ROBENZIDENE, AN INHIBITOR OF OXIDATIVE PHOSPHORYLATION

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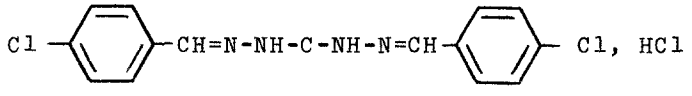
Robenzidene at 20 μM, inhibits the ADP stimulated respiration of intact rat liver mitochondria and induces respiratory control in submitochondrial fragments. At concentrations of the order of 16 nmole per mg protein, it also inhibits the Cl-CCP induced ATPase of mitochondria and ATPase of mitochondrial fragments. These properties of robenzidene resemble those of other inhibitors of oxidative phosphorylation such as oligomycin and DCCD.

Robenzidene, 1,3-bis(p-chlorobenzylidenamino)guanidine hydrochloride, has recently been reported as an anticoccidial agent (1).



Robenzidene, 1,3-bis(p-chlorobenzylidene amino)guanidine hydrochloride

Its effects on oxidative phosphorylation have been examined, and some similarity has been found in the inhibition of mitochondrial ATPases by robenzidene and oligomycin (2,3).

MATERIALS AND METHODS

Rat liver mitochondria were isolated according to the procedure previously described (4). Submitochondrial particles of rat liver were isolated under hypotonic conditions (5) in the presence of 2 mM EDTA pH 8.5 (6).

Oxygen uptake was measured with a Clark membrane electrode (7). Adenosine-triphosphatase activities were assayed by the measurement of inorganic phosphate (8).

Robenzidene was obtained from Mr. William N. Cannon; valinomycin and nigericin from Dr. R. L. Hamill of our laboratories. All other reagents were commercial products from Sigma Biochemical Company.

RESULTS AND DISCUSSION

Effects of Robenzidene on Respiration

Robenzidene at 10 μg/ml (20 μM) inhibited the ADP dependent oxygen uptake from 214.66 ± 5.19 to 67.5 ± 3.24 natom per mg protein per min in oxidation of succinate and from 134.12 ± 5.07 to 40.56 ± 3.39 natom per mg protein per min in oxidation of malate-glutamate (Table 1). The corresponding ADP/O ratios decreased from 2.02 ± 0.01 to 0 for succinate and from 3.01 ± 0.03 to 1.65 ± 0.07 for malate-glutamate as substrates. Robenzidene, however, did not inhibit the respiration induced by valinomycin or Cl-CCP, m-chlorocarbonyl-cyanide-phenyl-hydrazone (Table 1). In the oxidation of succinate, the restored activity was about 84 to 80% of the Cl-CCP and valinomycin activated rates. The corresponding recoveries in oxidation of NADH-linked substrates were about 70%. Nevertheless, robenzidene did not inhibit the utilization of conserved energy for cation transport as induced by valinomycin (8) and the dissipation of energy as induced by Cl-CCP.

Oligomycin, 1 nmole/mg protein (6) and DCCD (dicyclohexyl-carbodimide), 226 nmole/mg (10) are known to restore the respiratory control in submitochondrial particles, although DCCD was only effective with the oxidation of NADH but not succinate (10). With either substrate, robenzidene 35 nmole/mg protein, exerted significant respiratory control[+] (RC = 2.1 for NADH; 1.2 for succinate) which

[+] Respiratory control, RC = ratio of respiratory rate in the absence and the presence of inhibitor.

TABLE 1

EFFECTS OF ROBENZIDENE ON RESPIRATION OF INTACT MITOCHONDRIA

Substrate	Additions	Rate of Respiration (natom oxygen/mg protein/min)	
Succinate	None	59.98 ± 0	(5)
	Robenzidene	78.06 ± 9.4	(3)
	ADP	214.66 ± 5.19	(12)
	Robenzidene + ADP	67.50 ± 3.24	(7)
	Cl-CCP	297.98 ± 3.4	(3)
	Robenzidene + Cl-CCP	250.38 ± 5.8	(3)
	Valinomycin	274.18, 274.18	
	Robenzidene + Valinomycin	219.91, 239.90	
Glutamate	None	26.02 ± 2.35	(4)
	Robenzidene	32.40 ± 6.69	(3)
	Valinomycin	197.01 ± 4.20	(4)
	Robenzidene + Valinomycin	139.88 ± 1.84	(3)
Glutamate-Malate	None	43.78, 37.34	
	Robenzidene	29.61, 45.07	
	ADP	134.12 ± 5.07	(6)
	Robenzidene + ADP	40.56 ± 3.39	(4)
	Cl-CCP	103.01, 103.01	
	Robenzidene + Cl-CCP	74.68 ± 77.26	

Reaction mixture contains 0.25 M sucrose, 10 mM KCl, 10 mM Tris-phosphate pH 7.4 and mitochondria equivalent to 2.5 mg protein in a total volume of 1.7 ml. Other reagents were 20 μM robenzidene, 1 μg valinomycin, 185 nmole ADP (10 mM glutamate-malate as substrate), 280 nmole ADP (10 mM succinate as substrate) and 5 μM Cl-CCP.

TABLE 2

EFFECTS OF ROBENZIDENE ON RESPIRATION OF SONICATED MITOCHONDRIAL PARTICLES

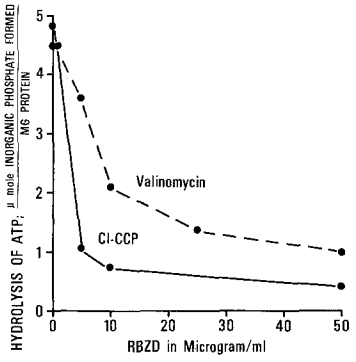
Substrates	Additions	Rate of Respiration natom oxygen/mg protein/min	Probability
NADH	None	210.61 ± 3.78 (6)	
	Robenzidene	104.02 ± 3.71 (3)	<0.001
	Robenzidene + Cl-CCP	216.27 ± 5.35 (3)	n.s.
	Oligomycin	85.48 ± 4.49 (3)	<0.001
	Oligomycin + Cl-CCP	253.34 ± 17.02 (3)	<0.05
Succinate	None	140.58 ± 3.96 (6)	
	Robenzidene	118.43 ± 6.75 (3)	<0.05
	Robenzidene + Cl-CCP	155.51 ± 8.43 (3)	n.s.
	Oligomycin	96.81 ± 4.12 (3)	<0.001
	Oligomycin + Cl-CCP	156.54 ± 7.21 (3)	n.s.

Reaction mixture consists of 0.25 M sucrose, 10 mM Tris-acetate, pH 7.4 and submitochondrial particles equivalent to 1.15 mg protein in a total volume of 1.7 ml. Either 1.3 mM NADH or 10 mM succinate-Tris, pH 7.4 was added to initiated respiration. Other additions were made as indicated: 20 μM robenzidene, 1 μg oligomycin and 1.5 μM Cl-CCP.

was released by the subsequent addition of 5 μM Cl-CCP (Table 2). Oligomycin, 2 nmole/mg protein, consistently showed better respiratory control (RC = 3 for NADH; 1.5 for succinate) than robenzidene.

Effects of robenzidene on ATPase

Lardy, et al. (2) first reported the inhibition of mitochondrial ATPase by oligomycin. Similar effects have been reported for DCCD in mitochondria (11) and in submitochondrial particles (10). The effects of robenzidene on the Cl-CCP and valinomycin induced ATPases in intact mitochondria are shown in Fig. 1. Robenzidene at 5 μg/mg protein, inhibited the Cl-CCP induced ATPase from 4.8 to 1.1 μmole/mg protein, whereas it decreased the valinomycin induced ATPase from 4.5 to 3.6 μmole/mg protein. At the moment, we cannot account for the less inhibitory effect of robenzidene on the latter ATPase. Perhaps, robenzidene has a greater influence on the Cl-CCP activated site than on the reaction between the phosphorylated and the non-phosphorylated high energy component which is responsible for ion translocation.



Effects of robenzidene on the Cl-CCP and valinomycin induced ATPases of rat liver mitochondria.

Medium of 1 ml consists of 0.25 M sucrose, 30 mM KCl 10 mM Tris-Cl pH 7.4 and 6 mM ATP-Tris, pH 7.4. Triplicate samples contained either 1 μg valinomycin or 0.03 μg Cl-CCP and various concentrations of robenzidene (RBZD). Mitochondria equivalent to 1 mg protein was added to initiate the reaction which was continued for 10 minutes at 30°C. The liberated inorganic phosphate in the trichloroacetic acid extract was determined.

Oligomycin and robenzidene inhibited the hydrolysis of ATP in the submitochondrial particles from 3.21 ± 0.06 to 0.07 and 0.93 ± 0.18 μmole Pi/mg protein, respectively (Table 3). As for comparison, the ionophores (valinomycin and nigericin) and electron transport inhibitor (antimycin A) did not affect the ATPase of submitochondrial particles although rotenone inhibited a little. The inhibition of robenzidene on submitochondrial ATPase proved its action different

TABLE 3

INHIBITORY EFFECT OF ROBENZIDENE AND OTHER AGENTS ON ATPase IN SUBMITOCHONDRIAL PARTICLES

Additions	P_i formed μmole/mg protein x 10 min	p
None	3.21 ± 0.06	
Oligomycin	0.07 ± 0	<0.001
Robenzidene	0.93 ± 0.18	<0.01
Valinomycin	3.04 ± 0.04	n.s.
Nigericin	3.05 ± 0.02	n.s.
Rotenone	2.89 ± 0.04	<0.05
Antimycin A	3.22 ± 0	n.s.

Medium of 1 ml consists of 75 mM sucrose, 60 mM KCl, 20 mM Tris-Cl, pH 7.4, 1.5 mM $MgCl_2$ and 6 mM ATP-Tris pH 7.4. Other reagents were 33 μM robenzidene, 1 μg oligomycin, 1 μg valinomycin, 1 μg nigericin, 2 μg antimycin A and 10 μM rotenone. Submitochondrial fragment of rat liver equivalent to 0.4 mg protein was added to start the incubation for 10 min at 30°C. Each value represents average of 3 samples.

from those of atractyloside (12) and bongkrekic acid (5). The latter two agents inhibited only the ATPase of intact mitochondria but not that of mitochondrial fragments indicating their effects on adenosine nucleotide transport. An examination on the nature of inhibition

caused by robenzidene on the mitochondrial ATPase is currently underway. The availability of robenzidene by chemical synthesis provides an opportunity for structural and activity relation study.

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