A Comparison of the Effects of *NN'*-Dicyclohexylcarbodi-imide, Oligomycin A and Aurovertin on Energy-Linked Reactions in Mitochondria and Submitochondrial Particles

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1. The effects of dicyclohexylcarbodi-imide, oligomycin A and aurovertin on enzyme systems related to respiratory-chain phosphorylation were compared. Dicyclohexylcarbodi-imide and oligomycin A have very similar functional effects, giving 50% inhibition of ATP-utilizing and ATP-generating systems at concentrations below 0.8 nmole/mg. of submitochondrial-particle protein. Aurovertin is a more potent inhibitor of ATP synthesis, giving 50% inhibition at 0.2 nmole/mg. of protein. However, aurovertin is a less potent inhibitor of ATP-utilizing systems: the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase is 50% inhibited at 3.0 nmoles/mg. of protein and the ATP-driven reduction of NAD⁺ by succinate is 50% inhibited at 0.95 nmole/mg. of protein. 2. With EDTA-particles (prepared by subjecting mitochondria to ultrasonic radiation at pH 9 in the presence of 2 mM-EDTA) the maximum stimulation of the ATP-driven partial reactions is effected by similar concentrations of oligomycin A and dicyclohexylcarbodi-imide, but the latter is less effective. The stimulatory effects of suboptimum concentrations of dicyclohexylcarbodi-imide and oligomycin A are additive. Aurovertin does not stimulate these reactions or interfere with the stimulation by the other inhibitors. 3. Dicyclohexylcarbodi-imide and oligomycin A stimulate the aerobic energy-linked nicotinamide nucleotide transhydrogenase of EDTA-particles, but the optimum concentration is higher than that required for the ATP-driven partial reactions. Aurovertin has no effect on this reaction. 4. The site of action of dicyclohexylcarbodi-imide is in CF₀, the mitochondrial fraction that confers oligomycin sensitivity on F₁ mitochondrial adenosine triphosphatase.

Aurovertin, DCCD† and the oligomycins A, B, C and D (rutamycin) have been shown to inhibit the synthesis of ATP associated with mitochondrial electron transport (Lardy, Connelly & Johnson, 1964; Beechey, Holloway, Knight & Robertson, 1966; Lardy, Johnson & McMurray, 1958; Lardy, Witonsky & Johnson, 1965), and also to inhibit those partial reactions of oxidative phosphorylation that are driven by ATP, e.g. ATP-driven reduction of NAD⁺ by succinate, ATP-driven energy-linked nicotinamide nucleotide transhydrogenase, uncoupler-

stimulated ATPase, [³²P]P_i-ATP exchange etc. The sites of action of these inhibitors have been postulated to be at, or near, the level of the hypothetical phosphorylated high-energy intermediate that is thought to react with bound ADP to form ATP. It seems that DCCD and oligomycin exert their effects at the same site (Beechey, Robertson, Holloway & Knight, 1967a). However, the relative position of the sites of inhibition by oligomycin and aurovertin is not yet clear. Lardy *et al.* (1964) suggested that oligomycin acts on the ATP side of the aurovertin site of inhibition, on the basis of results showing quantitative differences between the effects of the two inhibitors on the ATPase activities stimulated in mitochondria by uncoupling and swelling agents. Ernster, Lee & Janda (1967), however, postulated that it is aurovertin that acts closer to the reaction that catalyses ATP synthesis. This conclusion was based on the observations that, in contrast with oligomycin, (1) aurovertin is a less potent inhibitor of ATP-driven partial reactions

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‡ Abbreviations: DCCD, dicyclohexylcarbodi-imide; SMP, submitochondrial particles; HBHM, heavy-layer ox heart mitochondria; HBHM(N), heavy-layer ox heart mitochondria prepared by the Nagarse proteinase method; HBHM(B), heavy-layer ox heart mitochondria prepared by the blender method; ATPase, adenosine triphosphatase.

(Lenaz, 1965) (this might explain the findings of Lardy *et al.* 1964, above), (2) aurovertin is unable to stimulate energy-linked reactions in EDTA-particles and (3) aurovertin does not inhibit NADH oxidase activity in EDTA-particles. Further support for this location of aurovertin action comes from the finding that aurovertin inhibits the ATPase activity of the coupling factor, F_1 , which is thought to catalyse the terminal reaction of oxidative phosphorylation (Kagawa & Racker, 1966a; Robertson, Beechey, Holloway & Knight, 1967), and from the unpublished work of H. A. Lardy & B. Chance (quoted by Ernster *et al.* 1967) that aurovertin combines with and inhibits F_1 .

In this paper we have compared quantitatively the amounts of aurovertin, DCCD and oligomycin A required to inhibit various energy-linked reactions in an attempt to establish whether they act at the same or different sites. The hypothetical mechanism proposed for the reaction sequence that they inhibit is discussed.

METHODS

Molecular weights of inhibitors. The molecular formulae and molecular weights of the three inhibitors used in this study are as follows: aurovertin, mol. wt. 476, formula $C_{25}H_{32}O_9$; DCCD, mol. wt. 206, formula $C_{13}H_{22}N_2$; oligomycin A, mol. wt. 446, formula $C_{28}H_{46}O_4$ (Beechey, Williams, Holloway, Knight & Robertson, 1967b).

Methods of addition of inhibitors. The inhibitors were dissolved in either ethanol or dimethylformamide. Samples of oligomycin A and aurovertin were added to the assay system containing mitochondrial protein 3 min. before the final addition of a substrate. Samples of DCCD were added to suspensions of mitochondrial protein 16–24 hr. before assays were carried out. At the end of this preincubation at 0°, a sample of mitochondrial protein containing DCCD was pipetted into the assay system followed after 3 min. by substrate (Beechey *et al.* 1967a).

Assays. The conditions for assay of respiration rates, ATP-driven reduction of NAD^+ by succinate, ATP-driven energy-linked nicotinamide nucleotide transhydrogenase, aerobically driven energy-linked nicotinamide nucleotide transhydrogenase and non-energy-linked nicotinamide nucleotide transhydrogenase have been described by Beechey *et al.* (1967a). The ATPase assay used was based on the method of Pullman, Penefsky, Datta & Racker (1960). Assay conditions are described in the legends to the Tables and Figures.

Preparation of mitochondria, SMP and EDTA-particles. The preparation of mitochondria and SMP from ox heart has been described by Beechey *et al.* (1967a). EDTA-particles were prepared by the method of Lee, Azzone & Ernster (1964) from HBHM. The HBHM had been prepared the previous day either by the Nagarse proteinase method described by Beinert (1964) or by the blender method of Sanadi & Fluharty (1963), and had been stored in 0.25 M-sucrose–10 mM-tris sulphate buffer, pH 7.7, at 0° overnight. EDTA was added to a final concentration of 2 mM and the pH was adjusted to 9. The mitochondrial suspension was then subjected to ultrasonic radiation. The

resulting submitochondrial particles, EDTA-particles, were isolated by differential centrifugation. They were suspended in a solution containing 0.25 M-sucrose–10 mM-tris sulphate buffer, pH 7.7.

Cytochrome $a + a_3$ estimation. A solution containing SMP (10–15 mg. of protein), 10 μ g. of antimycin A, 300 μ moles of tris sulphate buffer, pH 7.6, and 15 mg. of sodium cholate was made up to 3 ml. with water at room temperature (Blair, Oda, Green & Fernandez-Moran, 1963). The solution was divided equally between two 1 cm.-light-path spectrophotometer cells, and a difference spectrum was recorded from 630 m μ to 605 m μ before and after the addition of $Na_2S_2O_4$ to one cell. A Cary 14 recording spectrophotometer was used with a 0.0–1 extinction slidewire. The difference in extinction at 605 m μ was measured assuming that the extinction at 630 m μ was unaffected by the addition of $Na_2S_2O_4$. The haem a content in the cytochrome $a + a_3$ was calculated on the basis of ΔE_{max} (reduced minus oxidized) at 605 m μ 12.0 cm.²/ μ mole (Van Gelder & Slater, 1963).

Assay of protein. Protein was estimated colorimetrically by the biuret method of Gornall, Bardawill & David (1949), after solubilization of the protein with 1.5% (w/v) potassium cholate.

Soluble ATPase preparation. Soluble ATPase from an acetone-dried powder of mitochondria was purified to the stage of the second $(NH_4)_2SO_4$ fractionation by the method of Selwyn (1967). It was stored in 50% (v/v) glycerol at –20° until used. During the preparation, one half of the supernatant material obtained after centrifugation of the homogenized acetone-dried powder was treated overnight with 5 nmole of DCCD/mg. of protein at room temperature. This material was then fractionated with $(NH_4)_2SO_4$, as was the control material.

Preparation of CF_0 . CF_0 was prepared by the method of Kagawa & Racker (1966b). At the T-particle stage of the preparation, one half of the material was treated overnight with 5 nmole of DCCD/mg. of protein and subsequently fractionated in the same way as the control material.

RESULTS

Effect of inhibitors on ADP-stimulated respiration. The concentrations of inhibitors required to inhibit ADP-stimulated respiration in HBHM(N) are shown in Fig. 1. Aurovertin gives more than 90% inhibition at a concentration of 0.35 nmole/mg. of protein, oligomycin A at 0.55 nmole/mg. of protein and DCCD at 1.0 nmole/mg. of protein.

Effect of inhibitors on the ATP-driven reduction of NAD^+ by succinate. The data illustrated in Fig. 2 show that ATP-driven reduction of NAD^+ by succinate catalysed by SMP is more than 90% inhibited by oligomycin A and DCCD at concentrations of 0.6 and 0.8 nmole/mg. of protein of SMP respectively. The reaction is much less sensitive to aurovertin, however, which gives 87% inhibition at 3 nmole/mg. of protein. Other experiments have shown that aurovertin completely inhibits the reaction at 5 nmole/mg. of protein.

Effect of inhibitors on the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase. The

Fig. 1. Inhibition of the ADP-stimulated respiration of mitochondria. Different amounts of DCCD dissolved in ethanol were added to 1 ml. samples of HBHM(N) containing 30 mg. of protein. The mitochondria, which were suspended in 0.25 M-sucrose-10 mM-tris sulphate, pH 7.7, were incubated at 0° overnight. Untreated HBHM(N) were also stored overnight at 0° and used for experiments with aurovertin and oligomycin A, which were added at the time of assay. Respiration rates were measured in an oxygen electrode: 0.1 ml. of mitochondrial suspension was added to an air-saturated reaction mixture containing (final concns.) 0.25 M-sucrose, 7.5 mM-potassium phosphate buffer, pH 7.4, 10 mM-MgSO₄, 2.5 mM-potassium L-glutamate, 2.5 mM-potassium L-malate and 5 mM-potassium malonate. Aurovertin or oligomycin was added as a solution in less than 5 μ l. of ethanol. The total volume was 4.16 ml. and the temperature 30°. The respiration rate was measured for 3 min., and then 20 μ l. of 40 mM-ADP was added and the respiration rate was measured again. The differences between the respiration rates in the presence of ADP (state 3; Chance & Williams, 1955) and in the absence of ADP (state 4) are plotted against the inhibitor concentrations present in the assays. $\blacktriangle$, Aurovertin; $\bullet$, oligomycin A; $\square$, DCCD.

results presented in Fig. 3 show the effect of the three inhibitors on the energy-linked nicotinamide nucleotide transhydrogenase driven by ATP in SMP. Higher concentrations of inhibitors are required to inhibit this reaction than to inhibit the ATP-driven reduction of NAD⁺ by succinate. About 1.6 nmol of oligomycin A/mg. of protein and 1.2 nmol of DCCD/mg. of protein cause 90% inhibition of this transhydrogenase reaction. Aurovertin is a much weaker inhibitor; at 20 nmol/mg. of protein it causes 80% inhibition of the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase. The non-energy-linked nicotinamide nucleotide transhydrogenase system is not affected by these inhibitors.

Effect of inhibitors on the ATPase activity of uncoupled HBHM(N). The effects of the three

Fig. 2. Inhibition of ATP-driven reduction of NAD⁺ by succinate in SMP. In each assay, 1.37 mg. of protein of SMP was used. DCCD was preincubated overnight with the SMP. Aurovertin and oligomycin A were incubated with the SMP in the assay cuvette for 3 min., and then ATP was added to start the reaction. $\blacktriangle$, Aurovertin; $\bullet$, oligomycin A; $\square$, DCCD.

Fig. 3. Inhibition of ATP-driven energy-linked nicotinamide nucleotide transhydrogenase in SMP. In each assay 1.12 mg. of protein of SMP was used. DCCD was preincubated overnight with the SMP. Aurovertin and oligomycin A were incubated with the SMP in the assay cuvette for 3 min.; then NADP⁺ was added, followed 1 min. later by ATP to start the reaction. The non-energy-linked transhydrogenase (----) has not been subtracted, and is not affected by inhibitors. $\blacktriangle$, Aurovertin; $\bullet$, oligomycin A; $\square$, DCCD.

inhibitors on the ATPase activity of HBHM(N) induced by the uncoupling agent 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (Beechey, 1966) are shown in Fig. 4. Maximum inhibition, usually greater than 85%, of the ATPase activity is observed in the presence of 0.6 nmol of oligomycin A/mg. of protein or 0.45 nmol of DCCD/mg. of protein. In contrast with this, the maximum inhibition caused by aurovertin is just over 50%, and this at 50 nmol/mg. of protein.

Fig. 4. Inhibition of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole-stimulated ATPase in HBHM(N). DCCD was preincubated overnight with the mitochondria. Aurovertin and oligomycin A were incubated with the mitochondria in the assay cuvette, as described below. Mitochondria (0.3 mg. of protein) were preincubated at 30° for 3 min. in a solution containing 0.25 M-sucrose, 25 μ moles of tris sulphate buffer, pH 7.4, 0.3 μ mole of NADH, 1 μ mole of KCN, 50 μ g. of lactate dehydrogenase, 25 μ g. of pyruvate kinase and inhibitors at various concentrations in a volume of 0.84 ml. After 3 min. 0.1 nmole of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole was added. After a further 0.5 min. a prewarmed solution containing 2 μ moles of ATP, 2 μ moles of MgSO₄, 2 μ moles of phosphoenolpyruvate and 50 μ moles of tris sulphate buffer, pH 7.4, in 0.16 ml. was added to start the reaction. The NADH oxidation was followed spectrophotometrically. ▲, Aurovertin; ●, oligomycin A; □, DCCD.

Effects of inhibitors on EDTA-particles

Oligomycin A and DCCD stimulation of ATP-driven reduction of NAD⁺ by succinate in EDTA-particles. The observation that P/O ratios and ATP-driven reduction of NAD⁺ by succinate may be stimulated in EDTA-particles by the addition of low concentrations of oligomycin, rutamycin or DCCD has been made in many Laboratories (Lee & Ernster, 1965; Fessenden & Racker, 1966; Lam, Warshaw & Sanadi, 1966; Beechey *et al.* 1967a). Some typical results, showing the effect of adding low concentrations of inhibitors to EDTA-particles, are shown in Fig. 5(a). The EDTA-particles used in this experiment were prepared from HBHM(B). The control rate of ATP-driven reduction of NAD⁺ by succinate, 6 nmol of NAD⁺ reduced min.⁻¹ mg.⁻¹ of protein, is stimulated to a maximum rate of 18 nmol of NAD⁺ reduced min.⁻¹ mg.⁻¹ of protein by 0.2 nmole of oligomycin A/mg. of protein and to 9.5 nmol of NAD⁺ reduced min.⁻¹ mg.⁻¹ of protein by 0.2 nmole of DCCD/mg. of protein.

Fig. 5. Effect of inhibitors on ATP-driven reduction of NAD⁺ by succinate in EDTA-particles. Assay conditions were the same as those described in Fig. 2, except that the Mg²⁺ concentration was 2 mM. (a) The EDTA-particles were prepared from mitochondria isolated by the blender method. In each assay, 1.45 mg. of protein of particles was used. (b) The EDTA-particles were prepared from mitochondria isolated by the proteinase method. In each assay, 1.29 mg. of protein of particles was used. ▲, Aurovertin; ●, oligomycin A; □, DCCD.

Oligomycin A always gives a greater stimulation than DCCD. Aurovertin is not able to stimulate the reaction, and exhibits its normal inhibitory pattern (cf. Fig. 2). Another typical experiment of this type is shown in Fig. 5(b). The difference from the above experiment is that the EDTA-particles were prepared from HBHM(N). The control rate of ATP-driven reduction of NAD⁺ by succinate in these EDTA-particles (9 nmol min.⁻¹ mg.⁻¹ of protein) is stimulated by 0.5 nmole of oligomycin A/mg. of protein and by 0.6 nmole of DCCD/mg. of protein to 55 and 21 nmol of NADH formed min.⁻¹ mg.⁻¹ of protein respectively; aurovertin again does not stimulate the reaction.

Table 1. *Stimulating effect of DCCD and oligomycin A on the ATP-driven reduction of NAD⁺ by succinate in EDTA-particles*

Maximal stimulation by DCCD and oligomycin A was measured in experiments similar to those described in Fig. 5. The EDTA-particle preparations were made by the same method, from either HBHM(N) or HBHM(B).

Expt. no.	Method of preparing mitochondria	Concn. of inhibitor giving maximum stimulation in EDTA-particles (nmole/mg. of protein)		Haem <i>a</i> content of EDTA-particles (nmole/mg. of protein)	Rate of reduction of NAD ⁺ (nmole min. ⁻¹ mg. ⁻¹ of protein)	
		Oligomycin A	DCCD		With optimum concn. of oligomycin A	Without oligomycin A
1	Blender	0.3	0.25	—	45*	13
2	Blender	0.2	0.2	—	18	6
3	Blender	0.23	0.2	—	46	28
4	Blender	0.3	0.25	1.19	44	18
5	Blender	0.2	0.25	1.22	48	22
6	Proteinase	0.6	0.5	—	55*	9
7	Proteinase	0.6	0.6	—	37	6
8	Proteinase	0.3	0.4	1.24	94	32
9	Proteinase	0.35	0.55	1.29	49	9
10	Proteinase	0.37	0.32	1.30	105	23

* The average stimulation by oligomycin A of this reaction was 250% for EDTA-particles derived from HBHM(B) and 505% for EDTA-particles from HBHM(N).

Table 2. *Additive effects of DCCD and oligomycin A on the ATP-linked reduction of NAD⁺ by succinate in EDTA-particles*

EDTA-particles were prepared from HBHM(N). Samples of the EDTA-particles were incubated at 0° overnight with DCCD. They were assayed for ATP-linked reduction of NAD⁺ by succinate in the presence of various concentrations of oligomycin A, which was added 3 min. before the reaction was started. In each assay, 1.29 mg. of protein of EDTA-particles was used.

		Rate of formation of NADH (nmole min. ⁻¹ mg. ⁻¹ of protein)								
Concn. of oligomycin A (nmole/mg. of protein)	...	0	0.1	0.2	0.3	0.4	0.5	0.75	1.0	1.5
Concn. of DCCD (nmole/mg. of protein)										
0		8.8	12.0	23.0	43.0	45.0	56.5	13.4	4.9	1.5
0.1		10.5	—	28.6	—	53.0	—	9.4	—	—
0.2		12.8	—	36.9	—	32.5	—	7.8	—	—
0.3		14.7	—	—	—	—	—	—	—	—
0.4		17.0	—	31.9	—	22.4	—	6.4	—	—
0.5		19.4	—	26.8	—	12.3	—	3.9	—	—
0.75		19.4	—	13.1	—	5.4	—	1.9	—	—
1.0		8.7	—	4.5	—	1.4	—	0	—	—

The results of a series of such experiments are shown in Table 1. Three trends may be discerned in the results. First, EDTA-particles prepared from HBHM(N) require higher concentrations of inhibitors for maximal stimulation (0.3–0.6 nmole of inhibitor/mg. of protein) than EDTA-particles prepared from HBHM(B) (0.2–0.3 nmole of inhibitor/mg. of protein). Secondly, the average percentage stimulation (505%) and the maximal stimulated rates are greater in the EDTA-particles derived from HBHM(N) than in the EDTA-particles from HBHM(B), which were stimulated on an average by 250%. Thirdly, as mentioned

above, oligomycin A always gives a greater stimulation than DCCD. The haem *a* content of both preparations of EDTA-particles is the same, precluding any explanation of the first two of these phenomena on the lines that one preparation is richer in respiratory-chain content than the other.

Summation of the effects of DCCD and oligomycin A. Table 2 shows the results of an experiment in which the effects of adding various concentrations of DCCD and oligomycin A together to EDTA-particles were measured. The EDTA-particles were preincubated overnight with various concentrations of DCCD, and the oligomycin A was added to the

Table 3. Absence of additive effects of aurovertin and oligomycin A on the ATP-linked reduction of NAD^+ by succinate in EDTA-particles

Conditions were the same as those in Table 2. Aurovertin and oligomycin A were added to the assay mixture 3 min. before the reaction was started.

Concn. of oligomycin A (nmoles/mg. of protein) ... Concn. of aurovertin (nmoles/mg. of protein)	Rate of formation of NADH (nmoles min. ⁻¹ mg. ⁻¹ of protein)								
	0	0.1	0.2	0.3	0.4	0.5	0.75	1.0	1.5
0	8.5	12.0	23.0	43.0	45.0	56.6	13.4	4.9	1.5
0.15	7.4	—	—	—	—	—	—	—	—
0.33	3.4	—	14.6	—	42.5	—	9.3	—	—
0.66	3.8	—	12.4	—	31.5	—	7.6	—	—
1.33	2.5	—	6.0	—	14.1	—	8.4	—	—
3.3	0	—	—	—	—	—	—	—	—

system used for the assay of the ATP-driven reduction of NAD^+ by succinate. Two comments can be made about the results. First, for any given concentration of DCCD, the rate of reduction of NAD^+ is increased by the addition of oligomycin A up to a total concentration of 0.4–0.5 nmole of (oligomycin A + DCCD)/mg. of protein. Secondly, if the total inhibitor present exceeds 0.4–0.6 nmole of (oligomycin A + DCCD)/mg. of protein, then the rate of reduction of NAD^+ is decreased. These results are interpreted as showing that the sites of oligomycin A and DCCD action (both for stimulating and for inhibiting the ATP-driven reduction of NAD^+ by succinate) are either identical or close enough to exclude the approach of a second inhibitor molecule.

Independent effects of aurovertin and oligomycin A. The results of an experiment, similar to the one above, in which the combined effects of aurovertin and oligomycin A on the ATP-driven reduction of NAD^+ by succinate in EDTA-particles were studied, are shown in Table 3. The addition of aurovertin and oligomycin A together always gives a rate less than oligomycin alone, no matter what their total concentrations are. Also, the concentration of oligomycin A that gives maximal stimulation is not altered by the addition of aurovertin (cf. DCCD). These results concur with those presented in Figs. 5(a) and 5(b), and suggest that aurovertin does not interfere with the stimulatory action of oligomycin A. Hence these inhibitors must act at different sites.

Effects of aurovertin, DCCD and oligomycin A on the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase in EDTA-particles. Oligomycin A and DCCD are both capable of stimulating the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase reaction in EDTA-particles (Fig. 6). A similar result was obtained by Van Dam & Ter Welle (1966), using a fresh heart-muscle

Fig. 6. Effect of inhibitors on ATP-driven energy-linked nicotinamide nucleotide transhydrogenase in EDTA-particles. The EDTA-particles were made from HBHM(N). Assay conditions were the same as those described in Fig. 3. In each assay, 1.06 mg. of protein of particles was used. ▲, Aurovertin; ●, oligomycin A; □, DCCD.

preparation to which oligomycin was added. The concentration of DCCD or of oligomycin A needed for maximal stimulation is similar to the concentration required to stimulate ATP-driven reduction of NAD^+ by succinate in the same preparation (results in Table 1, Expt. 9).

Aurovertin has no stimulatory effect on the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase, though a marginal inhibitory effect was observed at 5 nmoles of aurovertin/mg. of protein (cf. Fig. 3).

Effect of aurovertin, DCCD and oligomycin A on the aerobic energy-linked nicotinamide nucleotide transhydrogenase in EDTA-particles. Lee & Ernster (1965) have shown that the rate of the aerobic energy-linked nicotinamide nucleotide transhydrogenase driven by succinate oxidation in EDTA-particles is stimulated by oligomycin A. The oligomycin was thought to prevent the breakdown of

high-energy intermediates by some apparently oligomycin-sensitive wasteful reaction. They implied that the minimum amount of oligomycin required for maximum stimulation of the aerobic energy-linked nicotinamide nucleotide transhydrogenase would be the amount that maximally stimulates ATP-driven energy-linked reactions or P/O ratios in EDTA-particles. Fig. 7 shows an experiment in which the amounts of oligomycin A and DCCD necessary for maximum stimulation of the aerobic energy-linked nicotinamide nucleotide transhydrogenase in EDTA-particles prepared from HBHM(B) were measured. The minimum concentration of oligomycin A that caused the maximum stimulation was 0.6 nmole/mg. of protein, and that of DCCD about 1.0 nmole/mg. of protein. This result may be compared with the amounts of the two inhibitors that gave the highest stimulation of the ATP-driven reduction of NAD^+ by succinate in the same EDTA-particle preparation (Table 1, Expt. 5): 0.2 nmole of oligomycin A/mg. of protein and 0.25 nmole of DCCD/mg. of protein.

The results in Fig. 7 also show that aurovertin neither inhibits nor stimulates the aerobic energy-linked nicotinamide nucleotide transhydrogenase. Since aurovertin is known to inhibit the oxidative-phosphorylation sequence, this result suggests that aurovertin acts between ATP and the high-energy intermediate which is utilized by the energy-linked transhydrogenase and which is generated from respiratory-chain phosphorylation in the above

experiment. Since oligomycin A inhibits the wasteful breakdown of this high-energy intermediate, it was decided to examine whether aurovertin prevented or affected the stimulation of the aerobic energy-linked nicotinamide nucleotide transhydrogenase (cf. Table 3) by oligomycin A. The results of such an experiment (Table 4) showed that aurovertin was unable to interfere with the effect of oligomycin at the site at which the latter stimulated.

Effect of inhibitors on mitochondrial factors associated with oxidative phosphorylation

The experiments discussed so far were carried out with HBHM, SMP or EDTA-particles. However, a number of factors have been isolated from mitochondria that are thought to be associated with the oxidative-phosphorylation process (for a review see Racker, 1965). We therefore examined whether the actions of these inhibitors on an intact oxidative-phosphorylation sequence could be correlated with their actions on the isolated factors.

Effect of DCCD on F_1 . A soluble ATPase resembling the coupling factor F_1 (Penefsky, Pullman, Datta & Racker, 1960) was isolated from mitochondria by Selwyn (1967). It shows all the expected interactions with CF_0 (Robertson *et al.* 1967), the factor that confers oligomycin sensitivity on F_1 (Kagawa & Racker, 1966b). It has been shown previously that, though oligomycin A has no effect on F_1 alone (Racker, 1962) or on Selwyn's (1967) soluble ATPase alone (Robertson *et al.* 1967), aurovertin inhibits the soluble ATPase by 60–70% (Kagawa & Racker, 1966a; Robertson *et al.* 1967; see also unpublished work of B. Chance & H. A. Lardy, quoted by Ernster *et al.* 1967). The results of an experiment in which the effect of DCCD on soluble ATPase was studied are reported in Table 5. During the isolation of the soluble ATPase, one half of the supernatant obtained after the homogenizing

Fig. 7. Effect of inhibitors on the aerobic energy-linked nicotinamide nucleotide transhydrogenase driven by succinate oxidation in EDTA-particles. The EDTA-particles were made from HBHM(B). In each assay, 0.95 mg. of protein of particles was used. DCCD was preincubated overnight with the particles. Aurovertin and oligomycin A were incubated with the particles in the assay cuvette for 2 min.; succinate was then added, and NADP^+ was added after a further 1 min. to start the reaction. $\blacktriangle$, Aurovertin; $\bullet$, oligomycin A; $\square$, DCCD.

Table 4. *Lack of effect of aurovertin on oligomycin stimulation of the aerobic energy-linked nicotinamide nucleotide transhydrogenase in EDTA-particles*

Assay conditions were similar to those in Fig. 6. EDTA-particles prepared from HBHM(B) (1.07 mg. of protein) were used. In Expt. 2, 10 nmol of aurovertin/mg. of protein were initially present, and 1 nmole of oligomycin A was added in both experiments after 2 min.

Expt. no.	Addition	NADPH formed (nmol min. ⁻¹ mg. ⁻¹ of protein)	
		– Oligomycin A	+ Oligomycin A
1	None	37.6	163
2	Aurovertin	41.9	157

Table 5. *Properties of DCCD-treated soluble ATPase*

ATPase activities were measured as described by Robertson *et al.* (1967). Oligomycin A (5nmol), Sigma soya-bean phospholipid in micellar form (Fleischer & Klouwen, 1961) (120nmol) and CF₀ (200 µg. of protein) were added 5 min. before the reactions were started. The soluble ATPase (32 µg. of protein) was added 3 min. before the reactions were started.

Additions	ATPase activity (nmol of ADP formed min. ⁻¹ 32 µg. ⁻¹ of protein)	
	Control soluble ATPase	DCCD-treated soluble ATPase
ATPase	55.7	53.7
ATPase + CF ₀	9.3	8.3
ATPase + CF ₀ + phospholipid	26.9	22.4
ATPase + CF ₀ + phospholipid + oligomycin A	5.3	4.8

Table 6. *Properties of DCCD-treated CF₀*

Assay conditions were similar to those described in Table 5.

Additions	ATPase activity (nmol of ADP formed min. ⁻¹ 32 µg. ⁻¹ of protein)	
	Control CF ₀ preparation	DCCD-treated CF ₀ preparation
ATPase	58.2	58.2
ATPase + CF ₀	11.5	15.3
ATPase + CF ₀ + phospholipid	30.7	20.4
ATPase + CF ₀ + phospholipid + oligomycin A	12.1	23.0

and centrifuging of the mitochondrial acetone-dried powder was treated with 5nmol of DCCD/mg. of protein and the soluble ATPase subsequently isolated. The soluble ATPase so obtained is indistinguishable from the control enzyme. The activity of both preparations is inhibited by CF₀ (cf. Kagawa & Racker, 1966b). The soluble ATPase + CF₀ activity is stimulated by pre-incubation of the CF₀ with phospholipid, and this stimulated activity is inhibited by oligomycin A. The conclusion may be drawn that DCCD, like oligomycin but unlike aurovertin, does not affect or interfere with the properties of the soluble ATPase. It is assumed that any effect of DCCD would not be removed or reversed during the isolation procedure (see below).

Effect of DCCD on CF₀. Kagawa & Racker (1966b) have demonstrated that the site of oligomycin inhibition on an ATPase system containing F₁, CF₀ and phospholipid is on the CF₀ complex. It was therefore decided to see whether DCCD acts on the CF₀ complex. The properties of a CF₀ preparation, isolated from T-particles that were treated with DCCD, are described in Table 6. Both the CF₀ from the DCCD-treated preparation (DCCD-CF₀) and the control CF₀ inhibited the

activity of soluble ATPase. The addition of phospholipid, however, stimulated the DCCD-CF₀ + soluble ATPase activity by only 32%; oligomycin A did not inhibit this activity. In contrast, the ATPase activity of the ATPase + control CF₀ mixture was stimulated by 170% by phospholipid. It is concluded from these results that (1) DCCD does not prevent the binding of CF₀ to soluble ATPase; (2) DCCD does not prevent the inhibition of the soluble ATPase activity by CF₀; (3) DCCD prevents the phospholipid stimulation of the CF₀ + soluble ATPase system; (4) DCCD has an irreversible effect on CF₀, since the DCCD effect was not lost during the preparation of CF₀ from T-particles (cf. Holloway, Robertson, Knight & Beechey, 1966), in contrast with the reversible binding of oligomycin D (Kagawa & Racker, 1966b); (5) DCCD has similar effects to oligomycin A in the CF₀ complex. Similar results to these, with an authentic F₁ preparation kindly sent to us by Professor E. Racker, have already been briefly reported (Robertson, Holloway, Knight & Beechey, 1966). These observations with DCCD and oligomycin A are quite different from those made with aurovertin, which has been shown to act on F₁ or the soluble ATPase itself, as discussed above.

Table 7. *Inhibitor potencies*

The concentrations of inhibitors giving 50% inhibition of the partial reactions of oxidative phosphorylation have been taken from Figs. 1, 2, 3 and 4.

Reaction	Concentration of inhibitors giving 50% inhibition (nmoles/mg. of protein)		
	Aurovertin	DCCD	Oligomycin A
ATP synthesis in HBHM	0.22	0.62	0.35
ATP-linked reduction of NAD ⁺ by succinate in SMP	0.95	0.45	0.40
ATP-driven energy-linked nicotinamide nucleotide transhydrogenase in SMP	3.0	0.75	0.65
Uncoupler-stimulated ATPase in HBHM	40.0	0.24	0.21

DISCUSSION

In Table 7 are listed the concentrations of DCCD, oligomycin and aurovertin that give 50% inhibition of (a) ATP synthesis (see also Fig. 1) and (b) the sequence working in the reverse direction, i.e. the ATP-driven energy-linked reactions (see also Figs. 2 and 3) and the uncoupler-stimulated ATPase (see also Fig. 4). There is a marked difference between the effects of DCCD and oligomycin on the one hand and aurovertin on the other. Aurovertin is the most potent inhibitor of ATP synthesis and the least potent inhibitor of the ATP-utilizing systems.

The concentrations of DCCD and oligomycin that give 50% inhibition of the above reactions lie within the same range, 0.21–0.75 nmole of inhibitor/mg. of protein (Table 7), DCCD being always marginally less potent than oligomycin A. The variation in potencies of DCCD and oligomycin A for the different reactions may be a reflection of the affinity of these reactions for the high-energy intermediate whose formation or utilization is blocked by the inhibitor. This explanation is similar to that given by Danielson & Ernster (1963), who suggested that 2,4-dinitrophenol was a more potent inhibitor of the ATP-driven reduction of NAD⁺ by succinate than of the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase because the former reaction must have a lower affinity for the common high-energy intermediate whose hydrolysis is mediated by 2,4-dinitrophenol. It can be seen from the data in Table 7 that the potencies of DCCD and oligomycin A vary in a parallel manner, suggesting that they inhibit the formation or utilization of the same intermediate. This conclusion is supported by the data in Table 2, where it is shown that the stimulatory properties of DCCD and oligomycin A are additive.

In contrast, the data in Table 7 show that the concentrations of aurovertin that inhibit the listed reactions do not parallel those of DCCD and oligomycin A. A 50% inhibition of ADP-stimulated respiration is found with 0.22 nmole of aurovertin/mg. of protein, 0.35 nmole of oligomycin A/mg. of

protein and 0.62 nmole of DCCD/mg. of protein. Lardy *et al.* (1964) have also shown that oligomycin and aurovertin are approximately equally potent inhibitors of glutamate oxidation, [³²P]P_i-ATP exchange and [¹⁸O]HPO₄²⁻-water exchange activities in rat liver mitochondria. These are all reaction systems that involve the oxidative-phosphorylation sequence working in the forward direction.

However, aurovertin is a less potent inhibitor than either DCCD or oligomycin A of the reverse sequence of oxidative phosphorylation, the ATP-driven reactions. The ATP-driven reduction of NAD⁺ by succinate is 50% inhibited by 0.95 nmole of aurovertin/mg. of protein (Fig. 2 and Table 7). This agrees well with the value quoted by Ernster *et al.* (1967), 1.25 nmoles/mg. of protein. However, Lenaz (1965) found that this system was even less sensitive to aurovertin (43% inhibition by 4.2 nmoles/mg. of protein). The ATP-driven energy-linked nicotinamide nucleotide transhydrogenase is 50% inhibited by 3.0 nmoles of aurovertin/mg. of protein (Fig. 3 and Table 7), a much greater concentration than is required to inhibit the forward reaction. This result also differs from that of Lenaz (1965) who found that this system was inhibited by neither 4.2 nor 420 nmoles of aurovertin/mg. of protein. In our hands these concentrations of aurovertin gave 60% and 80% inhibition. We cannot explain this difference in results. Since Lenaz (1965) found that the ATP-driven reduction of NAD⁺ by succinate is inhibited by aurovertin, it is not surprising that the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase is inhibited. The uncoupler-stimulated ATPase of mitochondria is another example of a reaction involving the reversal of the oxidative-phosphorylation sequence which is relatively insensitive to aurovertin. The 4,5,6,7-tetrachloro - 2 - trifluoromethylbenzimidazole - stimulated ATPase of mitochondria is 50% inhibited by 40 nmoles of aurovertin/mg. of protein (Fig. 4 and Table 7), and higher concentrations of aurovertin did not give significantly greater inhibitions. This result is similar to those obtained with the aurovertin inhibition of a soluble ATPase

isolated from mitochondria (Kagawa & Racker, 1966a; Robertson *et al.* 1967). The results illustrated in Fig. 4 are qualitatively similar to those of Lardy (1960) and Lardy *et al.* (1964), who showed that oligomycin gave more than 90% inhibition of the 2,4-dinitrophenol-stimulated ATPase activity of rat liver mitochondria, whereas high concentrations of aurovertin gave only 70% inhibition.

A comparison of the data shown in Fig. 7 and Table 1, Expt. 5 (which refer to the same particle preparation), shows that the minimum amounts of DCCD or oligomycin A required to give maximal stimulation of the aerobic energy-linked nicotinamide nucleotide transhydrogenase in EDTA-particles, i.e. 1.0 and 0.6 nmole/mg. of protein for DCCD and oligomycin respectively, can be several times greater than the amounts needed to give maximum stimulation of ATP-driven reduction of NAD^+ by succinate. This result differs from those of experiments described by Lee & Ernster (1965), who found that the P/O ratios, the ATP-driven reduction of NAD^+ by either succinate or ascorbate plus *NNN'N'*-tetramethyl-*p*-phenylenediamine and the aerobic energy-linked nicotinamide nucleotide transhydrogenase were all stimulated maximally by the same concentration of oligomycin, 0.3–0.4 nmole/mg. of protein in EDTA-particles. We feel that our observations are more consistent with the hypothesis put forward by Lee & Ernster (1965) that oligomycin acts preferentially at the damaged sites in EDTA-particles that lead to the hydrolysis of $\text{X} \sim \text{I}$ (a hypothetical non-phosphorylated high-energy intermediate in the synthesis of ATP), and that at somewhat higher concentrations oligomycin also acts at the intact sites that lead to the phosphorylation of $\text{X} \sim \text{I}$, normally leading to the formation of $\text{X} \sim \text{P}$ (a hypothetical phosphorylated high-

energy intermediate in the synthesis of ATP). Stimulation by oligomycin or DCCD of the partial reactions of oxidative phosphorylation is thought to be due to the inhibitors' increasing the steady-state concentration of $\text{X} \sim \text{I}$. In the aerobic energy-linked nicotinamide nucleotide transhydrogenase, maximum concentrations of $\text{X} \sim \text{I}$ will be present when both the hydrolytic and phosphorylytic fissions of $\text{X} \sim \text{I}$ are inhibited, i.e. at high inhibitor concentrations. However, in the ATP-driven reduction of NAD^+ by succinate, the $\text{X} \sim \text{I}$ concentration and hence the maximal stimulation will be dependent on the net effect of these reactions and will be observed when $\text{X} \sim \text{I}$ hydrolysis is maximally inhibited and the synthesis of $\text{X} \sim \text{I}$ from $\text{X} \sim \text{P}$ is minimally inhibited. Thus maximum stimulation of the ATP-driven reduction of NAD^+ by succinate will be observed at lower concentrations of inhibitor.

The data in Fig. 7 show that aurovertin neither inhibits nor stimulates the aerobic energy-linked nicotinamide nucleotide transhydrogenase, and the results illustrated in Figs. 5 and 6 show that aurovertin has no stimulatory effects on the ATP-driven partial reactions. Further, the results summarized in Table 3 show that aurovertin does not affect the optimum concentration of oligomycin needed to stimulate the ATP-driven reduction of NAD^+ by succinate. These observations suggest that aurovertin, unlike oligomycin A or DCCD, has no effect on the hydrolysis of $\text{X} \sim \text{I}$. Since oligomycin can still stimulate the aerobic energy-linked nicotinamide nucleotide transhydrogenase in the presence of aurovertin (Table 4) it follows that the sites of DCCD and oligomycin A action are nearer to the respiratory chain than the site of aurovertin inhibition (see Scheme 1).

A more direct approach to the differences in the

Scheme 1. Proposed sites of action of aurovertin, oligomycin and DCCD: (a) simplification of the scheme proposed by Lardy *et al.* (1964); (b) simplification of the scheme of Ernster *et al.* (1967). C is a member of the mitochondrial electron-transport chain; X and I are hypothetical intermediates and P indicates a phosphorylated intermediate. $\text{C} \sim \text{I}$, $\text{X} \sim \text{I}$, $\text{X} \sim \text{P}$ are hypothetical high-energy intermediates in the synthesis of ATP by mitochondria.

sites of action of inhibitors in the oxidative-phosphorylation sequence is to study the effects of the inhibitors on the properties of various mitochondrial fractions thought to be involved in the synthesis of ATP. Racker (1962) has shown that the ATPase activity of F_1 is not inhibited by oligomycin, and this has been confirmed by Robertson *et al.* (1967) using a mitochondrial ATPase fraction identical with F_1 , described by Selwyn (1962, 1967). The data listed in Table 5 show that F_1 prepared from DCCD-treated mitochondrial extracts is functionally similar to a control preparation, suggesting that DCCD does not act on this protein in the intact mitochondrion. However, it is noteworthy that Schatz, Penefsky & Racker (1967) stated that treatment of F_1 with DCCD results in an inactivation of the ATPase activity with little or no effect on the coupling activity, although no experimental details were given. Kagawa & Racker (1966a) and Robertson *et al.* (1967) demonstrated that aurovertin will inhibit up to 70% of the ATPase activity of F_1 . H. A. Lardy & B. Chance (unpublished work quoted by Ernster *et al.* 1967) have showed that aurovertin combines with and inhibits F_1 .

CF_0 isolated from DCCD-treated T-particles differs in one specific property from a control CF_0 preparation: the ATPase activity of an F_1 -DCCD- CF_0 complex cannot be stimulated by the addition of phospholipid (see Table 6). The loss of this property from CF_0 is noted also when the CF_0 has been isolated from DCCD-treated mitochondria and DCCD-treated SMP. Also, the phospholipid-stimulated ATPase activity of CF_0 - F_1 preparations isolated from DCCD-treated SMP is greatly inhibited (A. M. Robertson, C. T. Holloway, I. G. Knight & R. B. Beechey, unpublished work). Thus it would appear that the locus of DCCD action is in the CF_0 complex, as is that for oligomycin (Kagawa & Racker, 1966b).

Beechey *et al.* (1966, 1967a) emphasized the functional similarities between DCCD and oligomycin. Some of the data presented in the present paper extend these similarities: (1) the constant ratio of the inhibitory concentrations of the two inhibitors, listed in Table 7; (2) the summation of the stimulatory effects of DCCD and oligomycin A, presented in Table 2; (3) the lack of effect of the inhibitors on F_1 and the locus of inhibition being on CF_0 . Racker & Horstman (1967) have shown that both oligomycin D and DCCD will enhance the efficiency of oxidative phosphorylation in ASU-particles. The latter are a type of submitochondrial particle prepared by the ultrasonic treatment of mitochondria in a dilute solution of ammonia, followed by passage down a Sephadex G-50 column and subsequent treatment with urea.

It has been recognized for some time that auro-

vertin and oligomycin act at different sites in the oxidative-phosphorylation sequence (Lardy & McMurray, 1959; Lardy, 1960). Lardy *et al.* (1964) observed that aurovertin did not abolish the effect of ATP on the swelling of mitochondria stimulated by certain uncoupling agents. These workers proposed the existence of a new high-energy intermediate, $\sim W$, which was responsible for the transport of water into the mitochondrion. In contrast, oligomycin inhibited all ATPase activity. On the basis of these and other observations, Lardy *et al.* (1964) proposed that the sites of action of oligomycin and aurovertin are as shown in Scheme 1(a). Ernster *et al.* (1967) reinterpreted the ATPase results in view of the results of Lenaz (1965) and some new experimental data on the effects of aurovertin and oligomycin on partial reactions. These showed that aurovertin is a potent inhibitor of reactions involving the oxidative-phosphorylation sequence working in the forward direction and a relatively poor inhibitor of reactions involving a reversal of the oxidative-phosphorylation process. Ernster *et al.* (1967) proposed that the sites of action of oligomycin and aurovertin are as shown in Scheme 1(b). The present results have confirmed and extended the results of Ernster *et al.* (1967). Thus it is difficult to reconcile the absence of a stimulation by aurovertin on the aerobic energy-linked nicotinamide nucleotide transhydrogenase, either in the presence or in the absence of oligomycin (Table 4), with the scheme of Lardy *et al.* (1964) (Scheme 1a). However, this observation is in accord with the scheme of Ernster *et al.* (1967) (Scheme 1b). Also, the effect of aurovertin on the ATP-driven energy-linked nicotinamide nucleotide transhydrogenase (Fig. 3) is that to be expected from the effects of inhibition by aurovertin on the ATP-driven reduction of NAD^+ by succinate and also on the uncoupler-stimulated ATPase. The inhibition of F_1 ATPase activity by aurovertin and the identification of CF_0 as the locus of both oligomycin action (Kagawa & Racker, 1966a,b) and DCCD action (Table 6) are in agreement with the scheme proposed by Ernster *et al.* (1967).

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