Chloride-Dependent Uncoupling Mediated by Oligomycin in Rat Liver Mitochondria

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In rat liver mitochondria suspended in KCl medium and containing a low concentration of a K^+ -specific cationophore (valinomycin or Triton X-100), oligomycin was shown to induce uncoupling of oxidative phosphorylation, stimulation of adenosine triphosphatase activity, release of the respiratory control, decrease of energy-dependent changes in the fluorescence of the dye 8-anilino-1-naphthalenesulphonic acid and rapid swelling of mitochondria. Oligomycin caused none of the above effects when Br^- or NO_3^- was substituted for Cl^- as the major anionic species or when Na^+ replaced the K^+ . The same concentration of oligomycin that caused uncoupling and swelling slightly improved energy-conserving reactions when the cationophores were omitted. In the presence of KSCN, valinomycin or Triton X-100 by itself caused uncoupling and swelling which was not further enhanced by oligomycin. On the basis of the above results it is suggested that the energy dissipation resulting from the concerted action of the cationophores and oligomycin is connected with the simultaneous transport of K^+ and its counter ion and that oligomycin plays its role in the uncoupling by facilitating the permeation of Cl^- through the cristae membrane of the mitochondria.

Oligomycin has played a key role in the study of the mechanism of energy transformation in mitochondria. At high concentrations it inhibits the transducing mechanism, which is the connecting link between the respiratory chain and reversible ATPase* (Lardy *et al.*, 1958). At low concentrations it can effectively suppress energy leakage in non-phosphorylating submitochondrial particles, and thus confer on them the ability to carry out oxidative phosphorylation and other energy-dependent functions (Lee & Ernster, 1965; Fessenden & Racker, 1966; Lee & Ernster, 1968). The site of binding of oligomycin is the ATPase complex (Slater & Ter Welle, 1969; Tzagoloff *et al.*, 1968; Racker, 1973) but the molecular basis of its action is still unknown. Several compounds resemble oligomycin in their action on energy transfer, among them are the trialkyltin derivatives, which have been extensively investigated (Aldridge, 1958; Selwyn *et al.*, 1970; Stockdale *et al.*, 1970; Aldridge & Street, 1971). In spite of the similarity in action there is no resemblance between the structure of oligomycin (von Gehlen *et al.*, 1972) and that of the trialkyltin compounds. Stockdale *et al.* (1970) have shown that the trialkyltin compounds act like inhibitors of energy transfer on mitochondria suspended in sucrose whereas in the presence of KCl an uncoupler-like effect is also manifested which is superimposed on the inhibitory action. This latter type of action of the

trialkyltin compounds was attributed to their ability to mediate Cl^- - OH^- exchange through the mitochondrial membrane.

During an investigation of the effect of low (0.08 – $0.1 \mu\text{g}/\text{mg}$ of protein) concentration of oligomycin on rat liver mitochondria it was noted that under certain conditions oligomycin too uncouples, rather than inhibits, oxidative phosphorylation. The experiments described in the present paper were designed to test the possibility that the uncoupler-like action of oligomycin is due to its effect on the permeation of anions. It was found that in all probability this is indeed the case.

Experimental

Materials

Special materials were obtained from the following sources: ADP, ATP, oligomycin, 8-anilino-1-naphthalenesulphonic acid and hexokinase (from yeast, type III) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; valinomycin (A grade) and catalase (A grade, 40000 units/mg of protein) from Calbiochem, Los Angeles, Calif., U.S.A.; rotenone, from K & K Laboratories, Plainview, U.S.A., Triton X-100 from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.; $^{32}\text{P}_i$ from The Nuclear Research Centre, Beer-Sheva, Israel; $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was a generous gift from Dr. N. Nelson, The Technion, Haifa, Israel. Other chemicals were of analytical grade.

* Abbreviation: ATPase, adenosine triphosphatase.

All stock solutions and reaction mixtures were prepared with double-distilled water. Stock solutions of valinomycin and oligomycin were prepared in ethanol at a concentration of $2\mu\text{M}$ and $30\mu\text{g/ml}$ respectively.

Methods

Rat liver mitochondria. Mitochondria were isolated from liver homogenates prepared in 0.25M-sucrose–3mM-Tris–HCl buffer (pH 7.4)–1mM-EDTA neutralized by Tris (pH 7.4), by differential centrifugation, as described by Ernster & Löw (1955). The mitochondrial pellet was finally suspended in 0.25M-sucrose–3mM-Tris–HCl buffer (pH 7.4) in a concentration of approx. 40–50mg of protein/ml.

Respiration and respiratory control ratio. Respiration was measured polarographically with a Clark oxygen electrode and recorded with a Görsz model 520 recorder. The standard assay medium contained 4mM-Tris–HCl buffer (pH 7.4)–10mM-potassium phosphate buffer (pH 7.4), 100mM-uni-univalent salt (KCl, NaCl, KBr, KNO_3 , KSCN), 8mM- MgCl_2 , 0.1mM-EDTA neutralized by Tris (pH 7.4), 0.1 μM -rotenone and 5mM-sodium succinate in a final volume of 4ml, at 28°C . The experiment was initiated by the addition of mitochondria equivalent to a concentration of approx. 2mg of protein/ml. The respiratory control ratio (R_c) was obtained by dividing the rate of O_2 uptake in the presence of ADP (1mM) by the rate obtained in the absence of ADP.

Oxidative phosphorylation. The standard assay medium contained in 4mM-Tris–HCl buffer (pH 7.4), 10mM-potassium phosphate buffer (pH 7.4) containing $^{32}\text{P}_i$ (2×10^6 – 3×10^6 c.p.m./ μmol), 100mM-uni-univalent salt as indicated, 8mM- MgCl_2 , 0.1mM-EDTA neutralized by Tris (pH 7.4), 20mM-glucose, 0.5mg of hexokinase/ml, 1mM-ADP, 0.1 μM -rotenone and 5mM-sodium succinate in a final volume of 4ml, at 28°C . The experiment was initiated by the addition of mitochondria equivalent to a concentration of approx. 2mg of protein/ml. Respiration was measured polarographically with a Clark oxygen electrode attached to a Görsz model 520 recorder. After anaerobiosis had been reached, the reaction was stopped by adding 0.3ml of 50% (w/v) trichloroacetic acid. After centrifugation, 1ml of the supernatant was removed and the amount of $^{32}\text{P}_i$ was determined by the method of Avron (1960). For the calculation of the P/O ratio, the amount of P_i esterified in the time-interval between the addition of the mitochondria and anaerobiosis was divided by the O_2 uptake in the same time-interval.

Proton translocation. The assay medium contained 4mM-Tris–HCl buffer (pH 7.4), 100mM-uni-univalent salt as indicated, 50mM-sucrose, 0.1 μM -rotenone, 5mM-sodium succinate, 40 units of catalase and mitochondria equivalent to a concentration of

2mg of protein/ml, in a final volume of 4ml, at 28°C . The initial pH was adjusted to 7.4. Respiration was measured polarographically with a Clark oxygen electrode. Proton translocation was estimated by monitoring the pH changes, in the same reaction vessel, with a glass electrode connected to a Radiometer-26pH meter. Both O_2 uptake and pH changes were recorded with a Görsz model 520 double-channel recorder. For calibration, 5 or 10 μl of 0.1M-HCl or NaOH was injected into the reaction mixture and the deflexion of the recorder was measured.

Assay of ATPase activity. The reaction mixture consisted of 50mM-Tris–HCl buffer (pH 7.4), 5mM- MgCl_2 , 100mM-uni-univalent salt, as indicated, and a mitochondrial preparation containing approx. 2mg of protein/ml in a total volume of 4ml. The reaction was initiated by adding 1.2mM- ^{32}P ATP (1×10^5 – 2×10^5 c.p.m./ μmol) to the mixture. After incubation for 10min at 30°C the reaction was stopped with 0.5ml of 50% (w/v) trichloroacetic acid. After centrifugation, 1ml of the supernatant was removed and the $^{32}\text{P}_i$ liberated was determined (Avron, 1960); controls in which trichloroacetic acid was added before ATP yielded corrections of less than 10% for the counts contributed by $^{32}\text{P}_i$ present in the ^{32}P ATP preparation and liberated non-enzymically.

Measurement of volume changes. Mitochondrial swelling was studied by measuring changes in E_{520} in a Gilford model 2400 spectrophotometer in cuvettes of 1cm light-path against water as a reference. The standard assay medium contained 4mM-Tris–HCl buffer (pH 7.4), 100mM-uni-univalent salt, as indicated, 50mM-sucrose, 0.1 μM -rotenone and 5mM-sodium succinate. The reaction was initiated by adding mitochondria equivalent to 0.4–0.8 E_{520} unit.

Measurement of energy-dependent fluorescence changes (ΔF). Fluorescence intensity changes were recorded at room temperature with an Eppendorf photometer equipped with a fluorimetric attachment and connected to a Varian graphic recorder. A 313–366nm filter was used for the excitation light and a 420–300nm cut-off filter for the emitted light. The standard assay medium contained 4mM-Tris–HCl buffer (pH 7.4), 50mM-KCl, 8mM- MgCl_2 , 0.1mM-EDTA neutralized by Tris (pH 7.4), 5mM-sodium succinate, 0.1mM-rotenone and 15 μM -8-anilino-1-naphthalenesulphonic acid in a total volume of 3ml. The reaction was initiated by the addition of mitochondria equivalent to approx. 2mg of protein/ml. The fluorescence intensity was recorded in the aerobic (F_{ae}) and anaerobic (F_{an}) state, and ΔF is defined as $F_{an} - F_{ae}$ and is expressed in arbitrary units.

Radioactivity determination. The samples were diluted with Bray's (1960) solution and radioactivity was measured with a Packard Tri-Carb model 3320 liquid-scintillation spectrometer.

Table 1. *Effect of oligomycin on energy-dependent functions of mitochondria in the presence of cationophores*

For details see the Experimental section. The uni-univalent salt used in all experiments was KCl. When indicated 0.08–0.1 μg of oligomycin/mg of protein, 4nM-valinomycin and 80 μM-Triton X-100 were added.

Additions	Respiration (ng-atoms of O/min)		Respiratory control ratio	P/O ratio	ATP hydro- lysed (nmol/ ΔF _e (anaero- 10min per bic → aerobic mg of protein) transition)	
	Control	ADP (1mM)				
None	84	340	4.05	1.53	190	60
Oligomycin	84	290	3.96	1.72	200	90
Valinomycin	112	358	3.20	1.28	250	55
Triton X-100	112	360	3.20	1.35	300	55
Valinomycin plus oligomycin	190	310	1.65	0.18	480	30
Triton X-100 plus oligomycin	180	310	1.70	0.46	460	20

Protein determination. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results

In the experiment shown in Table 1 conditions were similar to those under which trialkyltin compounds were reported to uncouple oxidative phosphorylation and cause extensive swelling of mitochondria, i.e., K⁺ was the major cation and Cl⁻ the anion in the assay medium (Stockdale *et al.*, 1970; Selwyn *et al.*, 1970). When oligomycin (0.08–0.1 μg/mg of protein) was added to the mitochondria which respired on succinate, it slightly improved the state of mitochondrial energization as manifested by a higher energy-dependent fluorescence change (ΔF_e) of the probe 8-anilino-1-naphthalenesulphonic acid (cf. Ernster *et al.*, 1971; Nordenbrand & Ernster, 1971; Radda, 1971) and moderate enhancement of the P/O ratio. The effect on ATPase activity was marginal. However, when in addition to oligomycin either of the two K⁺-specific cationophores, valinomycin (Pressman *et al.*, 1967; Pressman, 1969) or Triton X-100 (Brierly *et al.*, 1972), were also included in the assay medium a striking decrease in P/O ratio and ΔF_e, and release of respiratory control and activation of ATPase occurred. In the absence of oligomycin the cationophores caused only slight uncoupling. Thus when the permeability to the major cation, K⁺, was increased by means of an appropriate cationophore, oligomycin, instead of ‘tightening’ the energy-transducing mechanism induced a strong energy-leak, as did the trialkyltin compounds mentioned above.

In an experiment complementary to the one described in Table 1, the effect of oligomycin was tested on the swelling of mitochondria suspended in KCl medium. As shown in Fig. 1, oligomycin induced swelling only in the presence of K⁺-specific cationophores. Thus the conditions for oligomycin-mediated swelling and uncoupling were similar. Since swelling can occur only when the major cation and the anion of the medium are permeable apparently oligomycin facilitated, by some mechanism, the translocation of Cl⁻ across the inner mito-

Fig. 1. *Oligomycin-induced swelling of rat-liver mitochondria*

For details see the Experimental section. The uni-univalent salt used in all experiments was KCl. When indicated 0.08–0.1 μg of oligomycin/mg of protein, 80 μM-Triton X-100 and 4nM-valinomycin were added. Control (○), valinomycin (Δ), Triton X-100 (□), oligomycin (●), Triton X-100 plus oligomycin (■), valinomycin plus oligomycin (▲).

Table 2. *Influence of the ionic composition of the medium on oligomycin-mediated swelling of mitochondria*

For details see the Experimental section. The concentrations of oligomycin, valinomycin and Triton X-100 were as stated in Table 1. The results are expressed as a percentage of the swelling observed in the presence of 100mM-potassium phosphate (pH 7.4) in 5 min.

Additions	Swelling (%)					Potassium acetate
	KCl	NaCl	KBr	KNO ₃	KSCN	
None	20	10	25	30	50	60
Valinomycin	40	12	50	45	120	200
Triton X-100	53	10	60	50	100	180
Oligomycin	40	10	20	30	40	65
Oligomycin plus valinomycin	170	10	70	45	120	210
Oligomycin plus Triton X-100	200	10	80	60	100	200

chondrial membrane. The ion specificity of oligomycin-induced swelling is shown in Table 2. Among the ions tested Cl^- , Br^- and NO_3^- are listed by Chappell & Robinson (1968) among the non-penetrant and acetate ions among the penetrant anions, in rat liver mitochondria. Papa *et al.* (1973) placed NO_3^- with regard to permeability between Cl^- and SCN^- . Pressman (1969) described SCN^- as a highly permeant anion in rat liver mitochondria and in lipid membranes.

The results presented in Table 2 reveal that irrespective of the ionic composition of the medium, oligomycin, in the absence of K^+ -specific cationophores, did not stimulate significantly the rate of swelling. In media that contained a potassium salt the K^+ -specific cationophores in the absence of oligomycin induced rapid swelling when the permeant SCN^- or acetate ion was the anion. The concerted action of oligomycin and a K^+ -specific cationophore produced striking acceleration of swelling in KCl, slight stimulation in KBr and KNO_3 and practically no change of the already rapid rate of swelling in

potassium acetate and KSCN. In NaCl no swelling occurred under any of the above circumstances, apparently because of lack of penetration of Na^+ .

All of the above results support the assumption that the swelling observed is a corollary of net ion transport from the medium into the matrix space through the cristae membrane when the membrane was made permeable to the cation by means of the appropriate cationophore and when the counter-ion was either intrinsically permeable or its permeability was specifically increased (like that of Cl^- by oligomycin). It is also apparent that a close connexion exists between the initiation of net ion transport as manifested by gross swelling (cf. Chappell & Crofts, 1966; Chappell, 1968) and uncoupling.

To give further support to this latter notion the influence of the ionic composition of the medium was tested on some of the energy-linked functions described in Table 1. As shown in Table 3, only a marginal decrease in the P/O ratio was elicited by the simultaneous action of valinomycin (or Triton X-100) and oligomycin in NaCl or KNO_3 medium compared

Table 3. *Influence of the ionic composition of the medium on oligomycin-mediated changes in oxidative phosphorylation by mitochondria*

For details see the Experimental section. The concentrations of oligomycin, valinomycin and Triton X-100 were as stated in Table 1.

Additions	P/O ratio			
	KCl	NaCl	KNO_3	KSCN
None	1.85	1.85	1.64	1.27
Valinomycin	1.53	1.79	1.40	0.40
Triton X-100	1.66	1.80	1.40	0.40
Oligomycin	2.00	1.89	1.38	1.16
Oligomycin plus valinomycin	0.46	1.58	1.21	0.30
Oligomycin plus Triton X-100	0.51	1.60	1.13	0.21

Table 4. *Influence of the ionic composition of the medium on oligomycin-mediated changes in respiratory control in mitochondria*

For details see the Experimental section. The concentrations of oligomycin, valinomycin and Triton X-100 were as stated in Table 1. Respiration is expressed in ng-atoms of O/min. Respiratory control ratio (R_c) is as defined in the Experimental section.

Major ionic

component of
the medium ...

Additions	KCl		NaCl		KBr		KSCN		Potassium acetate	
	Respiration without ADP	R_c	Respiration without ADP	R_c	Respiration without ADP	R_c	Respiration without ADP	R_c	Respiration without ADP	R_c
None	70	3.43	56	3.75	70	3.70	80	3.00	80	3.13
Valinomycin	98	2.45	70	3.40	98	2.80	160	1.20	180	1.40
Triton X-100	85	2.67	80	3.20	84	3.00	180	1.10	160	1.60
Oligomycin	70	3.30	70	3.40	84	3.20	76	2.80	90	2.80
Oligomycin plus valinomycin	180	1.24	80	3.10	90	2.70	180	1.10	200	1.25
Oligomycin plus Triton X-100	168	1.34	88	2.90	84	3.0	190	1.20	220	1.13

Table 5. *Influence of the ionic composition of the medium on oligomycin-mediated changes in energy-dependent fluorescence changes in mitochondria*

For details see the Experimental section. The concentrations of oligomycin, valinomycin and Triton X-100 were as in Table 1.

Additions	ΔF_c			
	KCl	NaCl	KNO ₃	KSCN
None	60	90	60	70
Valinomycin	55	80	45	40
Triton X-100	55	80	40	30
Oligomycin	90	70	50	30
Oligomycin plus valinomycin	30	65	45	30
Oligomycin plus Triton X-100	20	70	50	30

with the strong decrease observed in medium containing KCl. As with swelling, a cationophore alone caused uncoupling in KSCN medium. Release of respiratory control (Table 4) and changes in ΔF_c (Table 5) were maximum under the same conditions which also induced maximum uncoupling of oxidative phosphorylation (see Table 3) or maximum swelling (see Table 2). It should be noted in this context that SCN⁻ at high concentrations was shown to have deleterious effects on membranous systems (Hatefi & Hanstein, 1969). The similarity between the effect of the non-chaotropic acetate ion and of SCN⁻ on the respiratory control (see Table 4) makes it, however, unlikely that perturbation of the membrane structure is involved in the SCN⁻-mediated uncoupling described in the present study.

The experiments described in Fig. 1 and Table 2 strongly indicated that oligomycin facilitated pene-

tration of Cl⁻ through the inner membrane of the mitochondria. It was, however, not clear whether the Cl⁻ transport induced by oligomycin is uniport (cf. Mitchell, 1967a) or is mediated by Cl⁻-OH⁻ exchange as with the trialkyltin compounds (Stockdale *et al.*, 1970). To obtain suitable conditions for the demonstration of a possible oligomycin-facilitated Cl⁻-OH⁻ exchange, an anaerobic suspension of rat liver mitochondria was pulsed with O₂ in the presence of succinate as the respiratory substrate (Fig. 2). Under the above conditions OH⁻ ions are known to be produced in the matrix space of the mitochondria in equivalent amounts with the protons ejected simultaneously into the medium (Mitchell, 1967b). When the acidification of the medium reached its maximum, electron transport was arrested by KCN. In the absence of perturbations, the H⁺-OH⁻ gradient, as judged from the pH of the bulk phase, remained

fairly constant. As shown in Fig. 2 (curve 1a), neither the injection of oligomycin nor the subsequent addition of valinomycin elicited a change in the pH value. The results were, however, different when the sequence of the additions was reversed (Fig. 2, curve 2a). Now valinomycin caused acidification of the medium and oligomycin nearly restored the pH to its original value. The experiments described above were repeated under conditions when the suspending medium contained KNO_3 , instead of KCl . It is apparent that in KNO_3 medium oligomycin was not able to reverse the valinomycin-induced acidification (Fig. 2, curve 2b) and valinomycin caused acidification even when it was added after oligomycin (Fig. 2, curve 1b).

If we adopt Mitchell's (1969) view that the valinomycin-induced proton translocation is a result of 'release of electric back pressure on proton translocation', i.e. that it is due to the abolition of the membrane potential by the K^+ ions moving down the electrical gradient, then the results of the above-described experiments can be tentatively interpreted as follows: since oligomycin caused no alkalization in the absence of valinomycin the possibility of Cl^- - OH^- exchange can be excluded. The alkalization that was observed when oligomycin was added after valinomycin could then possibly be the result of the uniport Cl^- movement induced by oligomycin. The latter is expected to

suppress the electrogenic action of K^+ and thus to restore the membrane potential. In the KNO_3 medium oligomycin cannot initiate uniport anion movement and therefore neither will its presence prevent valinomycin-dependent proton translocation nor will it reverse the acidification induced by oligomycin.

Discussion

Höfer & Pressman (1966) have shown that according to the conditions of the experiment the K^+ -specific cationophore valinomycin either stimulated or uncoupled oxidative phosphorylation in rat liver mitochondria. The above authors explained the uncoupling effect of valinomycin in a K^+ -containing medium by assuming that the respiration-driven ion transport consumes all metabolically derived energy, leaving none to drive the energy-dependent phosphorylation.

An intimate connexion between the ion transport and uncoupling was also apparent from the results of the present investigation. Net ion transport as reflected by mitochondrial swelling (cf. Izzard & Tedeschi, 1973) was invariably connected with energy dissipation and the latter was expressed by characteristic changes in energy-linked parameters. The P/O ratio decreased, ATPase activity increased,

Fig. 2. Changes in the proton-gradient of respiration-inhibited mitochondria induced by valinomycin and oligomycin

For details see the Experimental section. The uni-univalent salt used was KCl in curves (1a) and (2a) and KNO_3 in curves (1b) and (2b). The O_2 pulse was generated by injecting $2\mu\text{mol}$ of H_2O_2 . The following additions were made when indicated with an arrow: 0.05mM -KCN, $0.1\mu\text{g}$ of oligomycin/mg of protein and 4nM -valinomycin. Retraced from recorder traces. The polarographic traces (respiration) are omitted.

respiratory control was released and energy-dependent changes in the fluorescence of the dye 8-anilino-1-naphthalenesulphonic acid were diminished. The ion transport resulted in uncoupling when both the cation and the anion used could pass through the cristae membrane regardless of whether the ion was intrinsically permeable or its permeability was induced by ionophores (i.e. by valinomycin or Triton X-100 for K⁺ ion and apparently by oligomycin for Cl⁻ ion). At the present state of investigation one cannot differentiate between the possibility that oligomycin exerted its effect by activating a pre-existing mitochondrial translocase or that it itself acted as an ionophore as do the trialkyltin compounds (Selwyn *et al.*, 1970). It is not unlikely that the effect of oligomycin on Cl⁻ transport may be only one of the manifestations of the interaction with ion-translocating systems. It is noteworthy that according to Mitchell (1969) ion-translocating systems are also involved in ATP synthesis by the reversible ATPase.

Although the swelling caused by net ion uptake may by itself have a deleterious effect on the integrity of the energy-conserving mechanism situated in the cristae membrane, it seems more likely that the energy dissipation connected with ion transport was the main cause for the uncoupling. The data presented in Fig. 2 suggest, though do not prove, that the oligomycin-induced Cl⁻ transport is uniport. Thus when in the presence of a K⁺-specific cationophore and oligomycin K⁺ will move across the inner membrane of mitochondria as a charged complex, simultaneously with Cl⁻, the net salt movement from the medium into the matrix will be electroneutral and will not cause a dissipation of energy. However, some of the accumulated anions will escape from the matrix and move along the potential gradient, which is more positive outside than inside. Such shuttling of the anion will partly abolish the electrochemical potential and as a corollary will have an adverse effect on all of the energy-linked processes that depend on the maintenance of the protonmotive force.

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