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ON THE MECHANISM OF ACTION OF POLYETHER XXVIII AT SITE I OF THE ELECTRON-TRANSFER CHAIN IN RAT LIVER MITOCHONDRIA

ALFONSO CARABEZ [a] and FRANCISCA SANDOVAL [b,*]

[a] *Centro de Investigaciones en Fisiologia Celular Seccion de Microscopia Electronica and* [b] *Departamento de Bioquimica Facultad de Medicina, UNAM, Apartado postal 70-600, Mexico City 20 D.F. (Mexico)*

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In rat liver mitochondria, the macrocyclic polyether, dibenzo-18-crown-6 (polyether XXVIII) inhibits the oxidation of NAD-dependent substrates, as stimulated by ADP, uncouplers and valinomycin plus K^+. It does not inhibit the oxidation of succinate. It is concluded that polyether XXVIII inhibits electron transfer in the NADH-CoQ span of the respiratory chain. This is a process that is reversed by menadione. Inhibition of oxidation of NAD-dependent substrates in K^+-depleted mitochondria induced by the polyether is reversed by concentrations of K^+ higher than 60 mM, and also by Li^+, a cation that does not complex with polyether XXVIII. As assayed by swelling mitochondria, reversal of the inhibition of electron transfer is accompanied by influx of monovalent cations. Polyether XXVIII also inhibits in submitochondrial particles the aerobic oxidation of NADH, but not that of succinate; this inhibition is also reversed by K^+ at high concentrations, and Li^+. The data are consistent with the hypothesis that a monovalent cation is required for maximal rates of electron transport in the NADH-CoQ span of the respiratory chain.

Introduction

It has been reported [1,2] that polyether XXVIII inhibits the coupled oxidation of NAD-dependent substrates in intact rat liver mitochondria. It also inhibits NADH oxidation in yeast mitochondria, submitochondrial particles, Keilin-Hartree preparations, and in complex I of Hatefi [3,4]. As polyether XXVIII forms clathrates with K^+, Rb^+ and Cs^+, but not with Li^+, in cell-free systems [5], it is probable that the afore-mentioned inhibition of respiration is a consequence of this property. In this work, this possibility has been explored, particularly since it has been reported that alkali metal ions are required for maximal rates of electron transport in the NADH-CoQ segment of the respiratory chain [6–8].

The stimulation of NADH oxidation by monovalent cations has been ascribed to their effect on the surface charge of the membrane which in turn modifies the affinity of the enzyme towards the negatively charged substrate [7]. However, this explanation is not entirely satisfactory, since positively charged lipophilic cations, which in principle would seem to exert a similar effect on the surface charge of the membrane, do not stimulate, but instead induce strong inhibition of the oxidation of NAD-dependent substrates in intact mitochondria [9,10] and NADH in submitochondrial particles [11]. The apparent controversy could indicate that in addition to modifications of the membrane surface charge, electron transport in the respiratory chain somehow depends on the presence of a monovalent cation. This has been studied by measuring the characteristics of the action of polyether XXVIII on electron transport. The results indicate that polyether XXVIII inhibits the

* To whom correspondence should be addressed.

Abbreviations: polyether XXVIII, dibenzo-18-crown-6; 1799, hexafluoroacetone : acetone 2 : 1 adduct; menadione, 2-methylnaphthoquinone.

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oxidation of NADH by chelation of a membrane-bound cation that is required for maximal rates of electron transport in the NADH-CoQ span of the respiratory chain.

Materials and Methods

Rat liver mitochondria were prepared according to the method of Schneider and Hogeboom [12] in 0.25 M sucrose, 1 mM EDTA, adjusted to pH 7.4 with Tris base. K^+-depleted mitochondria were prepared as described by Gómez Puyou et al. [6]. The K^+ content of the latter preparations was less than 15 nmol/mg protein, as determined by flame photometry in perchloric acid extracts (10% final concentration) of the mitochondrial preparation.

Oxygen uptake was measured polarographically using the conditions described in Results in a Yellow Springs Oxygraph (model 55). Rat liver EDTA/sub-mitochondrial particles were obtained as described by Lee et al. [13]. The K^+ content of these particles was 8 nmol/mg protein. Swelling of the mitochondria was recorded spectrophotometrically at 546 nm under the conditions described in Results. Protein was assayed by the biuret method [14].

Polyether XXVIII was kindly supplied by Dr. Frensdorf from E.I. DuPont de Nemours (Willmington, Delaware, U.S.A.) and recrystallized twice from ethanol/water (2 : 1, v/v). The uncoupler 1799 was a generous gift of Dr. E. Racker, Cornell University. All reagents used were of the highest purity commercially available.

Results

In agreement with previous reports [1,2], the results of Fig. 1A show that polyether XXVIII inhibits the ADP-stimulated oxygen uptake with β-hydroxybutyrate as substrate. Inhibition of respiration is also observed when it is stimulated by valinomycin or by uncouplers of oxidative phosphorylation with glutamate plus malate as substrates (Fig. 1B and C). In contrast, polyether XXVIII does not inhibit the oxidation of succinate as stimulated by ADP (or by uncouplers or by valinomycin + K^+, data not shown), rather a moderate enhancement of respiration is attained (Fig. 1D). These observations therefore suggest that polyether XXVIII interferes with processes that occur on the substrate side of cytochrome *b*.

One of the most obvious characteristics of polyether XXVIII is its ability to chelate K^+ [2,5]. Since influx of certain NAD-dependent substrates is apparently linked to the influx of K^+ [15,16], the inhibiting action of the polyether could be due to an impairment in the entrance of the substrate. However, this does not seem to be the explanation for the observed inhibition of respiration, since menadione reverses the action of polyether XXVIII (Fig. 2), and because inhibition is also observed in the presence of β-hydroxybutyrate (Fig. 1A). Rather, the data seem to suggest that polyether XXVIII affects electron transport in the NADH-CoQ span of the respiratory chain.

As it has been reported that K^+ is required for maximum rates of electron transport and phosphorylation [4,6,8,17], it was considered that the inhibiting action of the polyether XXVIII could be due to removal of intramitochondrial K^+ which could be required for maximal rates of NADH oxidation. The results of Fig. 3 indicate that the extensive swelling that occurs in the presence of valinomycin + K^+ and oxidizable substrates is reversed by the addition of polyether XXVIII. The partial release of accumulated intramitochondrial K^+ as mediated by valinomycin and K^+ parallels inhibition of respiration (cf. data of Figs. 2B and 3). Although the results may be consistent with the idea that polyether XXVIII induces release of cations and subsequent arrest of electron transport, it is equally possible that release of intramitochondrial K^+ is a consequence and not the cause of inhibition of electron transport.

To explore further this possible action of polyether XXVIII, its effect on the oxidation of NAD-dependent substrates by K^+-depleted mitochondria was tested. The results of Fig. 4A show that in mitochondria incubated with 7.5 mM K^+ and valinomycin, polyether XXVIII exerts a strong inhibition of respiration. However, if the mitochondria are incubated with Li^+, which under the experimental conditions employed induces stimulation of respiration (Fig. 4B), the inhibiting effect of the polyether is only 20% as compared to that obtained in the presence of K^+. Moreover, Li^+ reverses the inhibiting action of the polyether (Fig. 4C).

Thus, these experiments suggest that formation of

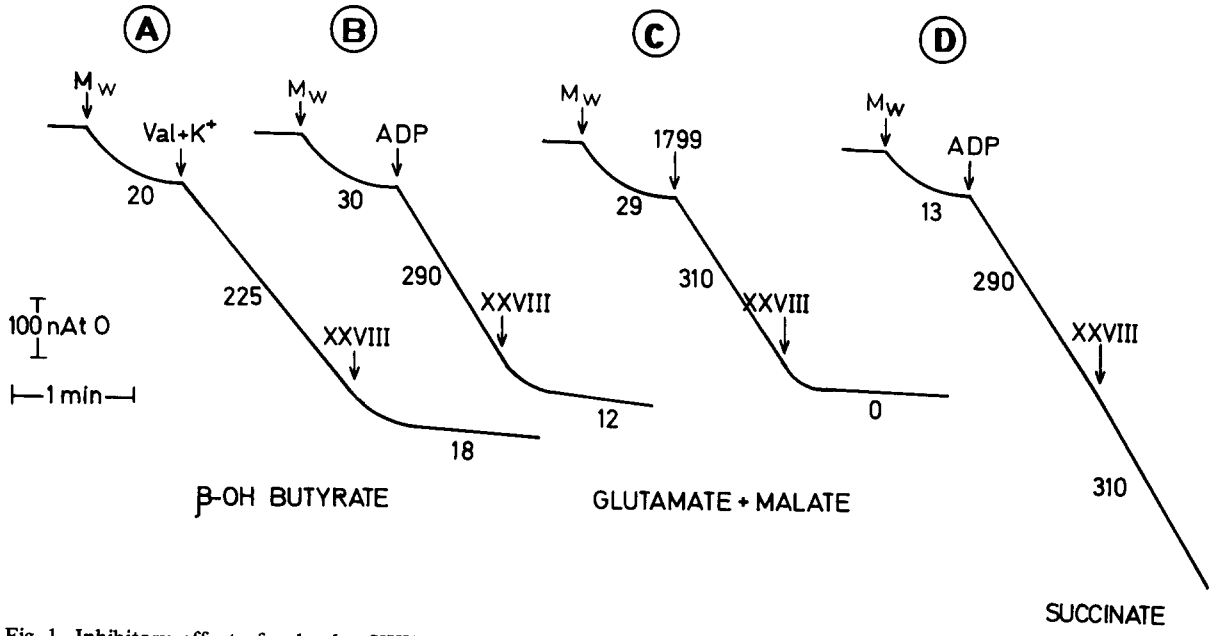

Fig. 1. Inhibitory effect of polyether XXVIII on mitochondrial respiration induced by different compounds in the presence of NAD-dependent substrates and succinate. The basic incubation media contained: 250 mM sucrose, 1 mM EDTA, 4 mM phosphate buffer, which had been neutralized with Na^+, 1 mM $MgCl_2$, 6 mM KCl, 3 mM oxidizable substrates, and 3 mg mitochondrial protein in a final volume of 3.0 ml. Where indicated, 66 μg polyether XXVIII/mg protein, 0.9 μg valinomycin (Val), 0.8 mM ADP, and 3 μg 1799 were added. With succinate as substrate 3 mM ADP was added. The numbers on the trace indicate ngatoms (nAt) O/min. M_w, washed mitochondria.

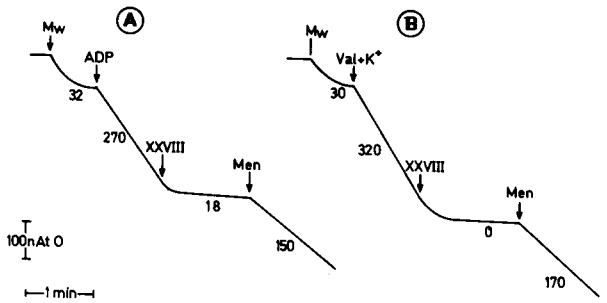

Fig. 2. Effect of menadione on the inhibition of mitochondrial oxygen uptake by polyether XXVIII. Experimental conditions as in Fig. 1. Oxidizable substrates were glutamate + malate, 3 mM each, and where shown 10^{-5} M menadione (Men) was added. Val, valinomycin.

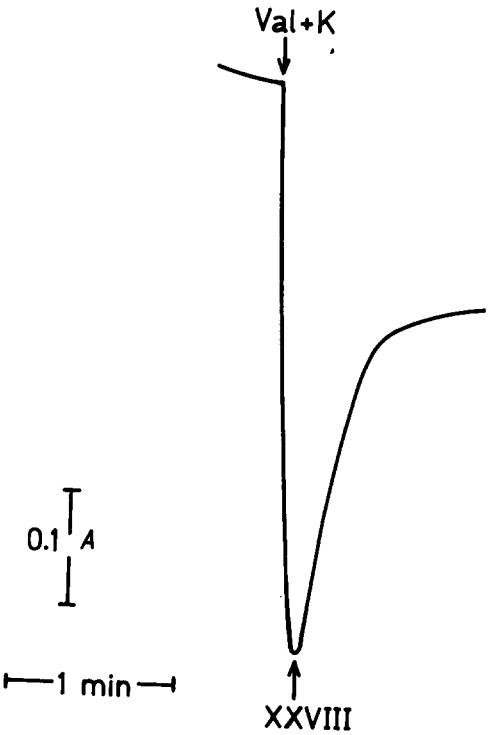

Fig. 3. Mitochondrial swelling induced by valinomycin (Val) in the presence of K^+. The experiment was carried out in the incubation mixture described in Fig. 1 with glutamate + malate as oxidizable substrates. Swelling was recorded at 546 nm.

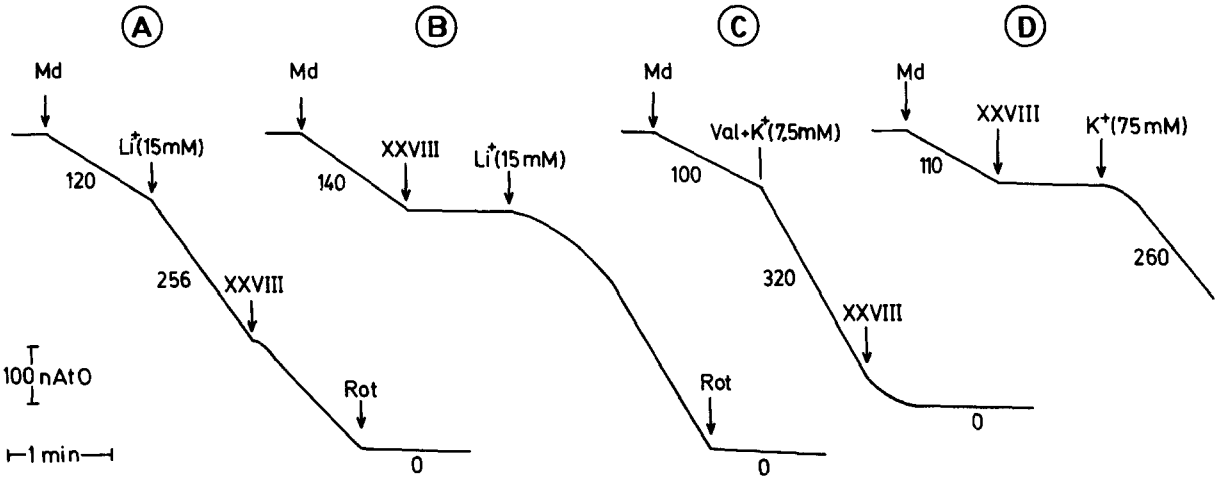

Fig. 4. Effect of polyether XXVIII on the aerobic oxidation of glutamate + malate by K^+-depleted mitochondria. The K^+ content of the mitochondrial preparation was 17 nmol/mg. The incubation conditions were as in Fig. 1 except that K^+ was omitted from the incubation mixture. Additions of polyether XXVIII, and valinomycin (Val) were also as in Fig. 1. Rotenone (Rot) (0.3 μg/ml) was also added to compare its effect with that of polyether XXVIII. Li^+ and K^+ were added at the indicated concentrations. Md, K^+-depleted mitochondria.

polyether XXVIII-metal ion complexes results in inhibition of electron transfer. In the presence of Li^+, which does not form complexes with polyether XXVIII [2,5], hardly any inhibition of respiration is observed. Moreover, the reversal of the inhibition of respiration by Li^+ further suggests that electron transfer in the NADH-CoQ span of the respiratory chain depends on the presence of monovalent cations, a conclusion that is further supported by the observation (Fig. 4D) that the inhibition of electron transport is also reversed by increasing the concentration of K^+ to 75 mM. Fig. 5 shows that under conditions in which high concentrations of K^+ induce reversal of the polyether XXVIII-induced inhibition of electron transport, extensive influx of cations takes place.

The action of polyether XXVIII was also studied by examining its effect on the aerobic oxidation of NADH by submitochondrial particles. As shown in Fig. 6, polyether XXVIII induces significant inhibition of the aerobic oxidation of NADH through a process that is released by Li^+ (trace A). If the respiration is stimulated by 7 mM K^+ (trace B), polyether XXVIII induces a marked decrease in the respiration rate. In contrast, trace C shows that preincubation of submitochondrial particles with polyether XXVIII diminishes the aerobic oxidation of NADH, and that this inhibition is released by 60 mM K^+. Polyether XVIII does not affect the aerobic oxidation of succinate (Fig. 6D) in agreement with the data in whole mitochondria.

The afore-mentioned observations strongly suggest that the chelation of an internal metal ion (K^+ in all probability) by polyether XXVIII induces inhibition of electron transport. However, the possibility exists that it is the metal ion complex of polyether XXVIII

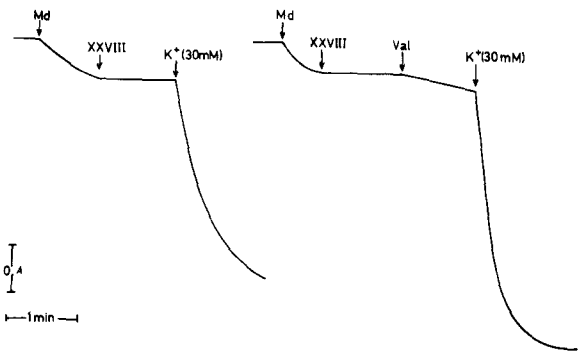

Fig. 5. Mitochondrial swelling of K^+-depleted mitochondria. Incubation conditions as in Fig. 3, except that K^+-depleted mitochondria were employed. The substrate was glutamate + malate. Other additions as shown. Val, valinomycin; Md, K^+-depleted mitochondria.

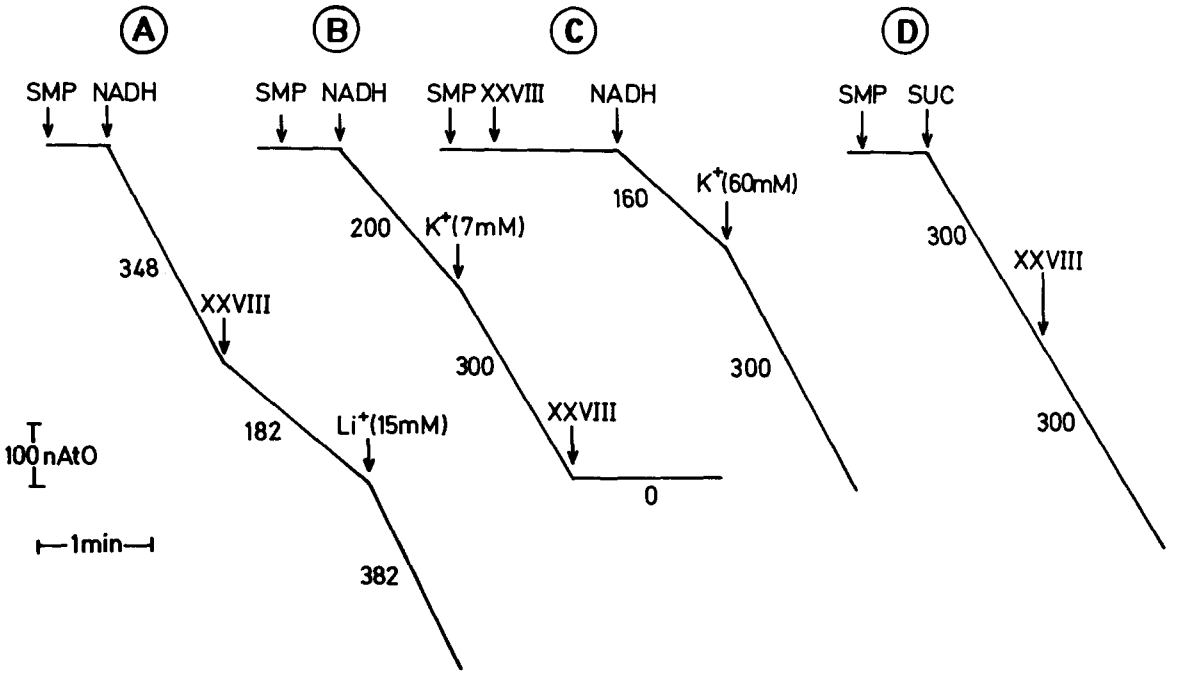

Fig. 6. Effect of polyether XXVIII on the oxidation of NADH by submitochondrial particles (SMP). The incubation mixture contained 250 mM sucrose, 1 mM EDTA adjusted to pH 7.4 with Tris base and 1.5 mg particle protein. Respiration was initiated by 0.75 mM NADH. Final volume 3.0 ml. K^+ or Li^+ was added where shown at the indicated concentrations. Polyether XXVIII was added (where shown) at 30 μg/mg protein. Trace D contained 3.3 mM succinate (SUC) instead of NADH.

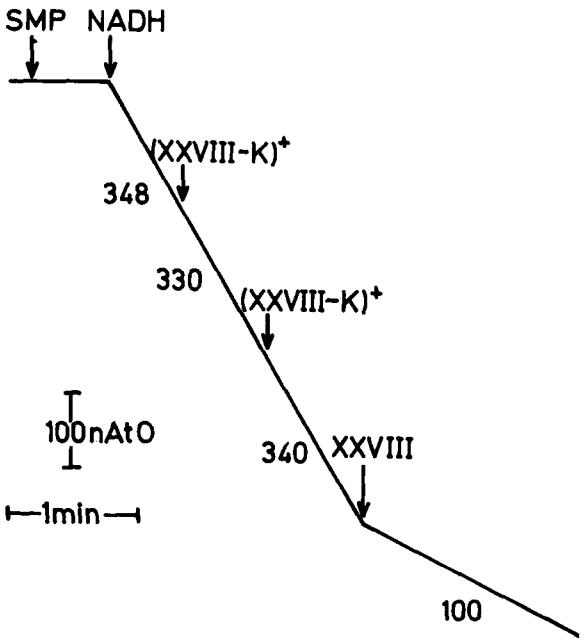

Fig. 7. Effect of polyether XXVIII-K^+ complex on the oxidation of NADH by submitochondrial particles (SMP). The conditions were as in Fig. 6. Polyether XXVIII-K^+ complex was added at each of the times shown at a concentration of 25 μg/mg. The polyether XXVIII-K^+ complex was prepared by suspending 20 mg of the free polyether in 1 ml of water. To the suspension a saturated solution of KCl was added dropwise until most of the crystals dissolved. The solution was filtered through a Millipore filter (0.45 μm). The concentration of the polyether in the filtrate was determined by measuring the absorbance at 275 and 280 nm. The concentration found was 9 mg polyether/ml solution. Free polyether XXVIII was added at a concentration of 30 μg/mg.

which is the species active in the inhibition of electron transport.

Fig. 7 shows that a previously formed (polyether XXVIII-K^+) complex has no effect on the aerobic oxidation of NADH by submitochondrial particles, while free polyether XXVIII does inhibit oxygen uptake in particles previously exposed to the polyether XXVIII-K^+ complex. Thus, it would appear that the chelation of a metal ion is the factor that induces inhibition of electron transport.

Discussion

Polyether XXVIII is a molecule that forms clathrates with K^+, Rb^+ and Cs^+, but not with Li^+ [2,5]; it also induces the translocation of metal ions across biological and model membranes [18,19]. In mitochondria polyether XXVIII has been reported to inhibit electron transport. In agreement with the latter findings, it is now shown that polyether XXVIII inhibits in mitochondria the oxidation of NAD-dependent substrates as stimulated by ADP, ionophores and uncouplers, as well as the oxidation of NADH by submitochondrial particles. On the other hand, polyether XXVIII does not exert a detrimental affect on electron transfer in the cytochrome *b* to oxygen segment of the respiration chain in both intact mitochondria and submitochondrial particles.

The inhibition of electron transfer in the NADH-CoQ span of the respiratory chain by polyether XXVIII differs from that induced by rotenone [20] and piericidin [21], since the data presented show that the inhibiting action of polyether XXVIII is highly dependent on the ionic composition of the incubation mixture. Indeed, the results presently described indicate that the inhibiting action of polyether XXVIII is reversed by relatively high concentrations of K^+, or by Li^+ at lower concentrations, characteristics that are not shared by rotenone or piericidin. The possibility that the polyether XXVIII-metal ion complex induces inhibition of electron transport may be ruled out by the lack of effect of the polyether XXVIII-K^+ complex on the aerobic oxidation of NADH by submitochondrial particles. Also, the alternative possibility that it is the free form of the polyether that induces arrest of electron transport may be discarded, since in the presence of Li^+ (which does not form complexes with crown XXVIII [5]) no inhibition of respiration is observed. Thus, it is logical to conclude that the inhibition of electron transport depends on the presence of a mitochondrial metal ion that may be chelated by polyether XXVIII and also, that a metal ion is required for maximal rates of electron transfer in the NADH-CoQ span of the respiratory chain.

It has been reported that maximal rates of electron transport in the NADH-CoQ segment may be induced by K^+ and other monovalent cations [6,817]. This effect of metal ions has been explained by their modification of the surface charge of the membrane [7]. However, if the latter idea is entirely correct, it would be expected that the neutralization of the negative charges of the membrane by other positively charged molecules would induce maximal rates of electron transfer. This is not true, since certain lipophilic cations, such as the alkylguanidines which modify the surface charge of the membrane [22–24], induce a strong inhibition of electron transfer in the NADH-CoQ span of the respiratory chain in whole mitochondria [10]. Thus, although part of the favorable effect of monovalent cations on electron transfer is certainly due to modification of the surface charge [7], it is very likely that monovalent cations affect other parameters involved in electron transport.

The present findings can be satisfactorily explained by assuming the existence of sites occupied by monovalent cations in the NADH-CoQ segment of the respiratory chain. The removal or chelation of these cations by polyether XXVIII (K^+ in all probability) would induce inhibition of electron transfer. This site can apparently be occupied by a number of cations; under our experimental conditions, the existence of this site becomes apparent from the lack of an inhibitory effect of polyether XXVIII on electron transfer in whole mitochondria, or submitochondrial particles incubated with Li^+. Moreover, Li^+ very effectively reverses the inhibiting action of polyether XXVIII, apparently by replacing the cation that had been removed by polyether XXVIII.

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