Bioenergetics (1972) 3, 429-443

Specific Inhibition by Macrocyclic Polyethers of Mitochondrial Electron Transport at Site I*

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Received 10 January 1972

Abstract

The macrocyclic polyethers dibenzo-18-crown-6 (XXVIII) and dicyclohexyl-18-crown-6 (XXXI) inhibit the valinomycin-mediated K^+ accumulation energized by glutamate, α-ketoglutarate, malate plus pyruvate or isocitrate but not that promoted by succinate, ascorbate plus TMPD or ATP. The polyethers inhibit the oxidation of the former group of substrates without preventing either the oxidation of succinate or ascorbate plus TMPD or the hydrolysis of ATP.

The substrate oxidation inhibited by the macrocyclic polyethers is relieved in intact mitochondria by increasing the concentration of K^+ in the medium. It is also completely reverted by supplementing the medium with valinomycin, Cs^+ and phosphate, or else by the addition of vitamin K_3.

In submitochondrial sonic particles the macrocyclic polyethers inhibit the oxidation of NADH as well as the ATP-driven reversal of electron flow at the site I of the electron transport chain. They also block the oxidation of NADH in non-phosphorylating Keilin-Hartree particles as well as in Hatefi's NADH-Coenzyme Q reductase. The polyethers do not inhibit electron transport in mitochondria from the yeast which lack the first coupling site.

The inhibition of electron transport by the polyethers do not require of the addition of alkali metal cations such as K^+ in intact mitochondria or other membrane preparations.

It is established that the macrocyclic polyethers XXVIII and XXXI, already characterized as mobile carrier molecules for K^+ in model lipid membranes, inhibit electron transport at site I of the electron transport chain from mitochondrial membranes.

* Supported in part by a Grant from the Research Corporation.

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It is suggested that the ability of the polyethers to coordinate alkali metal cations in aqueous versus lipid environments, but not K^+ transport *per se*, is related to their rotenone-like induced inhibition of electron flow in mitochondrial membranes.

Introduction

Previous observations by Pedersen [1, 2], Eisenman, Ciani and Szabo [3], Pressman [4] and Tosteson [5] have shown that macrocyclic polyethers XXVIII and XXXI (dibenzo, 18-crown-6 and dicyclohexyl, 18-crown-6 respectively), behave as K^+-mobile carriers in lipid bilayers, erithrocyte membranes and two phase watermock (water-bulk) lipid systems. Data by Lardy [6] have also shown that the macrocyclic polyethers synthesized by Pedersen [1] perturb the distribution of alkali metal cations and substrate oxidation in mitochondrial membranes.

The observations hereby presented indicate that the above macrocyclic polyethers specifically inhibit electron flow at the NADH-cytochrome segment of the mitochondrial respiratory chain. This inhibitory effect is exerted by the polyethers in intact mitochondria from rat liver and beef heart, in submitochondrial sonic particles, in Keilin-Hartree preparations and in Hatefi complex I (NADH-coenzyme Q reductase) from beef heart sarcosomes. The above results are evaluated with respect to the possible relation existent between K^+ transport, membrane K^+ requirements or cation complex-coordinates formed in the membrane and the ability of polyethers to inhibit electron flow at the NADH-cytochrome segment of the respiratory chain.

Materials and Methods

Membrane Preparations

Rat liver mitochondria were prepared as described by Johnson and Lardy [7]; beef heart sarcosomes were isolated as indicated by A. L. Smith [8]; yeast mitochondria from *S. cerevisiae* were prepared after Mattoon and Balcavage [9]; submitochondrial sonic membrane fragments were isolated as described by Ch. T. Gregg [10]; The Keilin-Hartree heart muscle preparations were prepared as described by King [11] and NADH-coenzyme Q reductase (Complex I of the respiratory chain) was semi-purified as shown by Hatefi and Rieske [12].

Measurement of Ion Movements, Oxygen Consumption and Light Scattering Changes in Mitochondria

A continuous recording of oxygen consumption, light scattering changes and variations in the extramitochondrial concentration of K^+

and protons was carried out by means of an apparatus designed, developed and constructed by Chance, Mayer, Pressman and Graham [13-15].

Measurement of Electron Transport Reactions and ATPase Activity

The energy linked reduction of NAD^+ by succinate was measured as shown by Lee and Ernster [16]. The NADH-coenzyme Q reductase activity was estimated as described by Hatefi and Rieske [12]. Oxidative phosphorylation was assayed as indicated by Chance and Williams [17]. ATPase activity was measured by the method of Lardy and Wellman [18] and inorganic phosphate was determined by the method of Sumner [19]. Protein was determined by the Biuret Method [20].

The macrocyclic polyethers XXVIII and XXXI were a generous gift from Dr. C. J. Pedersen, Dupont de Nemours Laboratory, Delaware. The antibiotic nigericin was kindly supplied by Dr. Marvin Gorman, The Eli Lilly laboratories.

Results

Effect of Polyethers XXVIII and XXXI on Ion Movements and Substrate Oxidation in Intact Mitochondria

A detailed study on the effect of different macrocyclic polyethers on mitochondrial oxidative phosphorylation has been published by Lardy [6]. Such information showed that both polyethers XVIII and XXXI caused an inhibition of glutamate oxidation parallel to the efflux of K^+ previously accumulated by valinomycin-like ionophores into mitochondria. Evidence described in Fig. 1 provided the clue to understand the possible mechanism of the respiratory inhibition induced by the polyethers in mitochondria. Panel A from Fig. 1 shows that 5×10^{-5} M polyether XXVIII inhibits glutamate oxidation simultaneous to an induced efflux of K^+ previously accumulated by 2×10^{-7} M valinomycin into mitochondria. The polyether does not cause a countermovement of protons parallel to the ejection of K^+ from the organelles. Moreover, the addition of 8×10^{-3} μmoles vitamin K_3 (menadione) after the polyether, not only completely overcomes the inhibited oxidation of glutamate but also allows valinomycin to stimulate a simultaneous uptake of K^+. In turn, anaerobiosis causes release of the accumulated K^+. Similar results are obtained when glutamate is substituted by α-ketoglutarate, L-malate or isocitrate. In contrast, when glutamate is replaced for by succinate in the medium (Panel B, Fig. 1), polyether XXVIII shows to be completely ineffective either to inhibit oxygen uptake or to reverse the valinomycin mediated K^+ movements. The addition of nigericin after the polyether catalyses a

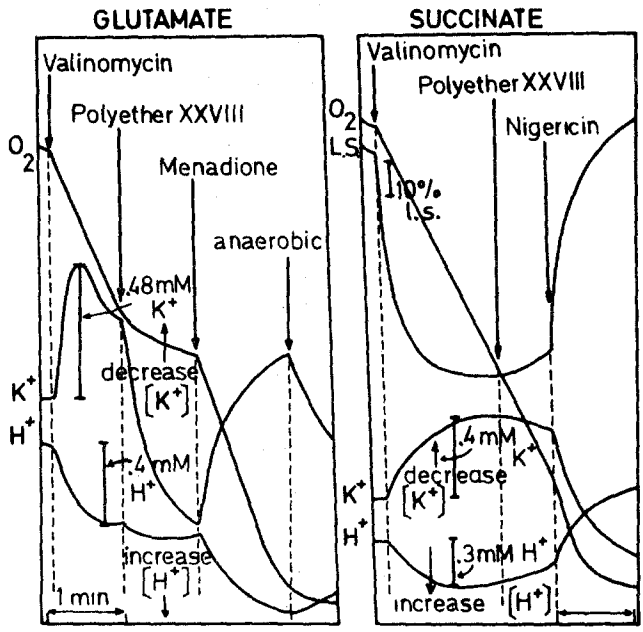

Figure 1. The effect of polyether XXVIII, menadione and nigericin on the oxidation of glutamate or succinate and the K^+ and H^+ movements respectively stimulated by valinomycin in intact mitochondria. The reaction mixture contained 5 mM KCl, 5 mM triethanolamine (TEA)–HCl (pH 7.4), 3 mM inorganic phosphate (TEA) pH 7.4, 220 mM sucrose, and 1.4 mg mitochondrial N in a 5 ml volume at 28° C. Valinomycin and polyether XXVIII were added at concentrations of 2×10^{-7} M and 5×10^{-5} M respectively; where indicated vitamin K_3 or menadione and nigericin were added at concentration of 8×10^{-3} μmoles and 1.4×10^{-6} M respectively. Panel *A* contained 10 mM glutamate (TEA) pH 7.4, and panel *B* 10 mM succinate (TEA) pH 7.4.

reversal of the K^+ and H^+ movements induced by valinomycin, and a discrete enhancement of succinate oxidation [21]. Almost identical negative inhibitory effects of the polyether are found when succinate is substituted by ascorbate plus TMPD. At a concentration of 5×10^{-5} M, polyether XXVIII does not affect the oxidative phosphorylation of glutamate (substrate from site I) [6], succinate (substrate from site II) [6], or ascorbate plus TMPD (substrate from site III) (0.9 P/O value without, and 0.83 with 5×10^{-5} M polyether XXVIII were found for the latter pair). Moreover, when ATP hydrolysis is used as energy source for alkali ion transport (Fig. 2), polyether XXVIII does not affect the oscilatory uptake of K^+ and the light scattering changes catalysed by valinomycin plus K^+. The further addition of nigericin causes a rapid loss of the accumulated K^+ and a reversal of the light scattering decrease

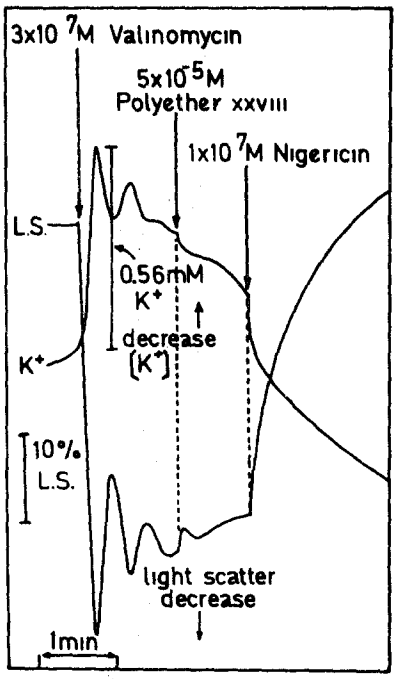

Figure 2. The effect of polyether XXVIII and nigericin on the ATP-energized K^+ movements and light scattering changes induced by valinomycin in intact mitochondria. The reaction mixture contained 5 mM KCl, 10 mM triethanolamine–HCl (pH 7.4), 200 mM sucrose 5 mM ATP-Tris (pH 7.4) and 1.2 mg mitochondrial N in 5 ml volume at 28°. Valinomycin, polyether XXVIII and nigericin were added at the indicated concentrations.

trace. In the presence of K^+ and at concentrations one or two orders of magnitude higher than 5×10^{-5} M, polyether XXVIII moderately uncouples the oxidative phosphorylation of succinate or ascorbate plus TMPD. At such concentrations, it also stimulates ATP hydrolysis in media containing alkali metal cations with the selectivity $K^+ > Rb^+ > Cs^+$. However, even at 10^{-3} M, the above compound does not inhibit succinate oxidation regardless of the addition of high concentrations of alkali metal ion. Thus, from the above results it is apparent that polyether XXVIII inhibits oxygen as well as K^+ uptake only when cation transport is energized by substrates oxidized at the level of the site I of the respiratory chain.

Dicyclohexyl, 18-crown, 6 (polyether XXXI) shows identical oxidizable substrate selectivity than dibenzo, 18-crown, 6 (polyether XXVIII) to inhibit oxygen uptake in intact mitochondria or submitochondrial membrane fragments. However, compound XXXI is

quantitatively less active than polyether XXVIII to inhibit glutamate oxidation. In fact the former compound moderately stimulates state IV respiration in the presence of succinate and K^+ even at concentrations (10^{-6} M) where polyether XXVIII shows negligible effects on succinate oxidation. The apparent valinomycin-like "uncoupling" effect [22] of polyether XXXI, may be related to its quantitatively greater ability, with respect to compound XXVIII, to transport K^+ across lipid bilayers of the Mueller-Rudin type [3]. Thus, because of the undesirable "uncoupling" effect of compound XXXI, polyether XXVIII was used in all experiments described below.

Effect of Different Anions and Cations on the Inhibition of Electron Transport by Polyethers in Intact Mitochondria

Figures 3-6 indicate that some of the cation chlatrating properties of polyether XXVIII [23] are related to its ability to inhibit substrate oxidation at site I of the electron transport chain. Similar to nigericin-like ionophores [24], the ionic selectivity of polyether XXVIII to inhibit oxygen uptake is dependent on the anion present in the medium (Fig. 3). The polyether does not show appreciable ionic discrimination to inhibit oxygen uptake in the presence of anions such as acetate or formate (lower tracing, Fig. 3); however, it clearly discriminates between K^+ and other alkali metal ions when inorganic orthophosphate is used as co-ion (upper tracing, Fig. 3). A previously postulated role of anions, as source of protons for facilitating the membrane exchange of alkali metal ions [24] could be the explanation for the above results. The oxygen uptake block mediated by the macrocyclic mobile carrier is also sensitive to the ionic gradients established across the mitochondrial membrane. As seen in Fig. 4, the increase in the concentration of extra-mitochondrial K^+, gradually reverts the respiratory inhibition induced by the polyether at the NADH-cytochrome segment of the respiratory chain. Moreover, Fig. 5 illustrates that 20 mM CsCl and valinomycin overcome the polyether induced-inhibition of glutamate oxidation previously stimulated by 2,4-dinitrophenol in the absence of added alkali metals. Twenty mM of cations such as Li^+ or Na^+ do not replace for Cs^+ in the above effect.

Effects of Polyether XXVIII on Electron Transport at Site I of the Respiratory Chain in Submitochondrial Membrane Fractions

The above results suggested that the site-specific inhibition of respiration induced by the macrocylic polyether was related with its ability to form coordination complexes with alkali metal ions in the membrane or with an induced transport of K^+ across the intact

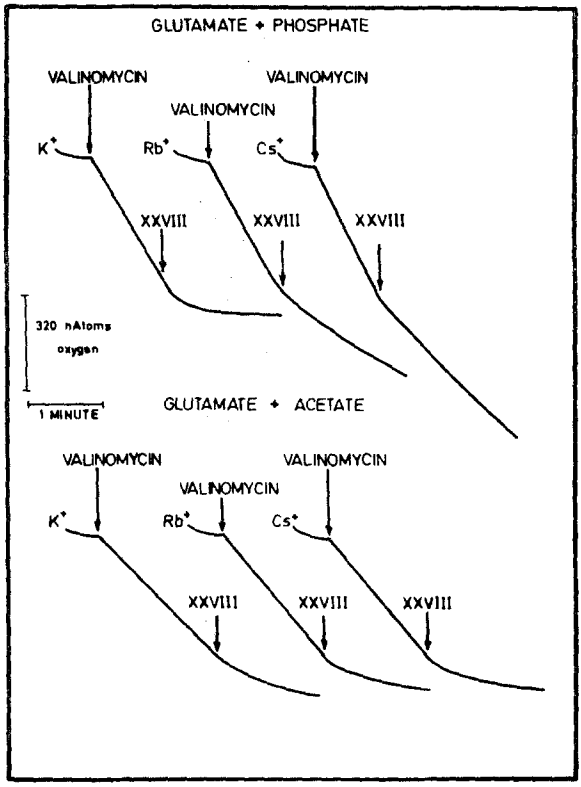

Figure 3. The effect of phosphate or acetate on the ionic selectivity of polyether XXVIII to inhibit the oxidation of glutamate stimulated by valinomycin in mitochondria. The reaction mixture contained 8 mM KCl, mM triethanolamine HCl, pH 7.4, 10 mM glutamate–(TEA) pH 7.4, 200 mM sucrose and 1.2 mg mitochondrial N. Valinomycin and polyether were added at concentrations of 2×15^{-7} M and 5×10^{-5} M respectively. Where indicated the TEA-salts of acetate of phosphate were present at a concentration of 5 mM.

mitochondrial membrane. The possibility that such inhibitory action could be directly exerted at the level of the electron transport mechanism in the respiratory chain was subsequently evaluated in different mitochondria membrane preparations. Figure 6 shows the inhibitory effect of polyether XXVIII on the oxidation of NADH by submitochondrial sonic particles. After the oxidation of NADH is coupled by oligomycin in these system [16] polyether XXXIV [1, 2] plus nigericin and K^+ uncouple substrate oxidation [25, 26] and polyether XXVIII subsequently inhibits oxygen uptake (Panel A, Fig. 6). The reversed electron transfer energized by ATP from succinate to NAD^+ is also inhibited by the macrocyclic polyether in submitochon-

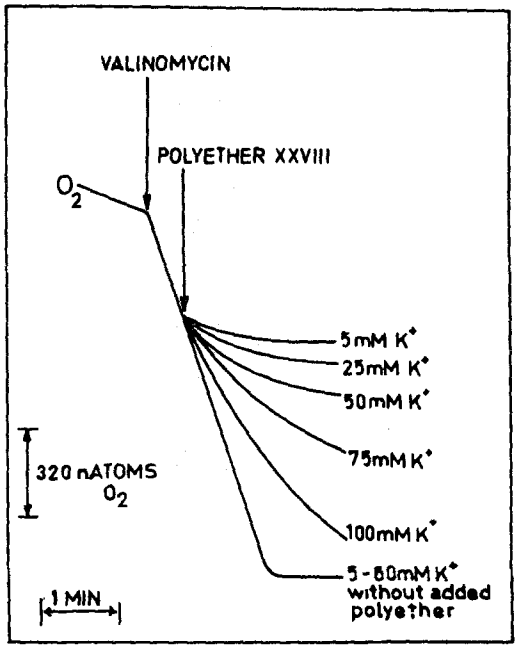

Figure 4. The reversal by increasing concentrations of KCl of the inhibition by polyether XXVIII of the oxidation of glutamate stimulated by valinomycin. The media was essentially the same to that described in Fig. 3 except for the addition of 5 mM phosphate as penetrant anion and the indicated concentrations of KCl.

drial sonic membrane fragments (Panel B, Fig. 6). Moreover, as shown in Table I, the oxidation of NADH occurring in Keilin-Hartree preparations and purified NADH Co Q reductase is also completely blocked by polyether XXVIII. It is also apparent that the respiratory chain inhibited by the polyether, catalyses an antimycin sensitive oxidation of vitamin K_3 [27] which bypasses the polyether inhibited site in intact mitochondria (Fig. 1A) as well as in mitochondrial membrane fragments (Table I).

Effect of Polyether XXVIII on Electron Transport from Mitochondria of Yeast S. cerevisiae

In contrast with the above results, when polyether XXVIII is tested as an inhibitor of electron transport in mitochondria from the yeast *Saccharomyces cerevisiae* which lack the first coupling site [28] and are insensitive to rotenone, it is observed that this preparation is also insensitive to the macrocyclic polyether (Panels A and B, Fig. 7).

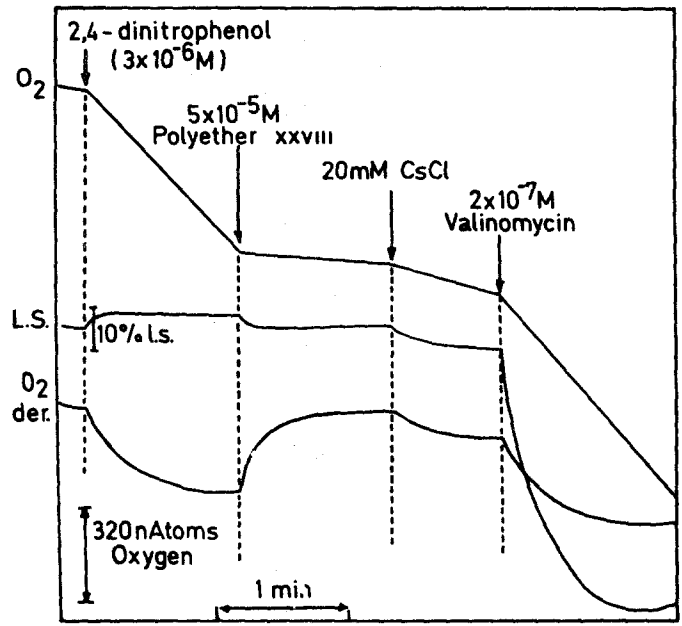

Figure 5. The reversal by Cs^+ plus valinomycin of the inhibition by polyether XXVIII of the mitochondrial oxidation of glutamate stimulated by 2,4-dinitrophenol, in medium initially lacking of added alkali ions. Except for the omission of KCl and the addition of 10 mM acetate instead of phosphate, as well as that of 15 mM choline-chloride the basic medium was essentially that from Fig. 3. Other additions were supplemented at the described concentrations.

Discussion

Macrocyclic polyethers XXVIII and XXXI appear as very interesting model compounds. Not only they complex and transport alkali metal cations through hydrophobic interfaces [1, 3-6, 29, 30] but, as shown in this work, they also inhibit electron flow at the NADH-cytochrome segment of the mitochondrial respiratory chain. The central question arising from this phenomenology is whether the above effects are: (a) circumstantially associated but completely unrelated in their respective mechanism, or (b) mechanistically related. The latter possibility seems to be very appealing as a working hypothesis. By analogy with nigericin, which may be considered as an example of a mobile carrier [21], it is apparent that polyether XXVIII inhibits electron flow, in intact mitochondria studies, associated with its ability to form coordination complexes with alkali metal cations. Similar to nigericin [15, 31], the oxygen uptake block catalysed by the polyether is reverted in intact mitochondria by the increase in the concentration of K^+ in the presence

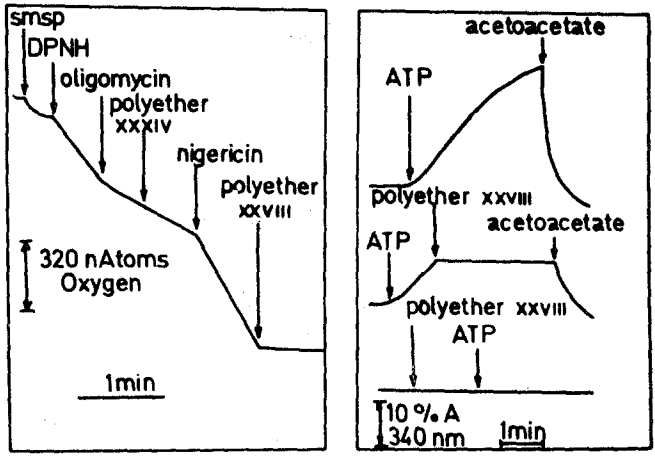

Figure 6. The inhibition by polyether XXVIII of the oxidation of NADH (Panel A) and the reversed electron transfer energized by ATP from succinate to NAD^+ (Panel B) in submitochondrial sonic particles. The reaction mixture from panel A contained: 15 mM KCl, 50 mM Tris-acetate (pH 7.4), 1.5 mM NADH, 1.0 μg/ml oligomycin, 8×10^{-6} M polyether XXXIV, 2.3×10^{-7} M nigericin, 5×10^{-5} M polyether XXVIII and beef heart submitochondrial sonic particles equivalent to 1.5 Mg N in a 3.0 ml volume at 25°. The media from panel B contained 20 mM Tris HCl pH 8.0; 4 mM $MgCl_2$; 0.25 sucrose, 1 mM KCN, 1 mM ATP and approximately 0.65 mg mitochondrial protein. The reaction was initiated by the addition of 10 mM succinate and 1.5 mM DNP.

of valinomycin, as well as by the possible competition induced for transport or complex formation between Cs^+ and K^+ (Figs. 4-5) in hydrophobic versus aqueous environments (see McLaughlin *et al.*, [29]). Moreover, parallel also to nigericin [24], anion translocation regulates the ionic selectivity of the respiratory block induced by the polyethers (Fig. 3). The above observations possibly implicate that the translocation of the K^+-polyether complex into the hydrophobic core of the membrane is dependent on the nature of the alkali metal cation present in the aqueous versus the hydrophobic environment, as well as on the distribution of cations, protons and anions across the membrane. Parallel to the above results, it is noteworthy that polyether XXVIII inhibits electron flow through an effect similar to that of rotenone. The fact that it inhibits the oxidation of NADH-linked substrates in intact mitochondria, without altering the phosphorylation and oxygen uptake linked to the oxidation of succinate or ascorbate plus TMPD, falls in this line. Also, in support to the above contention, is the observation that the polyether inhibits the reversal of electron transport coupled to NAD^+ reduction (Fig. 6B) as well as the oxidation of NADH is sonic membrane fragments, Keilin-Hartree preparations and NADH-Co Q reductase

TABLE I. The effect of macrocyclic polyether XXVIII on the oxidation of NADH by different mitochondrial membrane preparations

Membrane preparation	Oxygen uptake rate		
	Without additions	Plus rotenone	Plus polyether XXVIII
	nAtoms O_2 per min per 5 mg protein		
Submitochondrial sonic particles	405	0	20
Sonic particles plus menadione	400	396	403
EDTA sonic particles	380	42	80
Keilin-Hartree preparation	365	0	24
Hatefi complex I (NADH-Co Q reductase)*	350	0	0

The reaction mixture for assaying the oxidation of NADH in submitochondrial sonic particles and EDTA sonic particles was supplemented with 10 mM Tris-Cl^- pH 7.4, 250 mM sucrose, 1.5 mM NADH and approximately 2 mg N in 3 ml volume at 25°. Menadione was added at a concentration of 8×10^{-3} μmoles. NADH oxidation was assayed in the Keilin-Hartree preparation in a medium containing 20 mM KH_2PO_4 pH 7.8, 1.5 mM NADH, 9×10^{-6} M cytochrome *c*, 200 mM sucrose and 1.2 mg N in 3.0 ml volume. NADH oxidation was measured for the NADH-Co Q reductase preparation in a medium containing: 5 mM KH_2PO_4 pH 8.0, 5 mM NaN_3, 5 mM Tris-Cl^- pH 8.0, 1 mM histidine (Cl^-) pH 8.0, 1.5 mM NADH, 0.67 M sucrose 1.5 mg asolectin and 0.5 mg N of complex I in 1.0 ml volume. Rotenone and polyether XXVIII were added at 5×10^{-6} M and 5×10^{-4} M respectively.

* The activity of NADH-Co Q reductase is expressed in μmoles NADH oxidized/min/5 mg nitrogen.

(Fig. 6A and Table I), through and antimycin sensitive oxidation of vitamin K_3 which bypasses the polyether inhibited site.

Polyether XXVIII seems not to require of added alkali metal cations to inhibit electron transport in intact mitochondria or submitochondrial membrane fragments (Fig. 6, Table I). These data could lead to the wrong conclusion that K^+ or any other metal ion is not involved in the mechanism of electron flow inhibition mediated by the polyethers. However, in contrast with this latter point of view, data to be published elsewhere (Cárabez and Estrada-O., in preparation) indicate that in mitochondria, and only in the presence of added alkali ions such as K^+, but not Li^+ or Na^+, the polyether abates the fluorescence of ethidium bromide, a dye which monitors microscopic variation of charge surface in biological and model membranes [32, 33]. Rotenone reproduces the effects of polyethers XXVIII in the above parameter, without detectable K^+ requirements.

The results hereby presented point toward three alternatives regarding the association existing between polyether-cation complex formation and/or K^+ transport with respect to the flow of electrons at the NADH-cytochrome segment of the respiratory chain. Possibility (1)

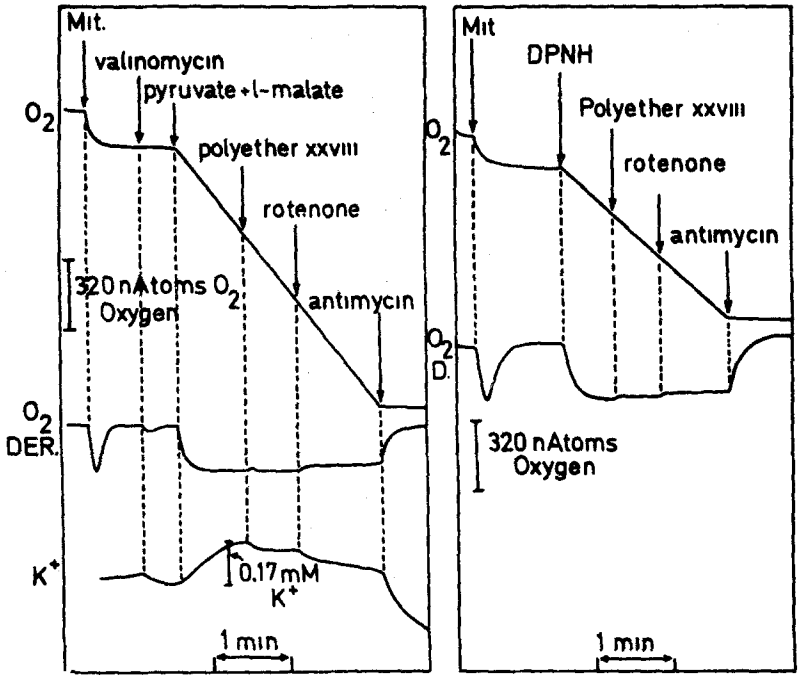

Figure 7. The effect of polyether XXVIII, rotenone and antimycin on the oxidation of pyruvate plus malate and K^+ movements induced by valinomycin as well as the oxidation of NADH by mitochondria from the yeast *Saccharomyces cerevisiae*. The basic reaction mixture in both experiments was essentially that from Fig. 3. Other additions where indicated were: 2×10^{-7} M valinomycin 8 mM of both pyruvate and L-malate, 1 mM DPNH, 5×10^{-5} M polyether XXVIII, 2×10^{-6} M rotenone, 8×10^{-7} M antimycin and yeast mitochondria equivalent to 2.6 mg N in 5 ml volume at 20°.

implicates that the ability of the macrocyclic polyether to coordinate either an electron carrier bound K^+ in the membrane lipid phase, or iron in a hydrophobic environment in a non-heme iron protein, is associated to its induced electron transport block. The latter possibility has also been suggested for rotenone mode of action in the respiratory chain [34]. Possibility (2) would indicate that the transport and membrane accumulation of the K^+-polyether complex could affect *per se* the operation of the NADH-cytochrome segment of the respiratory chain. The relatively high ability of this *locus* to microscopic variations in the physical properties of the membrane [35, 36], caused by the polyether-K^+ charged pair as well as by charged compounds such as the guanidines [37, 38], by Na^+ [39] or by lead ions [40], could be partly responsible for the effects observed. Possibility (3) implies that neither

K^+ is inextricably related with alkali ion requirements of the respiratory chain, nor with the intimate molecular mechanisms of the electron transport block mediated by macrocyclic polyethers. Instead, a given alkali metal cation would only be required for the proper solubility of the cation–polyether complex into the lipid phase and to its target site, at hydrophobic *loci* of the mitochondrial membrane. This hypothesis, which we strongly favour, implies that polyether XXVIII is sharing with rotenone a similar mechanism of respiratory inhibition and that the lipid versus the aqueous solvation of polyether XXVIII, is a function of the stability of the cation polyether complex in either phase, which depends on the nature of the chlatrated cation. In fact, the ionic selectivities of the aqueous association constants of polyether-cation complexes [29, 30, 41], are different from their permeability ratios determined from potential measurements in lipid bilayers [29]. Following this line it is possible that Cs^+ reverts the respiratory inhibition caused by the polyether in mitochondrial membranes (Fig. 5), by primarily removing this compound from its target site at the NADH-cytochrome segment of the respiratory chain.

A final consideration is worth noting in assessing the significance of the inhibition by neutral K^+-coordinating compounds of electron transport sites in energy transducing membranes. Previous observations by Baltschefsky and Arwidsson [42] showed that valinomycin inhibited photophosphorylation at only one of two possible sites in the cyclic electron transport of chromatophores from *Rhodospirillum rubrum*. Gromet-Elhanan [43] has also found that valinomycin or nonactin inhibit various photophosphorylating and photoreducing systems of *Rhodospirillum rubrum* chromatophores. Moreover, an inhibitory effect of valinomycin in ferricyanide reduction was observed by McCarthy [44] in chloroplasts and subchloroplast particles. Such inhibition was relieved by NH_4Cl. Moreover, similar to the macrocyclic polyethers in mitochondria, the inhibitory effect of valinomycin or nonactin in photophosphorylating membranes, was apparently independent of the presence of transportable ions such as K^+ [43]. We consider that it is more than a coincidence the parallelism existent between ionophores and electron transport in both membrane systems. Thus, similar alternatives to those suggested in the present manuscript concerning the inhibition of electron transport by macrocyclic polyethers in mitochondrial membranes, may also be considered to understand the inhibition of electron transport [43] by valinomycin or nonactin in photophosphorylating membranes.

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