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RHODAMINE 6G, INHIBITOR OF BOTH H^+-EJECTIONS FROM MITOCHONDRIA ENERGIZED WITH ATP AND WITH RESPIRATORY SUBSTRATES

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Summary

Rhodamine 6G inhibited ATP hydrolysis by oligomycin-sensitive ATPase, purified from rat liver mitochondria, in good accord with the dose-response curve for its inhibition of energy transduction of ATP synthesis in mitochondria, but it did not inhibit ATP hydrolysis by purified F_1. Rhodamine 6G also inhibited both H^+-ejections from mitochondria energized with respiratory substrates and with ATP.

The present findings show that the inhibitory effect of rhodamine 6G on energy transduction is not due to a modification of the transport system for adenine nucleotides, P_i, and respiratory substrates, and that the inhibition sites of rhodamine 6G are on components related with H^+-ejection by redox components and also on F_0.

Gear [1] found that rhodamine 6G inhibits energy transduction in oxidative phosphorylation and he concluded that it blocks adenine nucleotide translocase, in a similar manner to atractyloside [2—6].

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Abbreviation: F_0, oligomycin-sensitivity-conferring factor.

The present paper shows that the inhibitory effect of rhodamine 6G on energy transduction is not due to a modification of the transport systems for adenine nucleotides, P_i, and respiratory substrates, and that the inhibition sites of rhodamine 6G are on components related with H^+-ejection by redox components and also on F_0. Thus the inhibition by rhodamine 6G clearly differs from those of other well-known inhibitors of energy transduction in oxidative phosphorylation, such as oligomycin [7,8] and dicyclohexylcarbodiimide [7—9], because the inhibition site of these is on F_0 only.

Rhodamine 6G was purchased from Tokyokasei, Tokyo (Japan). Digitonin was obtained from Wako Pure Chemical Industries, Osaka, and purified by centrifuging a 4% (w/v) solution in deionized water at 10 000 × *g* for 10 min and evaporating the resulting supernatant to dryness in an evaporating dish above boiling water. Other reagents were as in Ref. 10.

Rat liver mitochondria were isolated as in Ref. 10. Oligomycin-sensitive ATPase and ATPase (F_1) were purified from rat liver mitochondria by the methods of Soper and Pedersen [11] and Pedersen and Catterall [12], respectively. Proton-ejection from mitochondria was measured as in Ref. 10. Measurement of binding of rhodamine 6G to mitochondria was as in Ref. 22 except that rhodamine 6G was determined spectrophotometrically by measuring A_{527}. Protein was estimated from the contents of cytochromes $a + a_3$ in mitochondria and submitochondrial particles as described previously [13]. Protein in oligomycin-sensitive ATPase and F_1 was measured by the method of Lowry et al. [14]. The amount of $^{32}P_i$-labelled substances was as in Ref. 10.

In good accord with the findings of Gear [1], Fig. 1 shows that 10 μM rhodamine 6G completely inhibited the increased rate of oxygen uptake with a phosphate acceptor (state 3) in mitochondria with succinate as substrate. This inhibition was completely released by addition of the uncoupler DNP (2,4-dini-

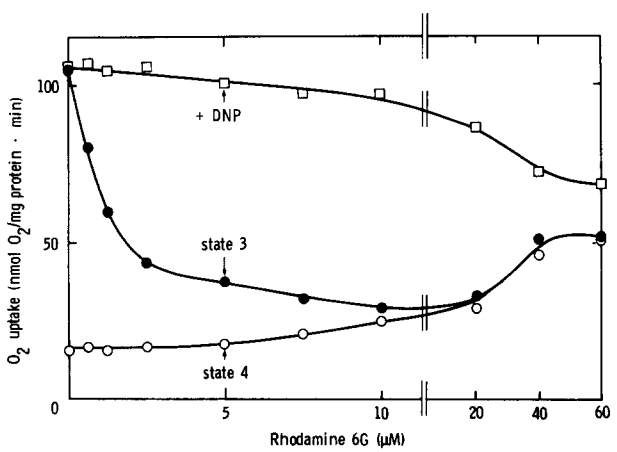

Fig. 1. Effect of rhodamine 6G concentration on oxygen uptake by mitochondria. Mitochondria (1 mg protein/ml) were preincubated for 3 min at 25°C in the presence of 10 mM succinate/2 μg of rotenone/ 5 mM $MgCl_2$/2 mM EDTA/15 mM KCl/50 mM sucrose/25 mM Tris/the indicated concentrations of rhodamine 6G in a final volume of 4.5 ml at pH 7.4, and then 20 mM potassium phosphate (state 4), 200 μM ADP (state 3) and 50 μM 2,4-dinitrophenol (DNP) were added. Oxygen uptake was measured polarographically with a Yellow Spring, Model YSI-53, oxygen monitor.

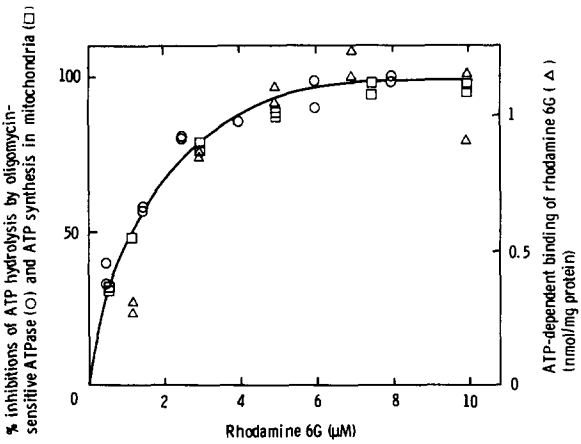

Fig. 2. Dose-response curves of the ATP-dependent binding of rhodamine 6G to mitochondria and of its inhibitions of ATP synthesis in mitochondria and ATP hydrolysis by oligomycin-sensitive ATPase. The amount of ATP-dependent binding of rhodamine 6G to mitochondria and ATP synthesis in the membranes were measured as described in the text. Oligomycin-sensitive ATPase (42 μg of protein) was preincubated for 5 min at 25°C in the presence of 50 mM Tris-acetic acid/3 mM $MgSO_4$/6 mM phospho*enol*pyruvate/32 μg of pyruvate kinase (8 units)/the indicated concentration of rhodamine 6G/±0.025 μM oligomycin, in a final volume of 1 ml at pH 7.4. The reaction was started by adding 4 μmol of ATP and was stopped 30 min later by adding 1 ml of 16% trichloroacetic acid. The P_i formed was determined by the method of Takahashi [23]. ATP hydrolysis by oligomycin-sensitive ATPase in the absence of rhodamine 6G was 2.2 μmol/mg protein per min. This activity was completely inhibited by 0.025 μM oligomycin.

trophenol). At a concentration of above 20 μM, rhodamine 6G stimulated state 4 respiration to about 50% of the activity of DNP-induced respiration. The inhibitory effect of rhodamine 6G on energy transduction was reversible since a washing of rhodamine 6G-treated mitochondria by centrifugation reversed the inhibition of state 3 respiration by the dye. Thus the dye may interact with mitochondria through non-covalent forces. Rhodamine 6G also

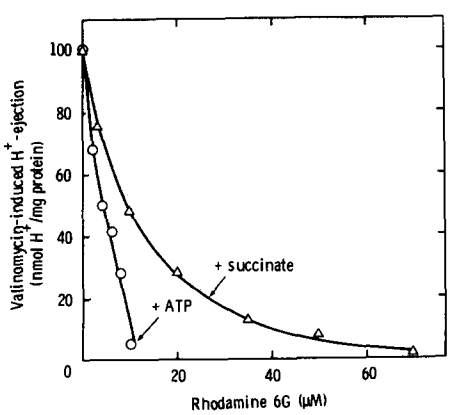

Fig. 3. Effect of rhodamine 6G concentration on valinomycin-induced H^+-ejection from mitochondria energized with succinate and with ATP. Conditions were as in Ref. 10 except that H^+-ejection from mitochondria energized with ATP was measured at pH 6.5 and 200 ng of valinomycin per mg protein was used.

inhibited ATP synthesis in submitochondrial particles, which are inside-out relative to the membranes of mitochondria [15—18], although ATP synthesis in mitochondria is more sensitive than that in submitochondrial particles to rhodamine 6G.

Energization of mitochondria with succinate and with ATP induced binding sites on the membranes for rhodamine 6G [19]. The energy-dependent binding of rhodamine 6G was not inhibited by ethidium or tetraphenylarsonium, which are anisotropic inhibitors of energy transduction [10,20—22]. Fig. 2 shows that, as in the case of ethidium [22] and tetraphenylarsonium [10], the dose-response curve for inhibition of ATP synthesis in mitochondria by rhodamine 6G approximately coincided with the dose-response curve of ATP-dependent binding of rhodamine 6G to the membranes, indicating that the energy-dependent binding of rhodamine 6G to the membranes inhibits energy transduction in oxidative phosphorylation. Rhodamine 6G also inhibited ATP-Pi exchange in mitochondria. Fig. 2 also shows that rhodamine 6G inhibited ATP hydrolysis by purified oligomycin-sensitive ATPase, and that the curve coincided with the dose-response curve for its inhibitions of ATP synthesis in mitochondria. Rhodamine 6G did not inhibit ATP hydrolysis by purified F_1 (ATPase).

Fig. 3 shows that rhodamine 6G inhibited valinomycin-induced H^+-ejection from mitochondria energized with succinate and with ATP. Rhodamine 6G also inhibited ethidium-induced and tetraphenylarsonium-induced H^+-ejection [10,20] from membranes energized with succinate and ATP. It should be noted that these H^+-ejections from membranes energized with succinate were inhibited by rhodamine 6G even at a concentration that did not increase state 4 respiration (Fig. 1). Therefore, these results are clearly different from those for other well-known inhibitors of energy transduction in oxidative phosphorylation, such as oligomycin [7,8], dicyclohexylcarbodiimide [7—9], and aurovertin [7], because the latter inhibit H^+-ejection from membranes energized with ATP, but not H^+-ejection from membranes energized with respiratory substrates.

Gear [1] proposed that rhodamine 6G blocks adenine nucleotide translocase. This proposal was based on his finding that rhodamine 6G inhibited the bindings of [^{14}C]ADP and [^{14}C]ATP at low nucleotide concentrations to mitochondria. However, this idea cannot explain his other finding that rhodamine 6G did not inhibit binding of adenine nucleotide at higher nucleotide concentrations to the membranes.

The present findings show that: (1) energy-dependent binding of rhodamine 6G to mitochondria occurred with either respiratory substrates or ATP (Fig. 2), (2) rhodamine 6G inhibited ATP synthesis in submitochondrial particles, which are inside-out relative to the membranes of mitochondria [15—18], though ATP synthesis in mitochondria is more sensitive to rhodamine 6G than that in submitochondrial particles, (3) rhodamine 6G also inhibited ATP hydrolysis by purified oligomycin-sensitive ATPase, in good accord with the dose-response curve for the inhibition of ATP synthesis in mitochondria (Fig. 2).

These results indicate that the inhibitory effect of rhodamine 6G on ATP synthesis in mitochondria is not due to modification of the transport systems for respiratory substrates, P_i, and adenine nucleotides. The finding of Gear that rhodamine 6G inhibited binding of adenine nucleotides at low nucleotide concentration to mitochondria, may be due to direct interaction between positively

charged rhodamine 6G and negatively charged adenine nucleotides. This possibility is supported by the fact that ADP and ATP caused a red shift in the absorbance maximum of rhodamine 6G.

Rhodamine 6G inhibited H^+-ejections both from mitochondria energized with ATP and from those energized with respiratory substrates (Fig. 3). These actions are different from those of other well-known inhibitors of energy transduction, such as oligomycin [7,8], dicyclohexylcarbodiimide [7—9], and aurovertin [7], because the latter inhibit H^+-ejection from mitochondria energized with ATP, but not H^+-ejection from membranes energized with respiratory substrates. This shows that rhodamine 6G inhibits two sites in the energy transfer system of oxidative phosphorylation. One is components related to H^+-ejection by redox complexes. The other inhibition site of rhodamine 6G may be on F_0, since rhodamine 6G inhibited ATP hydrolysis by oligomycin-sensitive ATPase, but it did not inhibit that in F_1. ATP-dependent binding of rhodamine 6G to mitochondria was inhibited by oligomycin or dicyclohexylcarbodiimide. Thus, the binding sites of rhodamine 6G in mitochondria energized with ATP (1.2 nmol per mg protein (Fig. 2)) are different from the binding sites of oligomycin or dicyclohexylcarbodiimide [9].

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