EFFECT OF AUROVERTIN ON ENERGY-LINKED PROCESSES RELATED TO OXIDATIVE PHOSPHORYLATION

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The effects of inhibitors on oxidative phosphorylation and on energy-linked reactions in mitochondria have provided a powerful tool for probing the mechanisms of these reactions (1). Among many "uncouplers" and "inhibitors" (2), the antibiotic, aurovertin (3), has become of particular interest. The studies of Lardy and his co-workers (4) (5) on the action of aurovertin have differentiated this inhibitor from oligomycin in respect to mode of action. Although both compounds inhibited oxidative phosphorylation in rat liver mitochondria, oligomycin, but not aurovertin, inhibited mitochondrial swelling and contraction, and some other reactions connected with ion transport, when these reactions were driven by ATP.

The present communication is concerned with some quantitative aspects of the effect of aurovertin on oxidative phosphorylation and on energy-linked reactions observed for the most part in submitochondrial particles.

The effect of aurovertin on translocation of Ca^{++} has been studied in HBHM*; on oxidative phosphorylation in ETP_H (6); on energy-linked transhydrogenation between pyridine nucleotides in ETP_H; and on energy-linked reduction of DPN by succinate in ETP_H.

Table I documents the inhibitory effect of aurovertin on oxidative phosphorylation. At a concentration of 2 μg per mg of protein aurovertin inhibited oxidative phosphorylation at all three sites. However, auro-

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*Abbreviations used are: HBHM, heavy beef heart mitochondria; ETP_H, phosphorylating submitochondrial particle; PMS, phenazine methosulfate; TMPD, (N,N,N',N') tetramethyl-p-phenylenediamine hydrochloride.

TABLE I

Effect of Aurovertin on Oxidative Phosphorylation in ETP_H

Substrate	Additions	O_2 µatoms	P_i µmoles	P/O
DPNH	None	5.42	9.28	1.70
	Aurovertin, 2 µg	4.76	0.56	0.12
Succinate	None	8.47	6.08	0.72
	Aurovertin, 2 µg	6.64	0.29	0.06
Ascorbate + PMS	None	10.57	5.61	0.53
	Aurovertin, 2 µg	10.24	0.44	0.04

Oxidative phosphorylation was measured in Warburg vessels at 37° for 20 min. The vessels contained in a total volume of 3.0 ml: sucrose, 750 µmoles; glucose, 50 µmoles; ^{32}P potassium phosphate (pH 7.4, about 25,000 cpm/µmole) 20 µmoles; $MgCl_2$, 15 µmoles; ADP, 5 µmoles; crystalline hexokinase, 100 µg; and ETP_H, 1 mg of protein. DPNH was generated in the system by the following components: DPN, 0.5 µmole; alcohol dehydrogenase, 0.5 mg; semicarbazide, 15 µmoles; and ethanol, 100 µmoles. Other substrates were: either succinate, 50 µmoles; or ascorbate, 40 µmoles, plus PMS, 3 mµmoles. After a 5 min period of thermal equilibration the reactions were initiated with the substrates.

The amount of P_i esterified was measured by counting the radioactivity in the lower phase after partition of the phosphates according to Lindberg and Ernster (9).

vertin had no (or very little) inhibitory effect on transhydrogenation even when present at very high levels (Table II) Moreover at a concentration of 2 µg per mg of protein, aurovertin inhibited by only 43% the ATP-mediated reduction of DPN by succinate, whereas at the same concentration it inhibited oxidative phosphorylation almost completely. The rate of reduction of DPN, mediated by the high energy intermediate(s) produced at the third site of oxidative phosphorylation, was not influenced by the presence of aurovertin (Table II). The translocation of Ca^{++} in HBHM was partially inhibited by aurovertin (high levels) when supported by ATP, but not when supported by the oxidation of succinate (Table III). At a concentration of 10 µg per mg of protein, aurovertin

TABLE II

Effect of Aurovertin on Transhydrogenation and Reduction of DPN by Succinate in ETP_H

Additions	Transhydrogenation		Reduction of DPN	
	Driven by ATP	Driven by succinate	Driven by ATP	Driven by ascorb + TMPD*
Exp. 1 - None	73	119	65	35
Oligomycin, 10 μg/mg	8**	119	0	-
Aurovertin, 2 μg/mg	-	-	37	-
Aurovertin, 20 μg/mg	67	128	0	32
Exp. 2 - None	83	94	105	61
Oligomycin, 10 μg/mg	26**	-	0	-
Aurovertin, 20 μg/mg	88	118	5	54

Transhydrogenation was measured spectrophotometrically at 37° according to Danielsson and Ernster (8). The assay system employed ethanol and alcohol dehydrogenase for the regeneration of DPNH (8). Reduction of DPN by succinate was measured spectrophotometrically at 37° according to Vallin and Low (10), except in the reaction driven by ATP in Experiment 1; in this case the procedure of Sanadi and Fluharty (11) was followed.

The results are expressed as μmoles of TPN reduced per min per mg of protein for the transhydrogenation reaction, and as μmoles of DPN reduced per min per mg of protein for the reduction of DPN by succinate.

*The experiments were performed in the presence of oligomycin.

**The rate in presence of oligomycin is considered to be the rate of the conventional non-energy dependent transhydrogenation.

TABLE III

Effect of Aurovertin on Ca^{++} Translocation in HBHM

Additions	Driven by ATP	Driven by succinate
None	159	182
Aurovertin, 2.5 μg/mg protein	128	200
Aurovertin, 5 μg/mg protein	101	192
Aurovertin, 10 μg/mg protein	87	188

The results are expressed as μmoles of Ca^{++} accumulated per min per mg of HBHM protein. Ca^{++} translocation was assayed at 37° according to Brierley et al. (12). The level of $^{45}CaCl_2$ used was 0.66 mM, the time of assay being 2 minutes.

inhibited the translocation reaction by 45% in the ATP-driven system. Figure 1 describes the extent to which the different reactions were affected by aurovertin. The different ATP-supported reactions showed a scalar sensitivity to the inhibitor. However, an exact comparison between the effects of aurovertin on Ca^{++} translocation and on the other processes studied can not be made because the former was observed in intact mitochondria, the latter in ETP_H. The discrepancy between the concentration required for complete inhibition of oxidative phosphorylation and that required for complete inhibition of the ATP-driven reduction of DPN by succinate, cannot be ascribed to a dilution effect, i.e. to the actual concentrations of inhibitor on a volume basis. In experiments in which the protein concentration ranged between 0.25 and 2 mg per flask, the lowest concentration of aurovertin which inhibited oxidative phosphorylation (90% inhibition) was in all cases about 1 μg per mg of protein.

The succinate-linked reduction of DPN, and the DPNH-linked reduction of TPN, can be differentiated on the basis of their varied susceptibilities to aurovertin. This, together with other observations (1) probably excludes the possibility that a common intermediate containing DPN is involved in both types of reactions (7).

These results are in agreement with the data of Lardy[c] on the reduction of endogenous DPN in intact mitochondria: the reaction was not inhibited by aurovertin when the energy was supplied by electron transfer, while the reaction driven by ATP was affected differently depending on the assay conditions.

We recognize that these observations summarized in Fig. 1 could be interpreted in terms of the model suggested by Lardy (4) (5), in which the "work functions" are not in the main path leading to phosphorylation. (Lardy's scheme implies that when "work functions" are energized by ATP the acceptor

[c] Personal communication.

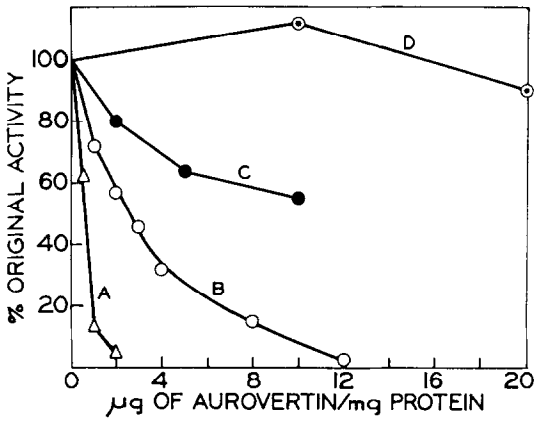

Fig. 1. Effect of Aurovertin on energy-linked functions. Details of the experimental procedures are given in the legends of Tables I, II and III. Curve A (Δ——Δ), oxidative phosphorylation (DPNH as substrate); Curve B (O——O), reduction of DPN by succinate, driven by ATP; Curve C (●——●), translocation of Ca^{++} driven by ATP; Curve D (⊙——⊙), transhydrogenation between pyridine nucleotides, driven by ATP.

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system does not interact directly with ATP but with an intermediate towards the ATP end of the phosphorylating pathway. An alternative scheme could be postulated where each acceptor system interacts directly with ATP). However, whatever version of this type of model is postulated, the observations presented here would now require that within each of the acceptor systems there is an aurovertin sensitive site (with the possible exception of the transhydrogenation system). The variable (scalar) sensitivity to aurovertin would then merely reflect differences in the sensitivities of these various sites. Another possible interpretation of the scalar sensitivity to aurovertin is based purely on kinetic considerations. The same high energy intermediate on the main path of oxidative phosphorylation would energize all the acceptor systems. Aurovertin would affect the kinetics of the interaction of the common high energy intermediate with the different acceptor systems. This interaction would take the form of a competitive inhibition. The site of inhibition would be the reaction generating the common high energy intermediate from the ATP side; and the extent of inhibition would depend on the relative affinities of

the energy-utilizing systems for the intermediate thus generated. According to Danielsson and Ernster (8) "the DPN-linked TPN reduction possesses a particularly low requirement" for the high energy donor in comparison with the succinate-linked reduction of DPN. We have to bear in mind however that Lardy found that aurovertin does not inhibit ATP hydrolysis competitively (4). Further study is needed to elucidate which of the possible interpretations is correct.

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