Chloride-Dependent Restoration of Coupling by Oligomycin in Rat Liver Mitochondria

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In rat liver mitochondria suspended in KCl medium, oligomycin interfered with the effect of uncouplers on energy conservation. It antagonized the effect of uncouplers that are weak acids (2,4-dinitrophenol etc.), but enhanced that of the lipid-penetrating cation *NN*-dimethyl-*N'**N'*-dibenzylammonium. Oligomycin caused none of the above effects when Br⁻ or NO₃⁻ was substituted for Cl⁻ as the major anionic species in the assay medium. The concentration of oligomycin that exerted the above-mentioned effects was lower than that necessary for the inhibition of energy transfer, but was in the range that induced Cl⁻ permeation through the cristae membrane. The possible connexion between the effect of oligomycin on Cl⁻ permeation and its interference with the action of uncouplers is discussed.

Oligomycin (Lardy *et al.*, 1958) and trialkyltin compounds (Stockdale *et al.*, 1970) are the most extensively studied inhibitors of energy transformation. According to a widely accepted concept (cf. Slater & TerWelle, 1969) an inhibitor of energy transfer, unlike an uncoupler, does not cause dissipation of energy, but acts by blocking the interaction between the respiratory chain and the functions catalysed by the mitochondrial ATPase*–ATP synthetase (EC 3.6.1.3). In media of high Cl⁻ content, however, the pattern of action of both of the above-mentioned compounds was shown to deviate considerably from that postulated for an inhibitor of energy transfer. Trialkyltin compounds caused swelling of the mitochondria and energy dissipation (Stockdale *et al.*, 1970) which was attributed by Selwyn *et al.* (1970) to their ability to mediate Cl⁻–OH⁻ exchange through the mitochondrial membrane. Paradoxically, under similar conditions where trialkyltin compounds acted like uncouplers, they suppressed the release of the respiratory control induced by the 'authentic' uncoupling agent 2,4-dinitrophenol (Stockdale *et al.*, 1970; Coleman & Palmer, 1971; Harris *et al.*, 1973; Dawson & Selwyn, 1974).

Ariel & Avi-Dor (1973) have shown that oligomycin, in a medium of high Cl⁻ content, acts in an uncoupler-like manner and induces swelling of mitochondria. However, in the case of oligomycin, these effects were only manifested under conditions in which the major counter-ion to Cl⁻ could permeate the cristae membrane; apparently, because the Cl⁻ transport facilitated by oligomycin is uniport and not an exchange reaction.

The aim of the present work was to determine whether a Cl⁻-dependent inhibition of uncoupled respiration could also be elicited by oligomycin.

* Abbreviation: ATPase, adenosine triphosphatase.

We describe experiments that show that in high Cl⁻ medium, oligomycin is capable either of diminishing or of enhancing the effect of uncouplers, according to their nature. Moreover, it is shown that the interference of oligomycin with the action of uncouplers is not restricted to the respiratory control, but can also be extended to other energy-linked mitochondrial functions.

Experimental

Materials

Special materials were obtained from the following sources: 3,3'-methylenebis (4-hydroxy-1,2-benzopyron) (dicoumarol) from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. This compound was dissolved in a few drops of 0.1 N-NaOH adjusted to pH 8.0 with HCl and diluted to 10 mM; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone from Sigma Chemical Co., St. Louis, Mo., U.S.A.; *NN*-dimethyl-*N'**N'*-dibenzylammonium chloride from K & K Laboratories, Plainview, N.Y., U.S.A. The sources of other materials was as given by Ariel & Avi-Dor (1973).

Methods

Preparation of rat liver mitochondria, radioactivity measurements, protein determination and the assay of respiration, oxidative phosphorylation and ATPase were performed as described by Ariel & Avi-Dor (1973), except for the composition of the assay medium used, which was as follows.

Respiration. For this assay 5 mM-Tris-HCl buffer (pH 7.4) was used when the major uni-univalent salt in the medium was KCl, or 5 mM-Tris-HNO₃ (pH 7.4). When the major uni-univalent salt in the

medium was KBr or KNO_3 . In addition 5 mM-potassium phosphate buffer (pH 7.4), 5 mM- MgCl_2 , 175 mM of a uni-univalent salt (KCl, KBr or KNO_3), as indicated in the legends to the Figures and Tables, 40 nmol of rotenone/mg of protein, 5 mM-sodium succinate and mitochondria in an amount equivalent to approx. 2 mg of protein/ml were added to the assay.

Oxidative phosphorylation. Buffers, uni-univalent salt, MgCl_2 , sodium succinate and mitochondria as in the assay medium for respiration were used plus 10 mM-potassium phosphate buffer (pH 7.4) containing $[\text{P}^{32}\text{P}]_i$ (2×10^6 c.p.m./ μmol), 0.1 mM-EDTA neutralized by Tris (pH 7.4), 20 mM-glucose, 0.5 mg of hexokinase/ml and 1 mM-ADP.

ATPase assay. Buffers, uni-univalent salt, MgCl_2 and mitochondria were as indicated in the assay medium for respiration plus 1.2 mM- $[\text{P}^{32}\text{P}]\text{ATP}$, (1×10^5 – 2×10^5 c.p.m./ μmol).

Results

Rat liver mitochondria, in KCl medium, were left to oxidize succinate in the presence and in the absence of oligomycin. The rate of respiration was measured as a function of 2,4-dinitrophenol concentration in the medium. The concentration of oligomycin (0.1 $\mu\text{g}/\text{mg}$ of protein) used in this experiment was of the same order of magnitude as that found to induce Cl^- permeation through the cristae membrane (Ariel & Avi-Dor, 1973). Fig. 1 shows that oligomycin was able to suppress to a large extent the respiratory stimulation caused by low concentrations of 2,4-dinitrophenol, but its effectiveness as an antagonist of this uncoupler gradually diminished as the concentration of 2,4-dinitrophenol increased.

Fig. 1. Effect of oligomycin on the respiration of mitochondria in the presence of various concentrations of 2,4-dinitrophenol

For details see under 'Respiration' in the Experimental section. The standard assay medium contained KCl as the major uni-univalent salt. $\circ$, Control; $\bullet$, 0.1 μg of oligomycin/mg of protein.

Titration of the above system with oligomycin, in the presence of a fixed amount of 2,4-dinitrophenol or ADP, revealed that approximately ten times lower concentrations of oligomycin were necessary to suppress the stimulatory effect of the uncoupler than that of ADP (Fig. 2). When uncouplers other than 2,4-dinitrophenol were used to release the respiratory control, it was found (Table 1) that oligomycin also inhibited the respiratory stimulation caused by dicoumarol or by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. In contrast, oligomycin enhanced the stimulation of the respiratory rate caused by *NN*-dimethyl-*N'*-dibenzylammonium chloride. It is noteworthy, that the first two uncouplers, like 2,4-dinitrophenol, are weak acids whereas the last one is the salt of a lipid-penetrating base (cf. Van Dam & Meyer, 1971). Uncoupler acids (Mitchell, 1968) and uncoupler bases (Skulachev, 1971) are thought to act by different mechanisms. The interference of oligomycin with the above action of uncouplers was Cl^- -dependent since it was not manifested in KBr or KNO_3 media (Table 1).

Further experiments revealed that antagonism and synergism between oligomycin and uncouplers are not restricted to the respiratory activity, but can also be demonstrated on oxidative phosphorylation

Fig. 2. Effect of 2,4-dinitrophenol and of ADP on the respiration of mitochondria in the presence of various concentrations of oligomycin

The conditions were as stated in Fig. 1. $\circ$, Control; $\bullet$, 5 μM -2,4-dinitrophenol; Δ , 100 μM -ADP.

Table 1. Effect of oligomycin on the rate of respiration in the presence of various uncouplers

For details see under 'Respiration' in the Experimental section. The standard assay medium contained: KCl, KBr or KNO₃ as the major uni-univalent salt. When indicated, the following additions were also made: 0.1 µg of oligomycin/mg of protein, 5 µM-2,4-dinitrophenol, 5 µM-3',3'-methylenebis-(4-hydroxy-1,2-benzopyron) (dicoumarol), 30nM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and 5mM-*NN*-dimethyl-*N'**N'*-dibenzylammonium chloride. Respiration is expressed in ng-atoms of O/min. *R_c*, the respiratory control ratio, indicates respiration in the presence of the additions indicated, divided by respiration without additions.

Additions	KCl		KBr		KNO ₃	
	Respiration	<i>R_c</i>	Respiration	<i>R_c</i>	Respiration	<i>R_c</i>
None	70	1.0	75	1.0	85	1.0
Oligomycin	70	1.0	75	1.0	90	1.1
2,4-Dinitrophenol	135	1.9	190	2.5	195	2.3
2,4-Dinitrophenol+oligomycin	80	1.1	225	3.0	180	2.1
Dicoumarol	165	2.4	180	2.4	204	2.4
Dicoumarol+oligomycin	90	1.3	175	2.3	235	2.8
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	195	2.8	185	2.4	360	4.2
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone+oligomycin	90	1.3	180	2.4	370	4.4
<i>NN</i> -Dimethyl- <i>N'</i> <i>N'</i> -dibenzylammonium chloride	116	1.7	102	1.4	87	1.0
<i>NN</i> -Dimethyl- <i>N'</i> <i>N'</i> -dibenzylammonium chloride+oligomycin	214	3.1	105	1.4	83	1.0

Fig. 3. Effect of oligomycin on the ATPase of mitochondria in the presence of various concentrations of 2,4-dinitrophenol

For details see under 'ATPase' assay in the Experimental section. ○, Control; ●, 0.1 µg of oligomycin/mg of protein.

and on ATPase. Fig. 3 shows a stepwise activation of ATPase by gradually increasing amounts of 2,4-dinitrophenol. It is apparent that oligomycin was able to reverse the effect of low concentration of the uncoupler. Table 2 summarizes the results of an experiment in which the concerted effect of oligomycin and uncouplers was assessed on oxidative phosphorylation and on ATPase, either in KCl- or in KBr-containing media. It is apparent that oligomycin was capable of restoring the oxidative phosphorylation and of inhibiting the ATPase when the perturbation on the above-mentioned functions was elicited by uncouplers of the weak acid class and KCl was the major uni-univalent salt in the assay medium. In the same medium, the uncoupling induced by the salt of the lipid-penetrant cation was enhanced by oligomycin. No appreciable interaction

could be demonstrated between oligomycin and uncouplers in media containing KBr.

Discussion

Ariel & Avi-Dor (1973) presented evidence in support of the view that oligomycin is capable of inducing permeation of Cl⁻ through the cristae membrane of rat liver mitochondria. The oligomycin-mediated Cl⁻ transport is apparently uniport and it causes swelling of the mitochondria in a medium in which the major counter ion to Cl⁻ is permeant and therefore a net ion transport can occur from the medium into the matrix space. This swelling was connected with energy dissipation. In the KCl medium used in the present work, uniport movement of Cl⁻ caused no swelling because simultaneous transport of non-penetrant K⁺ (Chappell & Haarhoff, 1967) could not take place. Hence oligomycin also caused no uncoupling, but it interfered with the action of uncouplers when the latter were added to this assay system. Since this interference occurred only when Cl⁻ was the major anionic species in the medium, it seems reasonable to connect the changes in the effectiveness of the uncouplers with an electrical gradient produced by uniport Cl⁻ movement. One parameter that may be influenced by an electrical gradient is the distribution of the uncouplers between the matrix and the outer space. An electrical gradient directed toward the matrix space is expected to facilitate the accumulation of the positively charged, lipid-penetrant *NN*-dimethyl-*N'**N'*-dibenzylammonium ion. An increased intramitochondrial concentration of the latter might account for the Cl⁻-dependent enhancement of its effect, which was ob-

Table 2. *Effect of oligomycin on oxidative phosphorylation and ATPase activity in the presence of various uncouplers*

For details see under 'Oxidative phosphorylation' and 'ATPase assay' in the Experimental section. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone was added at a concentration of 100 nM. All other additions were as indicated in Table 1. ATPase activity is expressed as nmol of ATP hydrolysed/10 min per mg of protein.

Additions	KCl		KBr		KNO ₃ P/O
	P/O	ATPase	P/O	ATPase	
None	1.8	100	1.6	188	1.7
Oligomycin	1.8	168	1.6	288	1.3
2,4-Dinitrophenol	0.6	937	0.5	469	0.5
2,4-Dinitrophenol+oligomycin	1.4	250	0.6	362	0.5
Dicoumarol	0.5	563	0.6	469	0.6
Dicoumarol+oligomycin	1.3	175	0.7	719	0.8
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone	0.4	788	0.5	406	—
Carbonyl cyanide <i>p</i> -trifluoromethoxyphenylhydrazone+oligomycin	1.1	269	0.6	469	—
<i>NN</i> -Dimethyl- <i>N'</i> - <i>N'</i> -dibenzylammonium chloride	1.1	238	1.2	350	1.5
<i>NN</i> -Dimethyl- <i>N'</i> - <i>N'</i> -dibenzylammonium chloride+oligomycin	0.5	438	1.3	313	1.6

served in the presence of oligomycin. In the case of the uncouplers that are weak acids the electrical gradient might induce an electrophoretic movement of the uncoupler anion from the matrix into the outer space. Under the relatively alkaline conditions prevailing in the matrix (Mitchell, 1968) a weak acid will tend to dissociate and thus the extrusion of the anion might lead to a fall in the total intramitochondrial concentration of the uncoupler and hence also to a decrease in its effectiveness. It is noteworthy in this context that Harris *et al.* (1973) have shown that under conditions in which trialkyltin compounds caused a Cl⁻-dependent inhibition of the uncoupled respiration, they also lowered the intramitochondrial concentration of the uncoupler, 2,4-dinitrophenol.

The findings described in the present and in a previous study (Ariel & Avi-Dor, 1973) agree with the view that the Cl⁻-dependent energy dissipation and interference with the action of the uncouplers do not result from a direct effect of oligomycin on energy conserving reactions. The above-mentioned phenomena are rather corollary to the facilitation of Cl⁻ permeation induced by oligomycin. A pertinent question is therefore whether the effect of oligomycin on Cl⁻ permeation is connected with its action on the mitochondrial ATPase complex (for nomenclature see Pedersen, 1975). Bertina *et al.* (1974) showed that the effect of oligomycin on the reactions connected with energy conservation can be accounted for quantitatively by the binding of this substance to specific mitochondrial sites of high affinity. It is noteworthy that the concentration of oligomycin that induces Cl⁻ permeation and that interferes with the action of uncouplers is of the same order of magnitude (0.05–0.1 µg or 0.06–0.12 nmol/mg of protein) as the concentration of the specific binding sites in rat liver mitochondria (0.125 nmol/mg of protein). Binding of oligomycin to these sites could

be responsible for the change in permeability of the cristae membrane to Cl⁻. The oligomycin-binding protein which, according to Tzagoloff *et al.* (1968), is a part of the ATPase complex, could be either by itself, or by interaction with other membrane proteins associated with the ATPase responsible for regulation of Cl⁻ permeation. That there exists a close interrelation between the mitochondrial energy transforming apparatus and translocases is indicated by the finding that trialkyltin compounds also inhibit the adenine nucleotide transport (Harris *et al.*, 1973; Cain *et al.*, 1974) and from the results of genetic experiments performed with *Saccharomyces cerevisiae*. These experiments show that the molecular basis for the resistance to oligomycin and to trialkyltin compounds may lie in the components of the oxidative phosphorylation complex which also have ion-translocating functions (Lancashire & Griffith, 1975).

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