warburg method, with air as gas phase and 0.2 ml of 2 M KOH in the center well. Phosphate uptake was determined by the isotope distribution method recommended by Lindberg and Ernster (15). In calculating P:O ratios the values for oxygen consumption

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were corrected by direct extrapolation for the 5-minute period of thermoequilibration.

Reduction of acetoacetate by succinate or by pyruvate plus malate (16) was assayed at 30° in a system containing, in a final volume of 2 ml, 0.01 M succinate or 0.01 M pyruvate + 0.01 M L-malate, 0.0042 M acetoacetate (prepared and standardized according to the method of Krebs and Eggleston (17)), 0.05 M KCl, 0.008 M $MgCl_2$ glycylglycine buffer, pH 7.5, and 0.05 M sucrose. The samples were fixed with 1 ml of 1.5 M perchloric acid and then centrifuged, and acetoacetate was determined by the method of Walker (18).

P_i-ATP exchange was measured according to the procedure of Löw *et al.* (10) with minor modifications (see legend of Fig. 2). ATPase activity was determined according to the method of Löw (9).

DPNH oxidase activity of the submitochondrial particles was determined by following the oxidation of DPNH at 340 mμ in a Beckman DK2 spectrophotometer. The assay system contained 1.33×10^{-4} M DPNH and 0.05 M phosphate buffer, pH 7.5, in a final volume of 3 ml. The temperature was 22°.

RESULTS

Effects of Rotenone on Electron Transfer

Oxidation of Pyridine Nucleotide-linked Substrates and Succinate—Rotenone was found to inhibit respiration with glutamate, but not with succinate, as substrate (Table I), in agreement with earlier reports (1–3). With the amount of mitochondria used, 5×10^{-8} M rotenone inhibited glutamate oxidation by approximately two-thirds without appreciably lowering the P:O ratio. Under the same conditions, succinate oxidation was not affected by a rotenone concentration of 10^{-6} M; the slight decrease in the P:O ratio with succinate at higher rotenone concentrations may be attributed to an inhibition of the further oxidation of fumarate, formed during succinate oxidation (*cf.* also Table V).

Rotenone also inhibited the oxidation of other pyridine nucleotide-linked substrates, such as pyruvate, malate, α-ketoglutarate, and β-hydroxybutyrate. As was found previously with Amytal, the inhibitory effect of rotenone was not relieved by an uncoupling concentration of 2,4-dinitrophenol (19, 20), and only to a low extent by added DPN and cytochrome *c* (21).

Oxidation of Extramitochondrial DPNH—Lehninger (22, 23) first demonstrated that liver mitochondria catalyze two types of oxidation of exogenous DPNH: one through the phosphorylating respiratory chain, and another, nonphosphorylating reaction that requires externally added cytochrome *c* and supposedly takes place at the surface of the mitochondria. The two pathways were found later to differ also with respect to their response to Amytal (6, 21, 24) and antimycin A (25–27), which inhibit only the "internal," phosphorylating pathway but not the "external," nonphosphorylating one.

Data in Table II compare the effects of rotenone and Amytal on the oxidation of exogenous DPNH. Continuous reduction of added DPN was achieved by means of ethanol and alcohol dehydrogenase. A relatively high concentration of DPN, 5 mM, was used, and 0.01 M fluoride was included in the reaction mixture, since these conditions have been shown by Maley (28) to yield maximal phosphorylation. Respiration and phosphorylation were measured in the absence and presence of added cytochrome *c*. In the absence of cytochrome *c*, the respiration was

TABLE I

Effect of rotenone on respiration and phosphorylation

Each vessel contained 0.01 M L-glutamate, succinate, or α-ketoglutarate, or 0.01 mM pyruvate + 0.01 mM L-malate, or 0.02 mM DL-β-hydroxybutyrate; 0.025 M P_i^{32}, pH 7.5; 0.008 M $MgCl_2$; 0.001 M ATP; 0.03 M glucose; 150 Kunitz-MacDonald units of yeast hexokinase; 0.05 M KCl; 0.05 M sucrose; and an amount of mitochondria containing 3.0 mg of protein. When indicated, 10^{-4} M 2,4-dinitrophenol, or 0.0015 M DPN and 1.6×10^{-5} M cytochrome *c*, were added. Final volume, 2 ml. Incubation took place at 30° with air as gas phase. Time measured, 17 minutes (Experiment 1) or 20 minutes (Experiment 2).

Experiment	Additions	Rotenone	Respiration	Phosphorylation
		M	μatoms O	μmoles P
1	L-Glutamate	0*	8.7	22.9
	L-Glutamate	5×10^{-8}	3.1	7.5
	L-Glutamate	10^{-7}	0.7	
	L-Glutamate	2×10^{-7}	0.6	
	L-Glutamate	10^{-6}	0.3	
	Succinate	0*	11.9	21.4
	Succinate	5×10^{-8}	12.8	22.5
	Succinate	10^{-7}	12.0	19.9
	Succinate	2×10^{-7}	11.7	21.0
	Succinate	10^{-6}	12.1	18.9
2	Pyruvate, L-malate	0	8.5	
	Pyruvate, L-malate	4×10^{-7}	0	
	α-Ketoglutarate	0	9.2	
	α-Ketoglutarate	4×10^{-7}	1.7	
	DL-β-Hydroxybutyrate	0	4.7	
	DL-β-Hydroxybutyrate	4×10^{-7}	0	
	L-Glutamate	0	10.7	
	L-Glutamate	4×10^{-7}	0	
	L-Glutamate, 2,4-dinitrophenol	4×10^{-7}	0	
	L-Glutamate, DPN, cytochrome *c*	4×10^{-7}	1.0	

* When 0.02 ml of ethanol was added, the respiration (microatoms of oxygen) and phosphorylation (micromoles of phosphate) were, respectively, 9.2 and 23.5 with L-glutamate, and 11.4 and 23.0 with succinate.

TABLE II

Comparison of effects of rotenone and Amytal on oxidation of extramitochondrial DPNH

The vessels contained 0.0125 M P_i, 0.005 M $MgCl_2$, 0.002 M ATP, 0.03 M glucose, 150 Kunitz-MacDonald units of hexokinase, 0.01 M KCl, 0.1 M sucrose, 0.005 M DPN, 0.042 M ethanol, 0.2 mg of crystalline alcohol dehydrogenase, 0.01 M NaF, and, when indicated, 1.6×10^{-5} M cytochrome *c*, 0.002 M Amytal, and 4×10^{-7} M rotenone. Final volume, 2 ml. Mitochondria containing 4.0 mg of protein were added to each vessel. Incubation took place at 30° for 30 minutes.

Additions	Without cytochrome *c*		With cytochrome *c*	
	Respiration	Phosphorylation	Respiration	Phosphorylation
	μatoms O	μmoles P	μatoms	μmoles P
None	2.3	4.1	5.9	5.7
Amytal	0.4	0.5	3.4	1.1
Rotenone	0.2	0.2	3.4	1.6

TABLE III

Bypass of rotenone-sensitive site of respiratory chain by vitamin K_3

Experimental conditions were as described in Table I; the substrate was L-glutamate. Mitochondria contained 3.86 mg of protein per vessel. The final concentrations of the additions were: rotenone, 4×10^{-7} M; antimycin A, 1.8×10^{-6} M; Dicumarol, 10^{-6} M; each quinone, 10^{-5} M. Incubation took place at 30° for 20 minutes.

Experiment	Additions	Respiration	Phosphorylation	P:O
		μatoms O	*μmoles P*	
1	None	12.0	30.6	2.6
	Rotenone	0.4		
	Vitamin K_3	15.1	33.9	2.2
	Rotenone, vitamin K_3	12.6	21.3	1.7
	1,4-Naphthoquinone	10.3	26.5	2.6
	Rotenone, 1,4-naphthoquinone	1.7	2.7	1.6
	1,4-Benzoquinone	12.5	30.2	2.4
	Rotenone, 1,4-benzoquinone	0.3		
	2-Methyl-1,4-benzoquinone	12.6	28.0	2.2
	Rotenone, 2-methyl-1,4-benzoquinone	0.9		
2	None	8.3	19.7	2.4
	Rotenone	0.5		
	Dicumarol	8.7	16.9	1.9
	Rotenone, vitamin K_3	10.6	13.7	1.3
	Rotenone, vitamin K_3, Dicumarol	2.4		
	Rotenone, vitamin K_3, antimycin A	1.9		
	Rotenone, vitamin K_1	0.5		
	Rotenone, vitamin K_2	0.5		
	Rotenone, coenzyme Q_0	2.0		
	Rotenone, coenzyme Q_9	0.1		

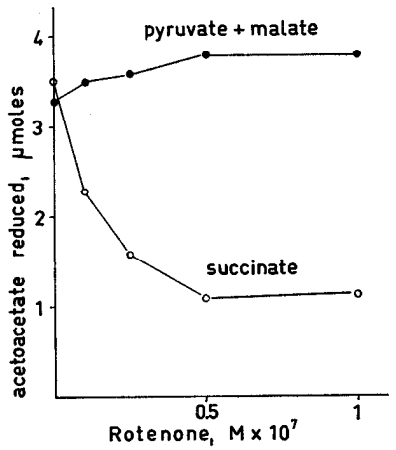

FIG. 1. Effect of rotenone on reduction of acetoacetate by succinate and by pyruvate + malate. Each vessel contained 0.0042 M acetoacetate, 0.02 M succinate or 0.01 M pyruvate + 0.01 M L-malate, 0.008 M $MgCl_2$, 0.02 M glycylglycine buffer, pH 7.5, 0.05 M KCl, 0.05 M sucrose, and an amount of mitochondria containing 3.9 mg of protein in a final volume of 2 ml. Incubation took place at 30° for 15 minutes.

relatively slow, and was accompanied by a phosphate uptake corresponding to a P:O ratio of approximately 1.8. Added cytochrome *c* substantially enhanced the respiration but induced only a slight increase in the net phosphate uptake; this increase in phosphate uptake probably was due to phosphorylation coupled to the oxidation of reduced cytochrome *c* by oxygen, as indicated by the studies of Maley (28). Both 2 mM Amytal and 4×10^{-7} rotenone inhibited virtually completely the respiration and phosphorylation occurring in the absence of added cytochrome *c* but left the cytochrome *c*-induced increase in these activities unaffected. Hence it can be concluded that rotenone, in the same way as Amytal, inhibits only the "internal," phosphorylating type of DPNH oxidation, and not its "external," nonphosphorylating counterpart.[1]

Bypass of Rotenone-sensitive Site by Vitamin K_3—Conover and Ernster (13, 29, 30) have demonstrated that the inhibition of respiration in rat liver mitochondria by Amytal can be overcome by the addition of a catalytic amount of vitamin K_3. A similar effect, as shown in Table III, occurred also in the case of the rotenone inhibition. As with Amytal, the vitamin K_3 effect was not duplicated by vitamin K_1 or K_2, or, to any significant extent, by identical concentrations of 1,4-naphthoquinone, *p*-benzoquinone, 2-methylbenzoquinone, 5,6-dimethoxy-2-methylbenzoquinone (coenzyme Q_0), or higher coenzyme Q homologues. The vitamin K_3-mediated respiration was inhibited by Dicumarol and antimycin A, and was connected with a phosphorylation of 1 P:O unit lower than that observed with the original system. These findings are consistent with the conclusion that vitamin K_3 induces a bypass of the rotenone-sensitive site of the respiratory chain by way of the Dicumarol-sensitive flavoenzyme called DT diaphorase (*cf.* 30), and that the electrons mediated by vitamin K_3 enter the terminal respiratory chain at the level of cytochrome *b*.

Succinate-linked Acetoacetate Reduction—It was recently reported from this laboratory that rat liver mitochondria catalyze an endergonic reduction of acetoacetate by succinate (13, 16, 31, 32). The reaction was shown to involve a backflow of electrons through the Amytal-sensitive site of the respiratory chain and to proceed at the expense of high energy bonds generated in the terminal oxidation of succinate. This reaction is also inhibited by rotenone (Fig. 1). Replacement of succinate by pyruvate + malate, which allowed a direct dismutation via DPN, resulted in an acetoacetate reduction that was insensitive to rotenone. These findings, which are analogous to those reported with Amytal (13, 16, 31, 32), further substantiate the concept that the succinate-linked acetoacetate reduction in isolated liver

[1] By the use of ethanol, alcohol dehydrogenase, and a relatively *low* (0.75 mM) concentration of *DPN* or, alternatively, a relatively *high* concentration (8 mM) of *DPNH*, Ernster *et al.* (6) found a very low phosphate uptake in the absence of cytochrome *c*, which was greatly increased by the addition of cytochrome *c*. Amytal, 2 mM, inhibited the respiration only partially in both the absence and presence of added cytochrome *c* and abolished the accompanying phosphorylation almost completely; similar findings were now made with rotenone. Since the same concentration of Amytal did not alter significantly the P:O ratio obtained with succinate, Ernster *et al.* (6) concluded that the inhibition of the cytochrome *c*-induced phosphorylation by Amytal could not be due to a true uncoupling effect, but rather meant that this phosphorylation actually originated from the "internal," phosphorylating pathway. Why, under the conditions used by Ernster *et al.* (6), externally added cytochrome *c* promoted the phosphorylation coupled to DPNH oxidation by the "internal" pathway, or why, in the experiments of Maley (28), a similar effect could be achieved by increasing the concentration of external, oxidized, DPN, or by fluoride, are questions that cannot readily be answered at this moment. They would seem to deserve future attention.

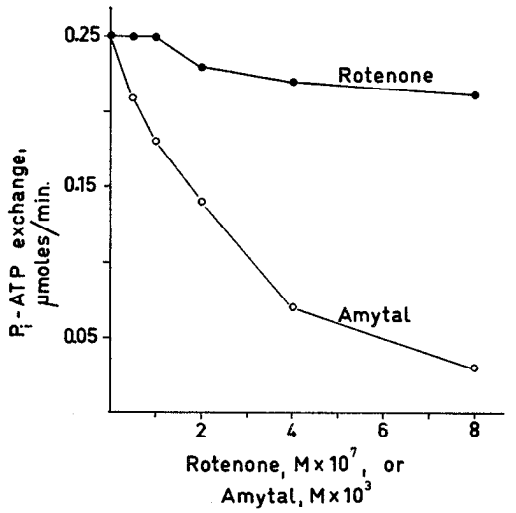

FIG. 2. Comparison of effects of rotenone and Amytal on P_i-ATP exchange. The assay system contained 0.01 M P_i^{32}, pH 7.5, 0.01 M ATP, 0.008 M $MgCl_2$, 0.05 M KCl, 0.05 M sucrose, and an amount of mitochondria containing 4.12 mg of protein in a final volume of 2 ml. Incubation was performed at 30°. Aliquots were removed after 5, 10, and 15 minutes, and the exchange rate was calculated by the method of Boyer, Luchsinger, and Falcone (34). The rates were virtually constant throughout the period measured.

P_i-ATP exchange by rotenone leveled off with increasing amounts of rotenone in a manner similar to, but even weaker than, that observed previously with antimycin A and cyanide (10, 34).

ATPase Reactions—Amytal was previously found to stimulate slightly the endogenous ATPase activity of rat liver mitochondria, and to exert an inhibition of the ATPase activity elicited by 10^{-4} M dinitrophenol (8). A strong inhibition of the dinitrophenol-induced ATPase by Amytal was demonstrated by Löw (9) to occur in the presence of an appropriate concentration of atebrin or chlorpromazine. These effects of Amytal are compared with those found with rotenone in Fig. 3. As can be seen, none of the effects of Amytal on the various ATPase reactions was duplicated by rotenone. In fact, rotenone appears to be the first respiratory inhibitor so far investigated that leaves mitochondrial ATPase reactions totally unaffected.

P:O Ratio with Succinate—It has been a general observation (35–37) that Amytal decreases the P:O ratio with succinate. It is seen in Table IV that rotenone, when added in an amount sufficient to inhibit DPN-linked electron transfer, lowered the P:O ratio to an extent that may be attributed to the inhibition of DPN-linked oxidations. Once this value was reached (as at 8×10^{-8} M rotenone in the present experiment), the P:O ratio did not decrease further even if the concentration of rotenone was increased 25-fold or more. On the other hand, Amytal,

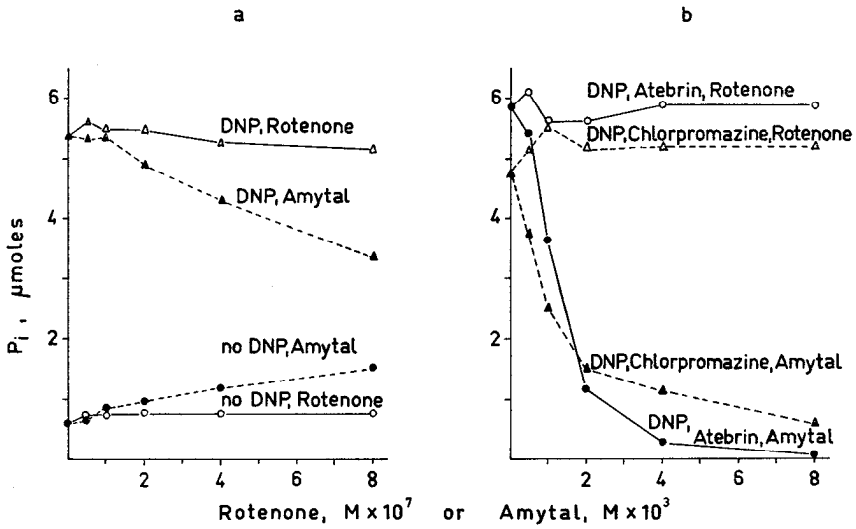

FIG. 3. Comparison of effects of rotenone and Amytal on ATPase activity under various conditions. The assay system contained 0.005 M ATP, 0.005 M Tris buffer, pH 7.5, 0.125 M sucrose, and, when indicated, 10^{-4} M dinitrophenol, 1.2×10^{-4} M chlorpromazine, and 1.7×10^{-3} M atebrin in a final volume of 2 ml. The amount of mitochondria added to each vessel contained 0.91 mg of protein. Incubation took place at 30° for 20 minutes. *DNP* denotes 2,4-dintrophenol.

mitochondria proceeds via the respiratory chain, and not, as has been argued (33), by a direct dismutation by way of DPN.

Effects of Rotenone and Amytal on Energy Transfer Reactions

P_i-ATP Exchange Reaction—Fig. 2 compares the effects of rotenone and Amytal on the rate of the P_i-ATP exchange. In confirmation of earlier results (10), 2 mM Amytal inhibited the P_i-ATP exchange by approximately 40%, and 8 mM Amytal, by almost 90%. On the other hand, rotenone induced only a slight inhibition of the exchange, even when added in large excess of the amount required for complete inhibition of DPN-linked electron transport (*cf.* Table I). The extent of inhibition of the

added either alone or in combination with rotenone, induced a progressive decrease in the P:O ratio.

Kinetics of Rotenone Inhibition

Binding of Rotenone to Mitochondria—Lindahl and Öberg (3) have observed that the inhibition by rotenone is not reversed when the respiration of gill filaments from rotenone-poisoned fishes is measured in a rotenone-free medium. This finding suggested that rotenone might be firmly bound to the mitochondria. This would be the opposite of the case of Amytal, which is readily removed from the mitochondria by washing.[2]

[2] O. Jalling, unpublished observation.

As shown in Table V, pretreatment of mitochondria with rotenone and subsequent washing (for details of pretreatment, see "Experimental Procedure") indeed resulted in a persisting inhibition of the respiration, whereas the Amytal-pretreated mitochondria respired at a rate equal to that of the washed control. It may be noted that the respiration of both the washed control and the Amytal-pretreated sample was lower than that of the original mitochondria, and was stimulated by added DPN. Apparently, the pretreatment procedure had caused a loss of DPN from the mitochondria. It is significant that, in spite of this fact, added DPN did not appreciably restore the respiration of the rotenone-pretreated sample. This observation, which is in accordance with previous findings with Amytal (21), shows that DPNH generated by intramitochondrial dehydrogenases reacts preferentially with the "internal" type of DPNH-cytochrome *c* reductase even after the respiration had been rendered dependent on added DPN.

TABLE IV

Comparison of effects of rotenone and Amytal on P:O ratio with succinate as substrate

Experimental conditions were as described in Table I; the substrate was 0.2 M succinate. Mitochondria contained 5.48 (Experiment 1) or 3.66 (Experiment 2) mg of protein per vessel. Incubation took place at 30° for 20 minutes.

Additions	P:O ratio	
	Experiment 1	Experiment 2
None	2.3	2.0
8×10^{-8} M rotenone	1.9	1.7
4×10^{-7} M rotenone	1.8	1.7
2×10^{-6} M rotenone	1.9	1.7
2×10^{-3} M Amytal	1.7	1.6
4×10^{-3} M Amytal	1.6	1.5
6×10^{-3} M Amytal	1.2	1.4
4×10^{-7} M rotenone + 2×10^{-3} M Amytal	1.8	1.6
4×10^{-7} M rotenone + 4×10^{-3} M Amytal	1.5	1.5
4×10^{-7} M rotenone + 6×10^{-3} M Amytal	1.0	1.1

TABLE V

Effect of pretreatment with rotenone and Amytal on respiration of mitochondria

Experimental conditions were as described in Table I; the substrate was 0.01 M L-glutamate; when indicated, 0.0015 M DPN was added. Mitochondria were pretreated, when indicated, with 0.002 M Amytal or 4×10^{-7} M rotenone as described in "Experimental Procedure." The amounts of mitochondria in the vessels were 3.66, 4.08, 3.81, and 5.27 mg of protein, respectively, for the samples that were untreated, washed, treated with Amytal, and treated with rotenone. Incubation took place at 30° for 10 minutes.

Treatment	Oxygen consumed	
	Without DPN	With DPN
	μatoms	
None	4.0	
Rewashed	1.7	3.5
Treated with Amytal, rewashed	1.6	3.9
Treated with rotenone, rewashed	0.0	0.5

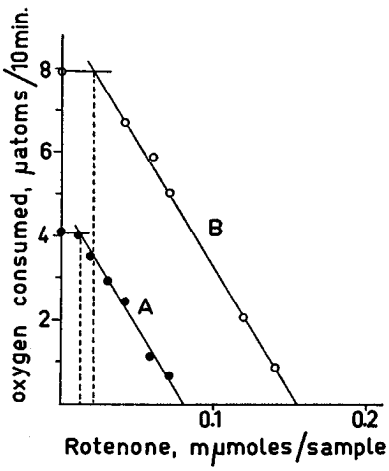

FIG. 4. Titration of rotenone-sensitive catalyst. Experimental conditions were as described in Table I, except that the substrate was 0.01 M pyruvate + 0.001 M L-malate. The amount of mitochondria added to each vessel contained 3.1 (*A*) or 6.2 (*B*) mg of protein. Time measured, 20 minutes.

Titration of Rotenone-sensitive Factor—Fig. 4 shows a titration of the "rotenone-sensitive factor," carried out by measuring the oxygen consumption (with pyruvate + malate as substrate) in the presence of varying amounts of rotenone and two different amounts of mitochondria (*A* and *B*). As could be anticipated, the respiratory values as functions of the amount of rotenone described straight lines and these were parallel for the two amounts of mitochondria used. From the intersection of the lines with the abscissa, the minimal amount of rotenone per unit of mitochondrial protein required for complete suppression of respiration can be estimated. This value, which may be taken as an expression of the amount of the rotenone-sensitive factor in the mitochondria, was 24.8 mμmoles of rotenone per g of protein (Fig. 4*A*), and 23.9 mμmoles of rotenone per g of protein (Fig. 4*B*). It is also possible from Fig. 4 to deduce the amounts of rotenone required between no inhibition (*dotted vertical lines*) and complete inhibition; this value is 0.068 mμmole in *A*, and 0.134 mμmole in *B*. If it is assumed that the rotenone-sensitive factor is an electron-transferring catalyst, the apparent "turnover number" of this catalyst can be calculated by dividing the rates of oxygen consumption in the two samples by the above values. This gives 6000 2-electron equivalents per mole per minute for *A*, and 5900 2-electron equivalents per mole per minute for *B*. With five preparations of mitochondria, and with pyruvate + malate or glutamate as substrate, the amount of the rotenone-sensitive factor varied between 24.7 and 28.0 mμmoles per g of protein, and its apparent turnover number ranged between 5400 and 7500 2-electron equivalents per mole per minute.

Comparison with Antimycin A and Oligomycin—In Fig. 5, rotenone and two other inhibitors, antimycin A and olgomycin (38, 39), are compared with regard to the above parameters (for previous titrations with these two compounds, see (39–42)). It can be seen that the amount of inhibitor required for complete (or, in the case of oligomycin, maximal[3]) inhibition was approximately twice as high with antimycin A, and about 8 to 9 times as high with oligomycin, as with rotenone. Another striking

[3] Oligomycin inhibits only that part of the respiration that is obligatorily coupled to phosphorylation (13, 39).

difference is that the ratio,

$$\frac{\text{amount of inhibitor required from no to maximal inhibition}}{\text{total amount of inhibitor required for maximal inhibition}}$$

is rather large with antimycin A (*cf.* also (40–42)) and oligomycin, whereas it is relatively small with rotenone. It appears, in other words, that whereas the rotenone-sensitive factor is present only in small excess of the total respiratory capacity, the antimycin A- and oligomycin-sensitive factors occur in large excess. An estimate of the turnover numbers was not possible from these data, because the experimental points were insufficient to determine the precise slopes of the lines.

Effect of Rotenone on Submitochondrial DPNH Oxidase

Submitochondrial preparations of DPNH oxidase of both the phosphorylating (43–45) and nonphosphorylating (45–47) types have been reported to possess a sensitivity to Amytal and antimycin A. A nonphosphorylating DPNH oxidase from rat liver mitochondria, prepared after mechanical disruption of the mitochondria by the method of Kielley and Kielley (14), has recently been studied in this laboratory in relation to these inhibitors (13, 48). The preparation exhibited an 80 to 90% sensitivity toward both Amytal and antimycin A. Data reported in Table VI compare the effect of rotenone in this preparation with the effect of antimycin A. It can be seen that 10^{-8} M rotenone gave maximal inhibition of the DPNH oxidase activity—an inhibition of approximately 80%—and that this concentration, as in the case of the intact mitochondria, was lower than the concentration of antimycin A needed for the same maximal inhibition.

The data in Table VI also show that pretreatment of the submitochondrial particles with either rotenone or antimycin A (for details of the pretreatment, see "Experimental Procedure") resulted in a persistent inhibition of the respiration just as in the case of the intact mitochondria. An attempt was made to restore the activity of the two preparations by combining them, since it was thought that the inhibited rotenone- and antimycin-sensitive factors of the respective preparations might be replaced by their uninhibited counterparts. Such a utilization of the antimycin-sensitive factor of an uninhibited Keilin and Hartree heart muscle preparation by an inhibited one has been demonstrated previously by Thorn (42). In the present case, however, no reactivation occurred with the combined rotenone- and antimycin A-inhibited preparations.

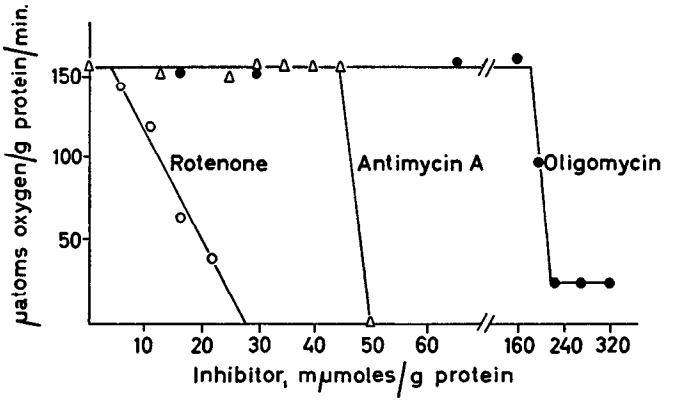

FIG. 5. Comparison of titration curves with rotenone, antimycin A, and oligomycin. Experimental conditions were as described in Table I; the substrate was 0.01 M L-glutamate. The amount of mitochondria added to each vessel contained 3.68 mg of protein. Time measured, 20 minutes.

TABLE VI

Effect of rotenone and antimycin A on submitochondrial DPNH oxidase

The assay system contained 4×10^{-4} M DPNH, 0.05 M phosphate buffer, pH 7.4, and enzyme preparation, containing 1.65 mg of protein, in a final volume of 3 ml. The reaction was started by the addition of the enzyme and followed at 340 mμ for 1 to 3 minutes in a Beckman DK2 spectrophotometer, with glass cuvettes of 1-cm light path. The temperature was 22°. In the pretreatment experiments, the enzyme preparation was treated with 1×10^{-6} M rotenone or 3×10^{-6} M antimycin A, as described in "Experimental Procedure."

Preparation	Inhibitor added in test	DPNH oxidase activity*
Untreated	None	214
	Rotenone, 3.3×10^{-9} M	212
	Rotenone, 6.7×10^{-9} M	202
	Rotenone, 10^{-8} M	42
	Rotenone, 1.3×10^{-8} M	41
	Antimycin A, 1.8×10^{-8} M	213
	Antimycin A, 3.7×10^{-8} M	179
	Antimycin A, 5.5×10^{-8} M	27
Pretreated with rotenone	None	50
Pretreated with antimycin A	None	46
Pretreated with rotenone and with antimycin A†	None	50

* Micromoles of DPNH oxidized per minute per g of protein.

† This assay was performed with a mixture of a rotenone-pretreated and an antimycin A-pretreated preparation, each added in one-half the amount previously used.

DISCUSSION

From the data reported in the present paper and from those already published by Fukami and Tomizawa (1, 2) and by Lindahl and Öberg (3, 4), the pattern of action of rotenone on *electron transport* seems to be identical with that of Amytal, and this identity suggests a common site of action of the two agents. However, there is an important difference between rotenone and Amytal with regard to their effects on certain *energy transfer* reactions. In sharp contrast with Amytal, rotenone inhibits the mitochondrial P_i-ATP exchange reaction to only a slight extent, and leaves the dinitrophenol-induced ATPase reaction and the phosphorylations accompanying the aerobic oxidation of succinate completely unaffected. These reactions are inhibited by Amytal even though the concentrations of Amytal required for these effects, as a rule, are higher than those needed for DPN-flavin-linked electron transport. That rotenone lacked similar effects was established by using amounts of rotenone in large (20- to 100-fold) excess of those needed to block DPN-flavin electron transport. In addition, Amytal has been shown to inhibit the ATP-dependent contraction of swollen mitochondria (49) and the relaxation of muscle fibers induced by the sarcotubular ATPase (50, 51). Also in these cases, the effect of Amytal is not duplicated by rotenone, as has been established by preliminary experiments in this laboratory. Evidently, rotenone blocks DPN-flavin-linked electron transfer in a more specific manner

than does Amytal, which inhibits also a number of energy transfer reactions.

The inhibition of the P_i-ATP exchange and dinitrophenol-induced ATPase reactions by Amytal has been taken as evidence for the involvement of an actual electron shuttle between DPN and flavin in these reactions (10). The flavin theory of oxidative phosphorylation (10, 52), involving a phosphorylated form of the reduced flavin as primary high energy intermediate, was partly based on the above interpretation of the Amytal effect. However, the present findings that the P_i-ATP exchange and the dinitrophenol-induced ATPase are unaffected when the DPN-flavin electron shuttle is completely inhibited by rotenone necessitate a different interpretation of the Amytal inhibition. Indeed, this conclusion is not in disagreement with the revised version of the flavin theory recently suggested by Ernster (16), in which an unknown radical other than phosphate is the partner of the reduced flavin in forming the primary high energy intermediate. This revised mechanism no longer requires an electron shuttle between DPN and flavin as an obligatory partial reaction of the P_i-ATP exchange and dinitrophenol-induced ATPase reactions.

Calculations of the minimal amount of rotenone required for complete inhibition of DPN-flavin-linked electron transport have given values ranging between 24 and 28 mμmoles per g of mitochondrial protein. This value is lower than the corresponding values for antimycin A and oligomycin (Fig. 5) and is, to our knowledge, the lowest value ever reported for an inhibitor of mitochondrial electron transport. In addition, the above value is considerably lower than the reported mitochondrial contents of various electron transfer catalysts, including pyridine nucleotides (53–57), flavins (53), quinones (58, 59), and cytochromes (53, 57). Significantly, the amount of rotenone required for complete inhibition of DPN-flavin-linked electron transfer is 10 to 20 times less than that fraction of the mitochondrial flavin that, according to Chance and Williams (53), becomes reduced in the presence of a DPN-linked substrate when respiration is blocked by antimycin A or anaerobiosis. Thus, if one assumes that the rotenone-sensitive factor is an electron-transferring catalyst, and that at least 1 molecule of rotenone is needed to block 1 molecule of the catalyst, it follows that this catalyst cannot be identical with the DPNH dehydrogenase flavoprotein. Furthermore, the apparent turnover number of the rotenone-sensitive catalyst is of the order of 5000 to 8000 2-electron equivalents per mole per minute, which, again, is very different from the turnover number of 1.3 million per mole of flavin per minute reported for the solubilized DPNH dehydrogenase (60).

Another point of interest arises from the findings reported in Fig. 4, according to which the extent of inhibition of respiration by a given amount of rotenone was almost directly proportional to the total amount of rotenone added. In other words, only a small amount of rotenone was needed to obtain an observable inhibition in relation to the amount required for complete inhibition. This situation is in striking contrast to that found with antimycin A, which gave a "sigmoid" type of titration curve, in agreement with previous findings of Ackermann and Potter (40) and Potter and Reif (41) and of Thorn (42). The oligomycin titration curve was also of the sigmoid type. The titration curve with antimycin A may be interpreted, in accordance with Thorn (42), as indicating that the antimycin-sensitive catalyst is present in the mitochondria at a capacity that is in a large excess of the capacity of the respiratory chain, *i.e.* that 90% or more of the catalyst may be blocked and full respiration can still proceed. By the same reasoning, then, it may be concluded that the rotenone-sensitive site is needed at nearly full capacity during maximal respiratory activity in the presence of phosphate and phosphate acceptor and, thus, that the rotenone-sensitive catalyst probably constitutes the rate-limiting factor of DPN-linked mitochondrial respiration under conditions of maximal respiration and phosphorylation.

SUMMARY

The effects of the fish poison, rotenone, on the respiration, phosphorylation, and related reactions of rat liver mitochondria have been investigated and compared with those of Amytal. In agreement with previous data in the literature, rotenone inhibits the aerobic oxidation of pyridine nucleotide-linked substrates but not that of succinate. The inhibition is not relieved by 2,4-dinitrophenol or by added diphosphopyridine nucleotide and cytochrome *c*, but is overcome by added vitamin K_3, which activates a bypass of the rotenone-sensitive site. Rotenone also inhibits the endergonic reduction of acetoacetate by succinate, but not the dismutative reduction of acetoacetate by pyruvate + malate. Oxidation of extramitochondrial DPNH is likewise inhibited by rotenone, yet only partially in the presence of added cytochrome *c*. The DPNH oxidase activity of submitochondrial particles is also highly sensitive to rotenone. All these effects are similar to those of Amytal.

In contrast to Amytal, rotenone leaves unaffected the mitochondrial inorganic orthophosphate-adenosine triphosphate exchange, and the resting as well as the 2,4-dinitrophenol-induced adenosine triphosphatase reactions, the latter in both the absence and presence of atebrin and chlorpromazine. Unlike Amytal, rotenone also does not lower the P:O ratio with succinate as substrate. It is concluded that rotenone blocks diphosphopyridine nucleotide-flavin-linked electron transport in a more specific manner than does Amytal, which inhibits also a number of energy transfer reactions.

Rotenone also differs from Amytal in that it is firmly bound to mitochondria and to submitochondrial particles. The extent of inhibition of respiration by rotenone is dependent on the amount, rather than the concentration, of rotenone added. The amount of rotenone required for complete inhibition of respiration is 24 to 28 mμmoles per g of mitochondrial protein, and the apparent turnover number of the rotenone-sensitive catalyst is 6000 to 8000 2-electron equivalents per minute per mole of catalyst. It is concluded that the rotenone-sensitive catalyst occurs at the lowest molar ratio among known components of the liver mitochondrial electron transport system, and probably constitutes the rate-limiting catalyst during maximal respiratory activity.

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