Antibiotics as Tools for Metabolic Studies. II. Inhibition of Phosphoryl Transfer in Mitochondria by Oligomycin and Aurovertin*

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The antibiotics oligomycin A and aurovertin are quantitatively equally effective in inhibiting oxidative phosphorylation, $^{32}P_i$-ATP exchange, and the exchange of ^{18}O between P_i and H_2O in rat liver mitochondria. The effects of partially inhibitory amounts of oligomycin and aurovertin are additive. Amounts of either oligomycin or aurovertin that inhibit up to half of the two exchange reactions are without effect on substrate oxidation and the associated phosphate uptake. Oligomycin A inhibits all induced ATPases. Aurovertin enhances ATP hydrolysis induced by selenite, selenate, and deoxycholate; it usually inhibits only slightly the hydrolysis of ATP in the presence of compounds related in structure to iodinated thyronines, palmitate, low concentrations of tellurite, and several antibiotics that affect ion transport in mitochondria; it inhibits partially the hydrolysis of ATP induced by DNP and a wide variety of agents that uncouple oxidative phosphorylation; it completely inhibits ATP hydrolysis induced by arsenate. The data presented here and in other papers lead to the conclusion that a category of high-energy compounds required for ion transport and for mitochondrial swelling and contraction phenomena may be generated irreversibly either from ATP or from intermediates generated by respiration. This category of intermediates is *not* on the main pathway of oxidative phosphorylation. Many uncoupling agents appear to catalyze the discharge of energy from this category of intermediates as well as discharging an early intermediate in oxidative phosphorylation. Evidence is presented concerning the site at which arsenate discharges a high-energy intermediate of oxidative phosphorylation.

The utility of specific inhibitors in studies of biological phenomena is well known and some of the advantages of the use of toxic antibiotics in this manner were presented in the first paper of this series (Lardy *et al.*, 1958).

The selective toxicity of the antibiotic oligomycin (Smith *et al.*, 1954) to aerobic organisms indicated that it might be of value in probing individual reactions in the process of oxidative phosphorylation (Lardy *et al.*, 1958; Lardy and McMurray, 1959, Lardy, 1961). It inhibits respiration of mitochondria, and, as in the case of guanidine (Hollunger, 1955), the inhibition may be reversed by uncoupling agents such as 2,4-dinitrophenol. It inhibits the adenosine triphosphatase activity of mitochondria induced by Ca^{2+}, dinitrophenol, thyroid hormones, or deoxycholic acid (Lardy *et al.*, 1958). Thus it appeared to inhibit a phosphate-transferring enzyme rather than one involved in electron transfer. More detailed studies with oligomycin led to the conclusion that the uncoupling effect of dinitrophenol is exerted prior to the step at which P_i is fixed in oxidative phosphorylation (Lardy and McMurray, 1959; Huijing and Slater, 1961). This antibiotic has been employed by many investigators in studies of oxidative phosphorylation, many biosynthetic processes, ion transport, and mitochondrial swelling and contraction.

Since the work cited, we have found that aurovertin (Baldwin *et al.*, 1964) inhibits oxidative phosphorylation in much the same manner as does oligomycin. Extended comparisons have shown, however, that the effects of these two types of antibiotics differ considerably in some test systems as will be described in this and the following paper (Connelly and Lardy, 1964b). These findings lead to the conclusion that the reactions of oxidative phosphorylation and of associated energy-transfer processes are more complicated than those visualized in most proposed mechanisms, particularly those involving only a single high-energy intermediate between the electron-transfer reaction and ATP. The data are most readily explained by assuming that the high-energy intermediates involved in mitochondrial swelling and contraction, and in ion transport, are not on the main path of oxidative phosphorylation but are derivable from intermediates on the latter.

EXPERIMENTAL

Homogenization and centrifugal fractionation of normal rat liver was conducted as described by Schneider (1948), except that the mitochondrial fraction was collected after 5 minutes at 17,000 × *g* and washed twice by suspending in 0.25 M sucrose equivalent to twice the original liver volume. The methods for measuring respiration and phosphorylation (Lardy and Wellman, 1952), ATPase (Lardy and Wellman, 1953), and $^{32}P_i$-ATP exchange (Boyer *et al.*, 1956) are those cited. Detailed experimental conditions will be presented in the protocols.

To determine loss of ^{18}O from orthophosphate, perchloric acid–treated mixtures[1] were centrifuged in the cold and the $MgNH_4PO_4$ was precipitated from the clear supernatant fraction. After the fraction was washed with cold dilute ammonia the residue was dissolved in a minimum of hydrochloric acid and applied to a column (6.0 mm diameter) containing 15 mm Norit-cellulose (coarse), 1:1; 1 mm Celite, and 20 mm Dowex 50W-X12 exchange resin (H^+ phase). The column was first washed with a minimum of water and then blown dry with air. The total volume of eluate was about 1 ml. After neutralizing to *p*H 4.6 with KOH, the eluate was diluted to three volumes with

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[1] Since these experiments were completed it has been found that the use of trichloroacetic acid as a protein precipitant leads to easier purification of the phosphate salts.

TABLE I
COMPARISON OF AUROVERTIN AND OLIGOMYCIN AS INHIBITORS OF MITOCHONDRIAL OXIDATION AND PHOSPHORYLATION; RESTORATION OF OXIDATION BY VARIOUS UNCOUPLING AGENTS[a]

Expt.	Substrate	Additions	Control		Oligomycin A		Aurovertin	
			$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O
1	Endogenous	(Normal rat liver M_w)	20	2	8	0	16	0.5
	DL-α-Glycerophosphate	(Same)	64	1.7	11	0	29	0.3
2	Endogenous	(Thyroid-fed rat liver M_w)[c]	35	0.7	5	0	22	0
	DL-α-Glycerophosphate	(Same)	300	1.4	113	0	140	0
3	Succinate		377	2.0	92	0	118	0
	+	1.6×10^{-5} M DNP	235	0.4	235	0	202	0
	+	3.3×10^{-5} M *O*-Me-Triac	300	1.7	147	0	125	0
	Choline		60	1.9	19	0	21	1.0
	+	1.6×10^{-5} M DNP	100	0	90	0	90	0
4	Choline		65	1.4	40	0	73	0.8
							44[b]	0
	+	10^{-5} M Usnic acid	138	0	131	0	142	0
5	DL-β-Hydroxybutyrate		162	2.8	14	0	29	0.8
	+	1.6×10^{-5} M DNP	130	0.7	108	0	132	0
	+	3.3×10^{-5} M *O*-Me-Triac	136	2.5	37	0	57	0
	+	5×10^{-4} M $CaCl_2$	170	2.7	24	0	20	0
6	DL-β-Hydroxybutyrate		185	2.4	0		50	0.4
	+	10^{-5} M Usnic Acid	120	0	110	0	95	0
7	DL-β-Hydroxybutyrate		230	2.4	35	0	25	0
	+	5×10^{-5} M Selenite	105	0	50	0	65	0
	+	10^{-4} M Tellurite	190	0.4	205	0	115	0
8	L-Glutamate		293	3.1	0		0	
	+	1.6×10^{-5} M DNP	295	1.9	181	0	206	0
	+	1.6×10^{-5} M DNT	133	0	136	0	122	0
9	L-Glutamate		375	3.1	0		21	3.0
	+	1.6×10^{-5} M DNP	390	2.1	175	0	180	0
	+	3.3×10^{-5} M *O*-Me-Triac	320	2.9	80	0.3	70	0.6
	+	10^{-5} M Dicoumarol	335	0.8	325	0	285	0.1
10	L-Glutamate		280	2.8	16[b]	0	30	0
	+	10^{-4} M $CaCl_2$	275	2.5	36[b]	0	30	0
	+	5×10^{-4} M $CaCl_2$	250	2.4	38[b]	0	35	0
11	L-Glutamate		325	3.4	24[b]	0	24[b]	0
	+	2 μg SQ 15859	350	0	365[b]	0.5	350[b]	0
	+	1.7×10^{-4} M TCAP	350	2.3	84[b]	0	166[b]	0
	+	3.4×10^{-4} M TCAP	360	1.9	144[b]	0	220[b]	0
12	α-Ketoglutarate		230	3.1	12	0	30	1.5
	+	3.3×10^{-5} M *O*-Me-Triac	240	2.6	53	0.7	73	0.3
13	α-Ketoglutarate		360	2.7	34	0.8	67	1.0
	+	Valinomycin 1 μg	425	0	435	0.4	440	0.1
	+	1.6×10^{-5} M DNP	368	1.5	305	0.6	315	0.6
	+	10^{-4} M DNP	290	0	290	0.7		

[a] The reaction mixture contained 2 mM ATP, 17 mM potassium phosphate, 5 mM $MgSO_4$, 17 mM glucose, yeast hexokinase (type III), and 0.5 ml of a suspension of rat liver mitochondria (originating from 0.5 g liver and containing 1.4–1.8 mg of N). Substrates were present in the following concentrations: DL-α-glycerophosphate, 13.3 mM; succinate, 7 mM; choline, 10 mM; DL-β-hydroxybutyrate, 13.3 mM; glutamate, 10 mM; α-ketoglutarate, 10 mM. All experiments were conducted at 30° and for 8–20 minutes, depending on the rate of substrate oxidation. [b] Oligomycin and aurovertin were added at 2 μg/flask except in those experiments marked with [b], where 4 μg was used. [c] The mitochondria used in experiment 2 were from rats fed 2% desiccated thyroid substance for one week; in all other experiments normal rat liver mitochondria were used.

ethanol and left in the cold overnight. Crystalline potassium phosphate containing some KCl was collected by filtration, washed with ethanol and ether, and dried *in vacuo* at 100° for 1–4 hours. ^{18}O content of the orthophosphate was determined by the method of Williams and Hager (1958).

The atom per cent excess values obtained were corrected for the recycling of ^{18}O (Boyer *et al.*, 1956; Cohn and Drysdale, 1955). It should be noted that the corrective treatments of Boyer and of Cohn[2] are equivalent.

For the determination of $^{32}P_i$-ATP exchange, P_i was separated from the reaction mixture according to the procedure of Hagihara and Lardy (1960). The $^{32}P_i$ exchange observed was corrected for recycling (Boyer *et al.*, 1956) on the assumption that both the β and γ phosphate moieties of ATP freely exchanged with orthophosphate.

[2] In the paper by Cohn and Drysdale (1955), $c^{1/4}$ is printed as 0.799 instead of 0.779.

Antibiotics and other compounds were generous gifts from the following: oligomycins A, B and C, the late Prof. W. H. Peterson, Prof. F. M. Strong, and Dr. George Foster, Department of Biochemistry, University of Wisconsin; rutamycin (Thompson *et al.*, 1961) and stendomycins (Thompson and Hughes, 1963), Dr. Robert Thompson, Eli Lilly and Co.; aurovertin ($C_{26}H_{34}O_9$), Dr. H. A. Nash and C. L. Baldwin, Pitman Moore Div., Dow Chemical Co.; valinomycin, Dr. L. C. Vining, Prairie Regional Laboratory, Saskatoon; 1,1,3-tricyano-2-amino-1-propene, Dr. Floyd Eberts, The Upjohn Co.; BA 180265, Dr. K. V. Rao, Pfizer Laboratories; warfarin and dicoumarol, Prof. Karl Paul Link; SQ 15,859, Dr. D. Perlman, The Squibb Institute. Triiodothyroacetic acid was purchased from Aldrich Chemical Co.; nucleotides, Pabst Laboratories; hexokinase (type III or crystallized), Sigma Chemical Co. Methyl ethers of thyroid hormones were synthesized as described by Tomita *et al.* (1961).

Unless otherwise specified, oligomycin A was employed for all experiments reported in this paper.

Rutamycin is a member of the oligomycin family of compounds, whose effects are similar to oligomycin A, B, and C (H. A. Lardy and P. Witonsky, paper in preparation); it has been recommended to several correspondents as the most readily available form of oligomycin.

Results

Mitochondrial Oxidation and Phosphorylation.—Aurovertin, like oligomycin, is a powerful inhibitor of mitochondrial oxidation because it blocks a reaction in the associated energy-coupling process. The oxidation of α-glycerophosphate, succinate, or choline is inhibited relatively less than that of NAD-linked substrates (Table I) because oxidation of the latter is more tightly coupled with phosphorylation. The oxidation of α-glycerophosphate was measured in liver mitochondria from both normal and thyroid-fed rats since the latter have an abundance of the mitochondrial dehydrogenase for this substrate (Lee *et al.*, 1959; Lardy *et al.*, 1960). The two antibiotics are approximately equally effective, on a weight basis, in blocking oxidation of the substrates shown in Table I and also proline, isocitrate, pyruvate (with or without catalytic amounts of fumarate), and malate. Neither oligomycin nor aurovertin uncouples phosphorylation; when used in amounts less than maximally inhibitory to respiration, near normal P/O ratios are obtained.

The inhibition of oxygen consumption by aurovertin or oligomycin is reversed by a large number of uncoupling agents (Table I). However, agents that stimulate ATPase differ greatly in their effectiveness in reversing this inhibition. Valinomycin and SQ 15,859 release aurovertin and oligomycin inhibition completely. Dicoumarol also very effectively reversed the respiratory inhibition. DNP and TCAP[3] restored aurovertin- or oligomycin-inhibited oxidation with most substrates to the rate achieved with the uncoupler alone. In several experiments, DNP and TCAP were relatively less effective in restoring glutamate oxidation. In these reversal experiments, DNP was employed at a concentration just sufficient to give maximum release of controlled respiration (Lardy and Wellman, 1952), which is about one-sixth the concentration required to elicit maximal ATPase activity (Fig. 1A).

Like oligomycin, aurovertin does not block the substrate-level phosphorylation associated with α-ketoglutarate oxidation. Thus in the presence of DNP to uncouple electron-transport phosphorylation, and of oligomycin to inhibit ATPase, good rates of α-ketoglutarate oxidation may be maintained with P/O values of from 0.5 to 1.0. This represents the substrate-level phosphorylation and is occasionally observed also with glutamate as a substrate because this amino acid may be oxidized via α-ketoglutarate (Borst, 1962; Krebs and Bellamy, 1960).

O-Methyl-Triac, which induces uncoupling and ATPase activity (Tomita *et al.*, 1961), has very little effect in restoring substrate oxidation in the presence of oligomycin or aurovertin.

Usnic acid uncouples phosphorylation but also inhibits respiration (Johnson *et al.*, 1950); it restores oxygen consumption in the presence of oligomycin and aurovertin to the rate with usnic acid alone. Usnic acid, like other uncoupling agents (T. Kagawa, D. Wilkin and H. A. Lardy, submitted to *The Journal of Biological Chemistry*) enhances the oxidation of choline above the rate achievable with glucose and hexokinase as the phosphate-acceptor system.

[3] Abbreviations used in this work: Triac, 3,3',5-triiodothyroacetate; *O*-Me-Triac, o-methyltriiodothyroacetate; TCAP, 1,1,3-tricyano-2-amino-1-propene; DBNP, 2,6-dibromo-4-nitrophenol; DNT, 2,6-dinitrothymol.

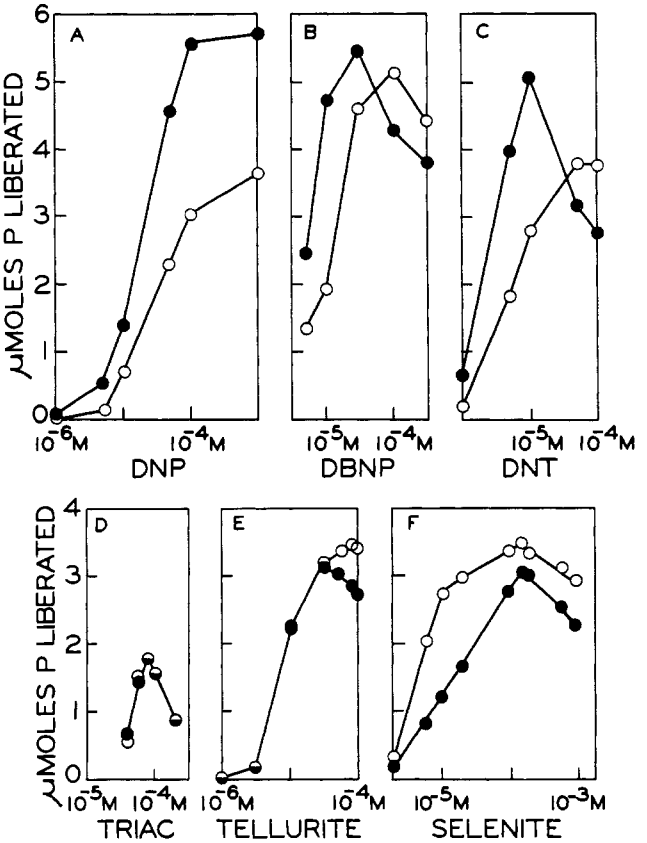


Fig. 1.—Influence of aurovertin on hydrolysis of ATP induced by various agents. The assays were conducted as described in Table II. Solid dots, without aurovertin; open circles, with 2 μg aurovertin/ml. Abbreviations are defined in footnote 3.

Ca^{2+} induces ATPase but, even at 5×10^{-4} M, does not significantly influence glutamate oxidation nor the associated phosphorylation under these experimental conditions. It was completely without effect in restoring oxidation inhibited by either oligomycin or aurovertin.

Both selenite and tellurite induce ATPase activity and both uncouple oxidative phosphorylation. Tellurite restored oligomycin-inhibited oxidation of β-hydroxybutyrate to the rate achieved with tellurite alone, but it was only partially effective in reversing the effect of aurovertin. In contrast, selenite at 5×10^{-5} M only very slightly enhanced respiration in the presence of oligomycin and aurovertin. At 10^{-5} M, selenite is an effective uncoupler of phosphorylation but was completely ineffective in reversing the inhibition by oligomycin or aurovertin.

The effects of oligomycin and aurovertin on mitochondrial oxidations are thus virtually identical.[4] Their effects cannot be differentiated by varying the substrates nor by the various uncoupling agents employed, with the possible exception of tellurite, which is being studied further.

Some additional aspects of respiratory inhibition by these two antibiotics will be presented under Relation of the Phosphorylation Block to Oxidation.

Adenosinetriphosphatase activity of rat liver mitochondria may be induced by a wide variety of agents. We have previously reported (Lardy *et al.*, 1958;

[4] Mitochondria from rat kidney, guinea pig liver and kidney, and from bovine spermatozoa are also equally susceptible to inhibition by oligomycin and aurovertin.

TABLE II
COMPARISON OF AUROVERTIN AND OLIGOMYCIN AS INHIBITORS OF MITOCHONDRIAL ATPASE INDUCED BY VARIOUS AGENTS[a]

Addition		μMoles P_i Liberated/0.2 mg N		
		Control	+ 2μg Oligomycin	+ 2 μg Aurovertin
None		0.23	0.17	0.24
2,4-Dinitrophenol	10^{-4} M	5.60	0.75	2.57
2,4-Dibromophenol	10^{-4} M	4.28	0.54	2.39
2,4,6-Tribromophenol	10^{-4} M	3.56	1.28	3.29
2,6-Dibromo-4-nitrophenol	10^{-4} M	2.99	0.54	4.11
2,6-Dibromo-4-aminophenol	10^{-4} M	0.58	0.15	0.44
2,6-Dinitrothymol	10^{-4} M	3.15	0.52	4.62
2,4-Dinitro-1-naphthol	10^{-5} M	3.91	0.32	2.64
2,4-Dinitrodiphenylamine	10^{-4} M	1.26	0.26	1.0
Dinitromesitylene	10^{-4} M	0.74	0.19	0.60
O-Iodophenol	10^{-4} M	0.44	0.12	0.26
2-Nitrofluorene	10^{-4} M	1.37	0.34	1.04
2-Nitro-*p*-cymene	10^{-4} M	0.60	0.27	0.39
O-Iodosobenzoate	10^{-4} M	1.20	0.11	1.57
3,4-Dichlorobenzoate[b]	10^{-4} M	1.04	0.08	0.53
2,3,5-Triiodobenzoate	10^{-4} M	1.21	0.21	0.71
2-Amino-3,5-diiodobenzoate	10^{-4} M	2.86	0.38	1.37
N-Propyl-3,5-diiodo-4-methoxybenzoate	10^{-4} M	0.74	0.16	0.44
N-Butyl-3,5-diiodo-4-hydroxybenzoate	10^{-4} M	1.28	0.33	1.28
4-Benzyloxy-3,5-diiodobenzoate	10^{-4} M	1.36	0.37	1.32
3,5-Diiodo-3′,5′-dimethoxy-4-*O*-methyl thyroformate	10^{-4} M	0.97	0.27	0.97
3,5-Diiodo-3′,5′-dimethoxy thyroformate	10^{-4} M	1.25	0.23	1.15
3,5-Diiodo-3′,5′-dimethyl thyropropionate	10^{-4} M	1.08	0.19	0.65
3,3′,5-Triiodothyropropionate	10^{-4} M	1.57	0.36	1.40
3,3′,5-Triiodothyronine	10^{-4} M	0.30	0.08	0.29
Warfarin	10^{-4} M	0.50	0.12	0.28
Dicoumarol		2.75	0.57	3.19
	10^{-5} M	3.78	0.28	2.00
TCAP	5×10^{-3} M	4.98		3.80
BA 180265A	1 μg/ml	3.35	0.21	1.60
Stendomycin B	50 μg/ml	1.88	0.28	1.22
SQ 15859[c]	1 μg/ml	4.93	1.03	4.80
Valinomycin	0.5 μg/ml	4.71	0.30	4.06
$CaCl_2$	3×10^{-4} M	2.37	0.0	2.27
	5×10^{-4} M	3.20	0.10	2.31
Palmitate	2×10^{-4} M	0.68	0.07	0.63
Deoxycholate	5×10^{-5} M	1.10	0.08	2.28
	8×10^{-5} M	2.21	0.10	3.45
	10^{-4} M	2.13	0.15	4.93
	5×10^{-4} M	1.94	0.23	2.83
	10^{-3} M	0.41	0.13	1.22
Selenate	10^{-5} M	0.95		2.60
Selenite	10^{-5} M	1.32		2.75
Arsenate	10^{-2} M	0.72	0.20	0.14

[a] The reaction mixture contained 6 mM ATP, 75 mM KCl, 10 mM Tris buffer, *p*H 7.4, and mitochondria from 0.05 g liver in 0.3 ml of 0.25 M sucrose; volume 1 ml; incubated at 30°. The values are average of duplicate determinations which agreed closely within a given experiment. The ATPase elicited by any given concentration of uncoupling agent varied appreciably among different batches of mitochondria. [b] 2,4-Dichlorobenzoate, 2,4-dinitrobenzoate, and 3,5-dinitrobenzoate did not enhance ATPase significantly. [c] SQ 15859 is an antibiotic similar to nonactin (Dutcher, 1961.)

Lardy, 1961) that oligomycin inhibits ATPase induced by these agents while aurovertin is only partially inhibitory with some and completely ineffective with others (Lardy, 1961). Table II summarizes data obtained with about one-half the effective ATPase inducers we have tested. With all inducers, oligomycin blocks 80–100% of the P_i release and aurovertin is far less effective, except with arsenate. In many cases aurovertin actually enhanced ATPase above the rate with the uncoupling agent alone. More detailed investigation disclosed a pattern of responses, typical examples of which are shown in Figure 1 and which may be categorized as follows:

(1) Aurovertin partially inhibits the ATPase induced by DNP over a wide range of concentrations. At low concentrations of DNP, and relatively low ATPase activity, aurovertin inhibits a rather high percentage of the induced activity (Fig. 1A). At 10^{-5}–10^{-4} M DNP, aurovertin at 2 μg/0.2 mg N inhibits about half the induced ATPase activity. Increasing the aurovertin concentration to 10 μg/0.2 mg N enhances the inhibition to about 75% but further increases are not additive. Aurovertin itself does not induce ATPase activity at these relatively high concentrations.

(2) Most inducers exhibit the phenomenon shown in Figure 1B,C for dibromonitrophenol and dinitrothymol. Aurovertin inhibits partially in the range of inducer concentration eliciting increasing ATPase activity. Above the optimum inducer concentration the two curves cross and aurovertin enhances ATP hydrolysis. It is this phenomenon that is responsible for some of the cases of apparent enhancement of P_i liberation by aurovertin in Table II. At extremely

TABLE III
LACK OF INFLUENCE OF PHOSPHORYL-ACCEPTOR SYSTEM ON AUROVERTIN INHIBITION OF OXIDATIVE PHOSPHORYLATION[a]

	Hexokinase + Glucose						Creatine Kinase + Creatine					
	Control		+Rutamycin		+Aurovertin		Control		+Rutamycin		+Aurovertin	
Substrate	$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O	$Q_{O_2}^{(N)}$	P/O
DL-β-Hydroxybutyrate	300	2.7	36	0	35	0	315	1.9	30	0	28	0
α-Ketoglutarate	495	3.6	36	0.7	56	2.1	335	2.3	49	0	60	0.4
L-Glutamate	550	2.9	8	0	27	0	382	1.8	62	0.2	74	0.5
L-Proline	333	2.8	39	0	38	0	234	1.5	39	0	40	0

[a] Conditions as in Table I. Creatine was employed at 16 mM with 0.2 mg creatine kinase. Phosphorus was determined in these experiments by the method of Lowry and Lopez (1946). Both hexokinase and creatine kinase were in excess of the amount required for the obtained rate of P_i uptake.

high concentrations of this type of inducer, the rate of ATP hydrolysis with or without aurovertin falls off nearly to zero.

(3) The ATPase activity induced by most compounds closely related in structure to the thyroid hormones (Table II) is not altered by aurovertin (Fig. 1D) but is completely susceptible to oligomycin. In some experiments, aurovertin inhibits partially the effect of iodinated thyronines and thyroacetates but this behavior is not readily repeatable. With Sq 15859, valinomycin (Lardy, 1961), and palmitate as inducers, aurovertin inhibited only very slightly, placing these compounds in the same general category as the thyroid hormones. The effects of these agents can be differentiated, however, in experiments with varying concentration of alkali metal cations (H. A. Lardy, data to be presented separately).

(4) This fourth category of ATPase induction, thus far found only with tellurite (Fig. 1E), resembles category 3 in the low-concentration range (aurovertin without effect) and category 2 above the concentration that induces maximal ATPase activity (aurovertin enhances activity when high concentrations of the inducer depress).

(5) ATPase induced by selenite (Fig. 1F), selenate, and deoxycholate (Table II) is, at all effective concentrations, enhanced by aurovertin. A more detailed study of selenite and selenate will be presented separately.

(6) Both oligomycin and aurovertin prevent the induction of ATPase by arsenate.

Unlike the similarity between oligomycin and aurovertin demonstrated by the oxidation measurements reported in the previous section, there is a clear differentiation between the effects of these two antibiotics on ATPase. An obvious difference between the two types of assays is the lower concentration of ATP in the oxidative experiments where hexokinase and glucose are present. In testing whether this might influence the effectiveness of aurovertin, we have found that this antibiotic does not inhibit ATP hydrolysis competitively. In a further attempt to determine whether ATP concentration might influence the effectiveness of aurovertin in inhibiting oxidation, experiments were conducted with two different acceptor systems which presumably maintain widely different ratios of ATP/ADP in the mitochondria (Table III). As was shown some years ago (Lardy and Wellman, 1952), the oxidation of most pyridine nucleotide–linked substrates proceeds more slowly during the coupled synthesis of phosphocreatine as compared with the synthesis of glucose-6-phosphate. The oxidation of β-hydroxybutyrate is not so influenced by the type of acceptor (Table III and Lardy and Wellman, 1952). If a higher ATP/ADP ratio is limiting respiration with the former substrates, one must consider the possibility that the rate-limiting net reaction in that case is more exergonic with β-hydroxybutyrate as the substrate. In any event, aurovertin was not less inhibitory to mitochondrial oxidations when creatine was the phosphoryl acceptor, indicating that ATP concentration is probably not correlated with the degree of inhibition by aurovertin.

Exchange Reactions.—The exchanges of $^{32}P_i$ with the phosphate of ATP and of oxygen between water and orthophosphate are considered to represent activities of enzymes involved in oxidative phosphorylation (Cohn, 1953; Boyer *et al.*, 1954, 1956). These exchanges in intact mitochondria and in submitochondrial particles were found (Lardy *et al.*, 1958; Lardy and McMurray, 1959) to be strongly inhibited by oligomycin. Aurovertin is equally effective in inhibiting both these exchange reactions (Fig. 2A,B).

The similarity of the concentration-response curves[5] for the two over-all reactions suggests that they are being blocked at a common point. Yet the ATPase data indicate different sites of action for these two antibiotics. In an attempt to clarify this dilemma, the effect of adding partially effective amounts of oligomycin and aurovertin was measured. If these two agents block at the same site their effects should be additive. If they block at different sites (O and A, respectively), but both are located on a single molecule or assembly, and if binding of the two antibiotics is mutually exclusive, they should also be additive. If the O and A sites are on separate, freely interacting molecules or assemblies, their effects should not be additive. As is shown in Figure 3, they are perfectly additive. Thus it is likely that a phosphorylating assembly that has bound either oligomycin or aurovertin cannot bind the second antibiotic. The perfect additivity further indicates that the O and A sites must be on the same molecule or molecular assembly and incapable of interacting with their counterpart on other molecules or assemblies.

Relation of the Phosphorylation Block to Oxidation.—Oligomycin (Lardy *et al.*, 1958; Huijing and Slater, 1961) and aurovertin inhibit only that portion of mitochondrial oxygen uptake which is tightly coupled to phosphorylation. With freshly prepared liver mitochondria exhibiting good respiratory control, this may be very nearly all the oxidation of pyridine nucleotide–linked substrates (Fig. 2C,D); with succinate, inhibition is much less (Lardy *et al.*, 1958, and Fig. 2E). A comparison of the influence of increasing oligomycin or aurovertin concentration on the exchange reactions and on respiration reveals a striking difference. The exchange reactions (Fig. 2A,B) are inhibited nearly linearly by increasing doses of either oligomycin or aurovertin up to about 1 μg/3ml of reaction mixture (0.9 mg mitochondrial N present). Even 0.1 μg/3ml

[5] The slight difference between the pairs of curves A and B probably results from an overcorrection of the reverse reaction for P_i-ATP exchange in which it is assumed that both β and γ phosphorus atoms participate equally.

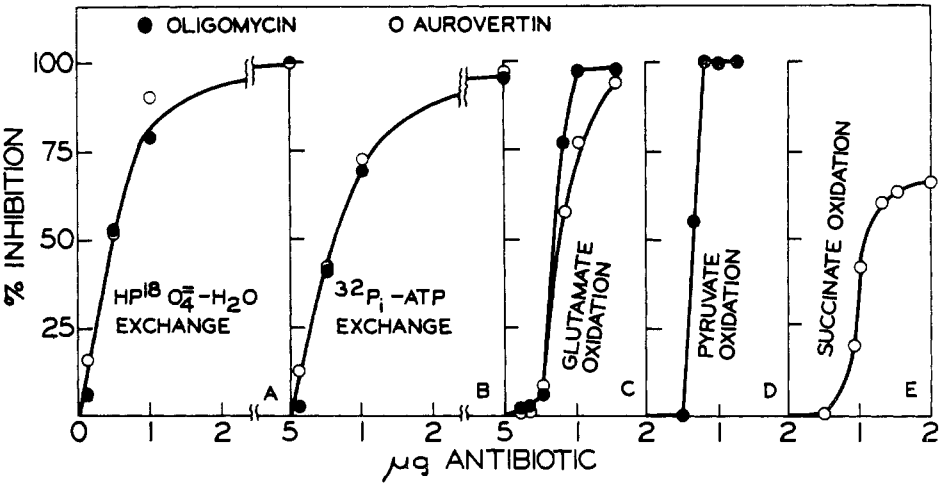


FIG. 2.—Inhibition of exchange reactions and of oxidation in mitochondria by oligomycin and aurovertin. (A) Exchange of ^{18}O between labeled orthophosphate and water. The reaction mixture contained 67 mM sucrose, 50 mM Tris-acetate, *p*H 7.2, 17 mM ATP, 17 mM $MgCl_2$, 30 mM potassium phosphate containing 0.472 atom % excess of ^{18}O. Mitochondria from 300 mg of rat liver in a final volume of 3.0 ml. Incubated 30 minutes at 25°. (B) Conditions as in A except that the orthophosphate contained 69,000 cpm of ^{32}P. (C-E) Respiratory experiments were carried out as described in Table I except that the amount of mitochondria was reduced to correspond to the exchange experiments. This amounted to 0.9 mg N on the average.

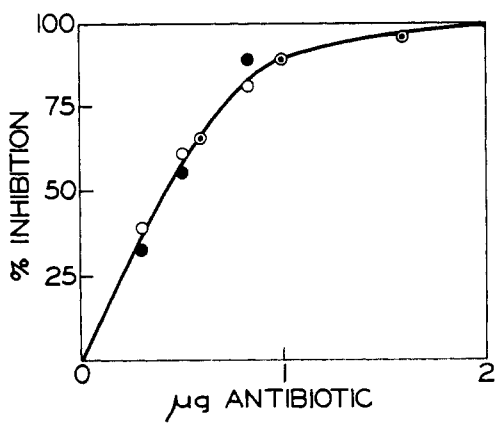


FIG. 3.—Summation of the inhibition by oligomycin and aurovertin of $^{32}P_i$-ATP exchange. Experimental conditions as in Fig. 2B. Solid dots, oligomycin; open circles, aurovertin; dotted circles, a mixture of 50% oligomycin and 50% aurovertin. The completely additive effect of these antibiotics was demonstrated in two additional experiments.

gives a discernible inhibition. In the oxidation assays (Fig. 2C,D,E), here conducted with the same concentration of mitochondria as the exchange reactions, the first 0.5 μg of antibiotic is completely, or nearly completely, without effect (cf. also Ernster *et al.*, 1963). As the concentrations of antibiotics are increased beyond the minimally effective amounts, inhibition is rapidly increased to the maximum level achievable. Thus it appears that about half the exchange activity may be inhibited without affecting oxidation significantly. If the exchanges measured are indeed a reflection of the action of enzymes that participate in oxidative phosphorylation, it may be concluded that the enzyme molecules of the respiratory chain are not bound irreversibly to a given set of phosphorylating enzyme molecules as represented by Green *et al.* (1963). Were this the case, respiration would also be inhibited linearly with increasing concentrations of oligomycin and aurovertin. If the exchange reactions are totally representative of oxidative phosphorylation reactions, and if there is any aggregation or assembly of the enzymes that participate in this process, the data indicate that the respiratory enzymes must be free to interact with any of the assemblies of phosphoryl-transferring enzymes. On the other hand, the data may also be interpreted as indicating that the half of the exchange activity with greatest affinity for oligomycin and aurovertin is not associated with oxidative phosphorylation. We have presented arguments (under Exchange Reactions) in support of the concept that the assemblies of phosphoryl-transferring enzymes do not freely interact with one another when individual steps of the over-all phosphoryl-transfer system are inhibited by oligomycin and/or aurovertin.

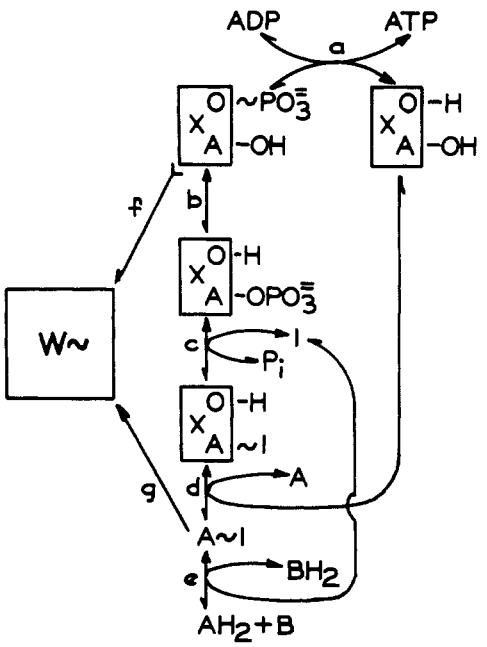
DISCUSSION

The data presented in this and other papers (Connelly and Lardy, 1964a,b) are difficult to explain in terms of current concepts of oxidative phosphorylation and the evidence suggesting (De Luca and Engstrom, 1961; Vasington and Murphy, 1962; Chappell *et al.*, 1962; Lehninger, 1962; Brierly *et al.*, 1963; Chance, 1963) that ion transport in mitochondria and the swelling-concentration phenomena are accomplished by high-energy intermediates of oxidative phosphorylation.

The points most difficult to rationalize may be enumerated as follows: Oligomycin inhibits and aurovertin fails to inhibit (Connelly and Lardy, 1964a,b) (1) the effect of ATP in preventing phosphate-induced swelling at *p*H 7.4; (2) the utilization of ATP for contraction of mitochondria swollen by thyroxine at *p*H 7.4; and (3) the utilization of ATP to support swelling of mitochondria at *p*H 6.1. (4) The hydrolysis of ATP (Table II) induced by a wide variety of agents that promote mitochondrial swelling is blocked by oligomycin but not by aurovertin; (5) most of these same agents reverse the inhibition of mitochondrial oxidation by *either* oligomycin or aurovertin. (6) Substances like $CaCl_2$, iodinated thyronines and compounds related in structure but with shorter side chains, and BA 180265A are relatively weak uncouplers of oxidative phosphorylation yet they induced ATP hydrolysis. This hydrolysis is completely susceptible

to oligomycin inhibition but only slightly, or not at all, to aurovertin. These substances do not catalytically reverse respiratory inhibition by either oligomycin or aurovertin. (7) The uncoupling of oxidative phosphorylation by valinomycin (Moore and Pressman, 1964), gramicidin, and Sq 15859, and the induction of ATPase by these agents as well as BA180,265A, is dependent on the presence of K^+ or in some cases on Na^+ (H. A. Lardy, data to be presented separately). A *portion* of the DNP-stimulated ATP hydrolysis in liver mitochondria is also dependent on Na^+ or K^+ (Lardy and Wellman, 1953; Myers and Slater, 1957). (8) The concentration of DNP required for maximal enhancement of mitochondrial oxidation in the absence of phosphate acceptor (10^{-5} M, Lardy and Wellman, 1952) is significantly less than that required to induce maximal rates of ATP hydrolysis (Fig. 1A).

A description of possible events, consistent with experimental findings, is presented in Scheme I. Double-barbed arrows indicate the direction of reactions during oxidative phosphorylation; single-barbed arrows the direction for ATP utilizing systems. The scheme depicts the sequence of reactions currently accepted for oxidative phosphorylation by several investigators (see Ernster and Lee, 1964) with some specific stipulations concerning the sites of inhibition by oligomycin and aurovertin. Furthermore, it proposes a separate category of high-energy intermediates (collectively labeled W), involved in ion transport, swelling, and contraction phenomena, that is distinct from the intermediates of oxidative phosphorylation. These W intermediates may be synthesized with the aid of energy derived from either electron transport or ATP.



Scheme 1

Reaction (a) is intended to indicate the sequence between ATP and the reaction inhibited by oligomycin. The data of Table II and Figure 3 indicate that this antibiotic must combine at a site different from, but close to, that bound by aurovertin, and both antibiotics must prevent loss of ^{18}O from orthophosphate. Oligomycin could combine with the phosphorylated protein, X~P, to prevent phosphoryl transfer to any acceptor, while aurovertin could block reactions (b) or (c). There is some evidence that inhibition of reaction (a) by oligomycin may depend on the extent to which a specific structural integrity is maintained (Kulka and Cooper, 1962; Wadkins, 1962; Wadkins and Lehninger, 1963). In keeping with this is the finding (S. Wang and H. A. Lardy, unpublished data) that a partially purified preparation of the solubilized DNP-stimulated ATPase of rat liver mitochondria (Lardy and Wellman, 1953) retained sensitivity to aurovertin but was not inhibited by oligomycin. This enzyme is cold-labile like the oligomycin-insensitive F_1 factor of Pullman *et al.* (1960). In the scheme X is depicted as possessing two sites for binding phosphate. Reactions (c) plus (b) could effect the exchange of ^{18}O between water and P_i, provided that OH^- can dissociate from the A site. The rate of the ^{18}O exchange is considerably greater than that of P_i-ATP exchange (Boyer *et al.*, 1956), yet the effect of both oligomycin and aurovertin is to inhibit each of these exchanges equally on a percentage basis. This is consistent with a process involving at least two steps, one of which accomplishes ^{18}O exchange and the second completes the ^{32}P exchange into ATP. If the loss of ^{18}O from the A site of $X^{18}OH$ were not extremely rapid relative to the rate of the ADP-ATP exchange reaction, ^{32}P exchange could occur without loss of ^{18}O. Partial retention of the ^{18}O bound at A could account for the small amount of ^{18}O transferred from arsenate to P_i in the experiments of Itada and Cohn (1963).

Agents that uncouple oxidative phosphorylation like DNP presumably react at the primary energy-coupling reaction (e) or at (d) but prior to the step where phosphate is fixed (Lardy and McMurray, 1959; Huijing and Slater, 1961). A concentration of 10^{-5} M DNP is sufficient to obtain maximal uncoupling in this region.

Reaction (f) designates the sum of reactions that utilize energy from ATP for mitochondrial swelling or contraction (points 1–3 above) and for ion transport. It represents also phosphoryl transfer enhanced by a variety of agents that induce ATPase but do not uncouple oxidative phosphorylation (point 6). It is active in all ATPase activities not inhibitable by aurovertin including the Mg^{2+} stimulated ATPase of aged mitochondria (Lardy and Wellman, 1953; Lardy, 1961). The relationship of this ATPase activity to cations will be presented separately. Agents like DNP and others whose effects are partially inhibited by aurovertin (Table II) probably affect ATP hydrolysis via hydrolysis of X~P and by discharging intermediate like A~I. The portion of ATP hydrolysis inhibited by aurovertin is probably representative of that occurring at the energy-transduction step associated with electron transport. Aurovertin, by blocking reaction (b) or (c), prevents transmission of the energy to this uncoupling site. The fact that most uncoupling agents are effective in discharging energy both in the vicinity of A~I (maximum effect with 10^{-5} M DNP) and via reaction (f) (maximum effect with 10^{-4} M DNP) leads to the suggestion that the catalysts involved in coupling electron transfer to ~P generation may be chemically similar to those involved in transferring energy from X~P to W. The former has, however, a greater affinity for DNP.

The energy required for formation of the W intermediates may be derived from electron transfer in the presence of either oligomycin or aurovertin (Connelly and Lardy, 1964a,b). This is presumed to be delivered via (g). Route (g) is designated as leaving the oxidative phosphorylation sequence prior to the participation of X since the latter is presumably bound by oligomycin and aurovertin in such a manner as to prevent its reacting with P_i. Fang *et al.* (1963; Fang and Rasmussen, 1964), have shown that parathyroid hormone enhances respiration of oligomycin-blocked mitochondria. This is presumed to occur because this hormone facilitates utilization of A~I for the generation of intermediates involved in ion transport. In

keeping with this concept, Sallis and DeLuca (1965) find that the hydrolysis of ATP induced by parathyroid hormone is strongly inhibited by aurovertin. That parathyroid hormone–stimulated ion transport does not utilize energy from ATP via reaction (f) may be deduced from the data of Rasmussen *et al.* (1964). Reaction (f) must not be reversible under the experimental conditions used or it would provide a by-pass for oxidative phosphorylation in the presence of aurovertin.

The site at which arsenate uncouples oxidative phosphorylation has remained obscure (Itada and Cohn, 1963; Ernster and Lee, 1964). Arsenate does not induce ATPase in the presence of oligomycin or aurovertin and respiratory inhibition by these agents is not relieved by arsenate (Table II, and Lardy, 1961; Huijing and Slater, 1961; Estabrook, 1961). This leads us to postulate that spontaneous hydrolysis of the high-energy arsenoyl intermediate occurs above reaction (b), possibly not until adenosine diphosphoarsenate is formed. If all carboxyl-arsenic anhydrides are as labile as that which is formed during triose phosphate oxidation in the presence of arsenate, it is unlikely that a carboxyl phosphate is an intermediate in oxidative phosphorylation, except possibly at the step immediately prior to ATP formation.

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