Antibiotics as Tools for Metabolic Studies. IV. Comparative Effectiveness of Oligomycins A, B, C, and Rutamycin as Inhibitors of Phosphoryl Transfer Reactions in Mitochondria*

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ABSTRACT: Oligomycins A, B, and C, and rutamycin are approximately equally effective in inhibiting mitochondrial oxidation of several different substrates and the exchange of radioactive orthophosphate with ATP.

Dinitrophenol reverses the respiratory inhibition by these compounds with equal facility. Oligomycin C is appreciably less effective than the other three congeners in inhibiting the hydrolysis of ATP induced by 2,4-dinitrophenol, 2,6-dinitrothymol, *O*-methyl-3,3′,5-triiodothyroacetic acid, or by storing mitochondria at 37°. Oligomycin A, stored in 50% ethanol at 5° for a few months, loses its ability to inhibit adenosinetriphosphatase and mitochondrial oxidations. Oligomycins B and C and rutamycin are stable under these storage conditions.

The oligomycin group of antibiotics has been widely used for studies of oxidative phosphorylation, membrane transport phenomena, and related energy-requiring processes. The availability of the pure oligomycins A, B, and C (Masamune *et al.*, 1958), and of rutamycin[1] (Thompson *et al.*, 1961), a new member of the oligomycin family, permitted a comparison of their effectiveness as inhibitors of phosphoryl transfer reactions in mitochondria. In general, all four compounds exert similar effects on phosphoryl transfer systems but appreciable quantitative differences in activity and stability have been found, and are reported here as an aid to other investigators using these agents.

TABLE I: Inhibition of Mitochondrial Oxidations by Various Oligomycins.[a]

		Q_{O_2} (N)							
		Oligomycin A $C_{24}H_{40}O_6$, 425		Oligomycin B $C_{22}H_{36}O_6$, 397		Oligomycin C $C_{28}H_{46}O_6$, 479		Rutamycin $C_{25}H_{42}O_6$, 439	
Substrate	Control	1 μg	2 μg	1 μg	2 μg	1 μg	2 μg	1 μg	2 μg
L-Glutamate	330	310	21	320	80	340	130		40
α-Ketoglutarate	306				48		55		55
Succinate	410		150		130		150		125
DL-β-Hydroxybutyrate	260	225	15	230	20	240	50	245	60
			15[b]		25[b]		4[b]		10[b]

[a] The reaction mixture contained 2 mM ATP, 17 mM potassium phosphate, 5 mM $MgSO_4$, 17 mM glucose, yeast hexokinase (Type III or crystalline), and 0.5 ml of a suspension of rat liver mitochondria (about 1.5 mg N) in 0.25 N sucrose. All substrates were present at 10 mM. Final volume 3.0 ml; temperature 30°. [b] 3 μg of oligomycin per flask.

The structures of the oligomycins are unknown, but they are probably macrolides (F. M. Strong and E. Van Tamelin, personal communication). They differ slightly in empirical formula (Table I) and in the number of active hydrogens and *C*-methyl groups (Masamune *et al.*, 1958; Thompson *et al.*, 1961) but differ considerably in their toxicity to mice and certain fungi (Marty and McCoy, 1959; Thompson *et al.*, 1961).

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[1] The name rutamycin for Lilly compound A272 has been adopted by the Council on Drugs of the American Medical Association.

TABLE II: Influence of Various Oligomycins on ATP-P_i Exchange.[a]

Amount of Oligomycin	μMoles $P_i \rightarrow$ ATP[b]			
	Oligomycin A	Oligomycin B	Oligomycin C	Rutamycin
None	2.23[c]			
0.03 μg	2.16	1.97	1.71	1.67
0.3 μg	0.063	0.22	0.17	0.086
3 μg	0.078	0.055	0.056	0.062

[a] The reaction mixture contained 10 mM ATP, 10 mM orthophosphate containing 1.8×10^6 cpm, 75 mM Tris buffer, *p*H 7.3, and mitochondria from 0.1 g of rat liver in 0.1 ml of 0.25 M sucrose. All components except $^{32}P_i$ were equilibrated at 30° for 5 minutes; final volume 1 ml.; incubated 10 minutes with $^{32}P_i$. [b] Calculated according to Boyer *et al.* (1956), assuming that only the terminal P of ATP exchanged. [c] This value derived from an incorporation of 18% of the $^{32}P_i$ into ATP.

et al. (1962) were employed. Magnesium salts were omitted which diminishes equilibration of ^{32}P between the β and γ positions of ATP (Wadkins, 1962). The calculations were therefore based on the assumption that exchange occurred only between P_i and the γ position of ATP. Radioactive ATP was determined according to Falcone and Witonsky (1964).

Results

Table I records the relative effectiveness of the different oligomycins in inhibiting oxidation of various substrates by rat liver mitochondria. In some experiments oligomycin C appeared to be slightly less effective than the other oligomycins. In these cases, increasing the concentration of oligomycin C resulted in as complete inhibition as was obtained with its congeners. Dinitrophenol reversed the inhibition of respiration by each of the oligomycins with equal effectiveness.

The various oligomycins also are approximately equally inhibitory to the P_i-ATP exchange reaction in intact mitochondria as is shown in Table II.

In contrast to the foregoing responses, the adenosine triphosphatase activity of mitochondria exhibits a selective susceptibility to the different oligomycins. Oligomycin C is consistently less inhibitory to ATPase induced by dinitrophenol, dinitrothymol, triiodothyroacetate (Triac),[2] and *O*-methyltriiodothyroacetate (Table

TABLE III: Comparison of Oligomycins as Inhibitors of Mitochondrial ATPase.[a]

ATPase Inducer	P_i Liberated per 0.2 mg N in Control (μmoles)	Inhibition of P_i Liberation (%)							
		Oligo. A		Oligo. B		Oligo. C		Rutamycin	
		1 μg	2 μg	1 μg	2 μg	1 μg	2 μg	1 μg	2 μg
5×10^{-5} M 2,4-Dinitrophenol	4.42	84	90	80	87	58	74	82	87
	4.16	89	94	86	92	41	73	80	88
6×10^{-6} M Dinitrothymol	4.24[b]	88	94	82	90	35	67	79	86
4×10^{-5} M *O*-Methyl Triac[c]	2.59	91	94	91	90	71	74	89	88
	2.44	89	94	85	89	38	55	77	81
10^{-4} M Triac	0.96	55[d]		42[d]		19[d]			
Valinomycin	4.30						89		97
Gramicidin	4.85						93		98
Aging 20 minutes at 37°	0.58	75	82	68	78	42	62	71	73
Aging 20 minutes at 37° + 1.5×10^{-3} M Mg	1.52	83	86	81	85	63	70	78	81

[a] The reaction mixture contained 6 mM ATP, 75 mM KCl, 10 mM Tris buffer, *p*H 7.4, and mitochondria (*ca.* 0.2 mg N) from 0.05 g liver in 0.3 ml of 0.25 M sucrose. Final volume 1 ml; incubated 10 minutes at 30°. The values recorded are averages of closely agreeing duplicates in each of the experiments. [b] In this experiment, aurovertin at 1 and 2 μg/ml inhibited 34 and 50% of the ATPase induced by 6×10^{-6} M dinitrothymol. [c] The effectiveness of oligomycin C in blocking ATPase induced by thyroid hormones and related structures varies with different mitochondrial preparations (cf. Lardy *et al.*, 1964; Connelly and Lardy, 1964). The two experiments recorded here represent the extremes of this observed variability. [d] Only 0.5 μg of the oligomycins were used in this experiment.

Experimental

The methods and materials used have been recorded in Paper II of this series (Lardy *et al.*, 1964). In the P-ATP exchange reaction, the conditions of Falcone

TABLE IV:[a] Comparative Stability of Oligomycins Evidenced by Their Effect on ATPase of Aged Mitochondria.

Additions	P_i Liberated per 0.2 mg N in Control	Inhibition of P_i Liberation (%)							
		Oligo. A		Oligo. B		Oligo. C		Rutamycin	
		1 μg	2 μg	1 μg	2 μg	1 μg	2 μg	1 μg	2 μg
None	1.61	−12	−10	93	96	75	81	88	94
1.5×10^{-3} M Mg^{2+}	3.86	− 3	− 2	79	86	44	58	70	79

[a] Conditions as in Table III except that only aged (20 minutes at 37°) mitochondria were used. The oligomycins used in this experiment had been stored 4 months at 5° as solutions containing 100 μg/ml in 50% ethanol. The minus signs indicate enhancement of ATPase above the control value.

III). The lesser effectiveness of oligomycin C cannot be explained on the basis of its higher molecular weight (a factor of only 20%), for 2 μg of oligomycin C is usually less effective than 1 μg of any of the other three oligomycins.

The finding that oligomycin C inhibited only part of the ATPase activity induced by these agents suggested that it might be functioning, like aurovertin, to inhibit the reversal of the oxidative phosphorylation sequence of reactions while not inhibiting the hydrolysis occurring in the *W* category (work performance in membrane transport) (Lardy *et al.*, 1964). Oligomycin C was therefore tested as an inhibitor of ATPase induced by valinomycin, gramicidin, and also by aging mitochondria at 37°. Whereas these activities are not significantly inhibited by aurovertin (Lardy, 1961, and unpublished data), they are blocked by oligomycin C (Table III), indicating that this agent is merely quantitatively less effective than other oligomycins and does not act in the manner of aurovertin.

During the course of investigations of the oligomycins it was observed that samples of oligomycin A lost activity during storage in 50% ethanol. This was never observed with oligomycin B or C, or with rutamycin. The experiment recorded in Table IV presents results with solutions of the four congeners made in 50% ethanol and stored under identical conditions. In 4 months at 5°, oligomycin A had lost all inhibitory properties while the other three congeners were as inhibitory as fresh solutions. Incidentally, this loss of activity did not seem to occur in absolute ethanol. During the storage of oligomycin A, loss of ability to inhibit ATPase was paralleled by loss of ability to inhibit mitochondrial oxidation in tightly coupled mitochondria.

Some of the discrepancies in the literature concerning the action of oligomycin may stem from this lability of oligomycin A.

[2] Abbreviation used in this work: Triac, 3,3′,5-triiodothyroacetate.

Added in Proof

Since this paper was submitted R. Michel, P. Huet, and M. Huet ((1964), *Compt. Rend. Soc. Biol. 158*, 994) have also reported that rutamycin inhibits mitochondrial ATPase.

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