J. Membrane Biol. 18, 201–218 (1974)

Model Translocators for Divalent and Monovalent Ion Transport in Phospholipid Membranes

II. The Effects of Ion Translocator X-537A on the Energy-Conserving Properties of Mitochondrial Membranes*

Sergio Estrada-O., Heliodoro Célis**, Esthela Calderón, Guadalupe Gallo and M. Montal

Department of Biochemistry, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, P.O. Box 14–740, México 14, D.F. Mexico

Received 5 October 1973; revised 24 April 1974

Summary. The antibiotic X-537A, already characterized as an ionophore for mono-, di- and trivalent cations across lipid membranes, inhibits ATP hydrolysis and glutamate oxidation stimulated in mitochondria by ion translocators such as monazomycin and beauvericin with the selectivity pattern: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$. The ionophore-mediated inhibition of both ATPase and respiration is fully reversed by concentration gradients of K^+ and H^+ imposed between intra-extra mitochondrial compartments. It is not reversed by modifying the concentrations of divalent or trivalent cations in the medium. These data as well as the substrate dependance of the respiratory inhibition indicate that X-537A inhibits energy transduction primarily by mediating the translocation of protons in exchange for K^+ rather than by complexing divalent cations. Because of its ability to catalyze net proton transfer, concentrations of X-537A above 5×10^{-6} M uncouple the respiratory control of intact mitochondria. At concentrations below 10^{-6} M, the antibiotic releases the oligomycin-induced respiratory control of submitochondrial sonic particles with an alkali ion and proton-dependent selectivity as that shown to transport ions across lipid bilayers. It also stimulates a lanthanide-sensitive, ruthenium red-insensitive uptake of Ca^{2+} in submitochondrial sonic particles apparently occurring through an antiport type of electroneutral exchange diffusion of Ca^{2+} out/$2H^+$ in.

The antibiotic X-537A is an ion-transporting membrane carrier of the widest ionic selectivity properties known. Not only is it able to transport alkali metal, divalent and trivalent cations through natural and model membranes but protons as well (Lardy, Graven & Estrada-O., 1967;

* Some of these experiments have been previously presented at a meeting on "Ca^{++} Binding Proteins" held at Warsaw, Poland (July 1973).

** Fellow of the CONACYT (México).

Pressman, 1968, 1970, 1972, 1973; Henderson, McGivan & Chappell, 1969; Shavit, Degani & San Pietro, 1970; Caswell & Pressman, 1972; Degani & Shavit, 1972; Entman, Gillete, Wallick, Pressman & Schwartz, 1972; Estrada-O., Céspedes & Calderón, 1972; Scarpa & Inesi, 1972; Fernández, Célis & Montal, 1973; Lin & Kuhn, 1973; Célis, Estrada-O. & Montal, 1974).

The Ca^{2+} fluxes mediated by X-537A in biological membranes have been ascribed to the antibiotic's ability to form mobile complexes with divalent cations (Johnson, Herrin, Liu & Paul, 1970; Pressman, 1970, 1973; Caswell & Pressman, 1972). Moreover, similar to the effects of the other nigericin-like ionophores, the H^+ and K^+ movements catalyzed by X-537A stimulate Ca^{2+} and phosphate uptake in intact mitochondria (Estrada-O. *et al.*, 1972). However, the mechanism by which X-537A affects the energy-conserving properties of mitochondrial membranes is not readily apparent. It is known that concentrations of the antibiotic below 5×10^{-6} M inhibit oxidizable substrate uptake and ATPase activity in intact mitochondria (Lardy *et al.*, 1967; Lin & Kuhn, 1973) and uncouple photophosphorylation in chloroplast membranes (Shavit *et al.*, 1970). On the other hand, concentrations above 5×10^{-6} M uncouple oxidative phosphorylation in intact mitochondria suspended in media free of added alkali or divalent cations (Estrada-O., Graven & Lardy, 1967; Henderson, 1971).

The present work is an attempt to correlate the effects of compound X-537A on the ion permeability of lipid bilayers (Célis *et al.*, 1974) with its action on substrate oxidation, ATPase activity and energy conservation in mitochondrial membranes. From this approach, possible mechanisms by which antibiotic X-537A affects energy transduction in mitochondrial and photosynthetic membranes emerge.

Materials and Methods

Mitochondria were prepared from livers of male rats weighing 150 g as described by Johnson and Lardy (1967). Submitochondrial particles derived by sonic disruption of beef heart mitochondria were prepared by the method of Fessenden and Racker (1967).

In some experiments, mitochondria were substantially depleted of more than 90% of the endogenous K^+ and Mg^{2+} content by the modification made by Gómez-Puyou, Sandoval, Tuena, Peña and Chávez (1969) to the method of Settlemire, Hunter and Brierley (1968).

A continuous recording of oxygen consumption and its derivative as well as K^+ and H^+ movements was carried out by means of an apparatus designed, developed and constructed by Chance, Mayer and Pressman (Pressman 1967); (Graven, Estrada-O., & Lardy, 1966). K^+ movements were measured by a glass cation-sensitive electrode (Eisenman, Rudin & Casby, 1957) Beckman No. 39047; protons by means of a Beckman

combination electrode 39030. Ca^{2+} movements were measured in submitochondrial sonic particles by means of a spectrophotometric method which uses murexide as metalochromic indicator (Ohnishi & Ebashi, 1963). ATPase activity was measured as described by Lardy and Wellman (1953). Inorganic phosphate from ATP hydrolysis was determined as described (Lindberg & Ernster, 1956). Protein was determined by the biuret method (Jacobs, Jacobs, Sanadi & Bradley, 1956). The antibiotic X-537A was very kindly provided by Dr. J. Berger of Hoffman La Roche Laboratories. All chemicals used were of the highest purity commercially available. Glass-redistilled water was used throughout.

Results

The Inhibition by X-537A of Substrate Oxidation and ATP Hydrolysis in Intact Mitochondria

Concentrations of X-537A below 10^{-6} M inhibit glutamate oxidation or ATP hydrolysis in intact mitochondria (Lardy *et al.*, 1967) without affecting oxidative phosphorylation *per se*. Some reports (Lin & Kun, 1973, 1974) have suggested that the antibiotic inhibits glutamate oxidation because of its ability to complex divalent cations such as Mg^{2+}. However, being aware of the wide ionic selectivity properties of the antibiotic in membranes (Estrada-O. *et al.*, 1973; Célis *et al.*, 1974) studies were undertaken to find out a possible association existent between the transport of ions mediated by X-537A and its aforementioned inhibitory effects.

The ionic selectivity of X-537A to inhibit substrate oxidation and ATP hydrolysis in intact mitochondria. Fig. 1 shows that the inhibition of glutamate oxidation and ATP hydrolysis by the carboxylic ionophore is dependent on its ability to translocate protons in exchange for alkali metal cations across membranes (Lardy *et al.*, 1967; Pressman, 1968). The antibiotic monazomycin which stimulates glutamate oxidation and ATP hydrolysis linked to an induced translocation of Li^+, Na^+, K^+, Rb^+ and Cs^+ in mitochondria (Estrada-O. & Gómez-Lojero, 1971) was used as a promoter of alkali ion uptake in the intact organelle. In turn, X-537A was added as an inhibitor of the substrate oxidation and ATPase stimulated by the former antibiotic and the different alkali ion added. It is clear (Fig. 1) that X-537A inhibits both the ion transport-dependent ATP hydrolysis and glutamate oxidation stimulated by monazomycin with the same ionic selectivity pattern ($Cs^+ > Rb^+ > K^+ > Na^+, Li^+$) exhibited to transport alkali ions across lipid bilayers (Célis *et al.*, 1974) or to complex alkali ions in bulk-phase systems (Pressman, 1968). An identical ionic selectivity sequence is also shown by X-537A to inhibit both ATPase or respiration when monazomycin is replaced by the antibiotic beauvericin (Dorschner & Lardy, 1968; Roeske, Isaac, Steinrauf & King, 1971; Estrada-O., Gómez-Lojero & Montal, 1972).

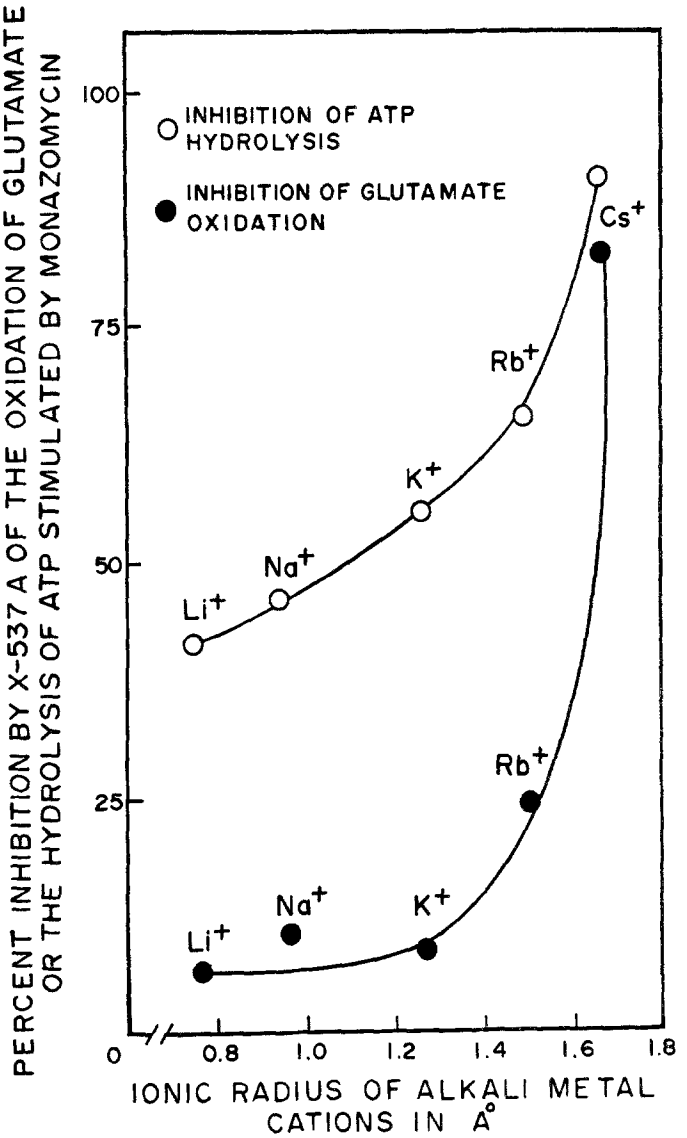


Fig. 1. Effect of X-537A on the hydrolysis of ATP and the oxidation of glutamate stimulated by monazomycin and different alkali metal ions in intact mitochondria. The oxidation of glutamate was measured in a medium which contained 10 mM triethanolamine-HCl (pH 7.4), 10 mM acetate adjusted with triethanolamine to pH 7.4, 225 mM sucrose, 10 mM glutamate-triethanolamine, 30 mM of the indicated alkali ions and 4 mg protein. The reaction mixture for measuring ATP hydrolysis contained 6 mM tris ATP (pH 7.4), 200 mM sucrose, 10 mM acetate triethanolamine, 4 mg protein and where indicated 30 mM of alkali metal cations. Monazomycin and X-537A were added at a concentration of 2×10^{-7} and 3×10^{-7} M, respectively

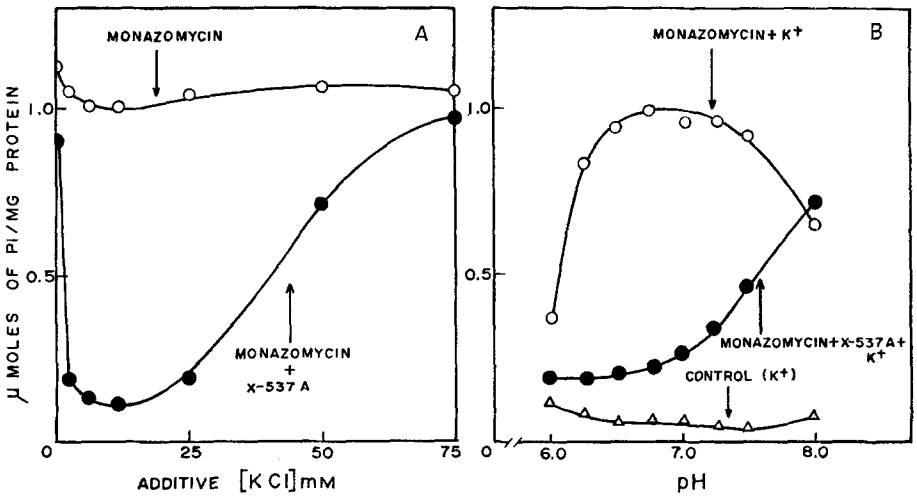


Fig. 2. Effect of changes in the concentration of K^+ (*A*) and the pH change (*B*) on the inhibition by X-537A of the hydrolysis of ATP stimulated by monazomycin in mitochondria. Except for the indicated concentrations of K^+ and the changes in the pH of the medium, conditions were similar to those of Fig. 1

Effect of imposed monovalent ion gradients on the inhibition of ATPase and respiration caused by X-537A in intact mitochondria. Figs. 2 and 3 illustrate that the inhibition by X-537A of the ATPase coupled to the K^+ transport mediated by monazomycin (Fig. 2 and Estrada-O. & Gómez-Lojero, 1971) is fully sensitive both to concentration differences imposed between intra- and extramitochondrial K^+ as well as to the ΔpH existent across the membrane. Fig. 2*A* shows that low concentrations of added K^+ (less than 7 mM) are required for the carboxylic antibiotic to block the ATPase stimulated by monazomycin. Likewise, an increase in the initial concentration of added monovalent cation, from 10 to 60 mM is accompanied by a progressive release of the inhibited ATP hydrolysis. Complete reversal of the inhibited state is observed above 75 mM K^+. The block of ATP hydrolysis by X-537A is also sensitive to the pH in the medium. As shown in Fig. 2*B*, at a constant concentration of added K^+, the gradual alkalinization of the extramitochondrial medium leads to a concomitant reversal of the inhibited ATPase.

Fig. 3 shows that the inhibition by X-537A of the glutamate oxidation stimulated by monazomycin is also an inverse function of the concentration of extramitochondrial K^+. The increment in the concentration of added K^+ releases the oxidizable substrate uptake block mediated by X-537A with maximal effects at 75 mM K^+; alkalinization of the medium at a constant

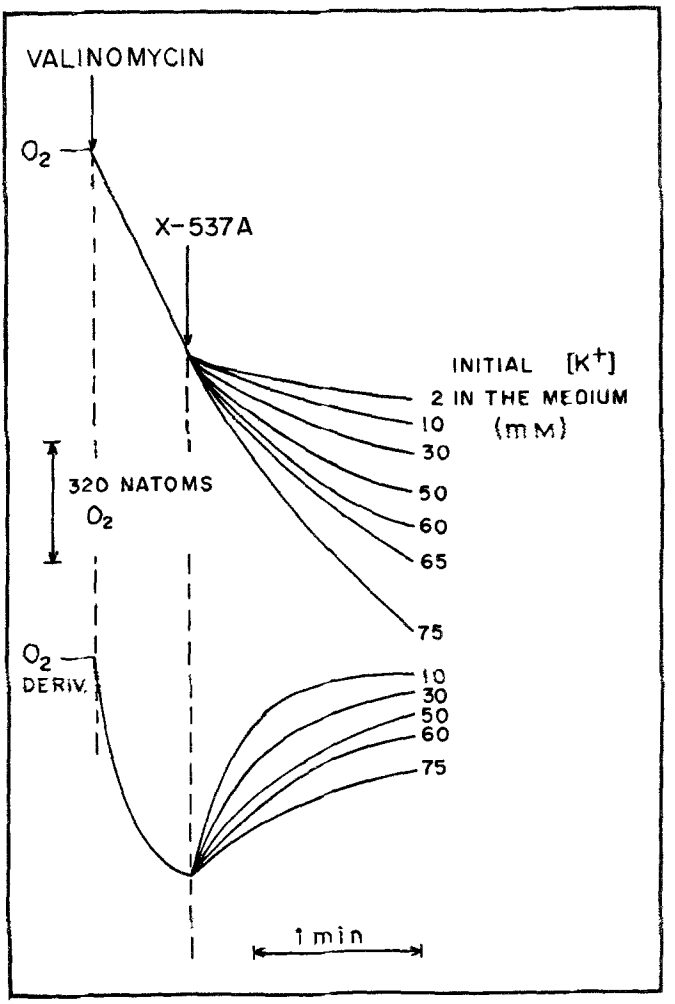


Fig. 3. Effect of increasing concentrations of K^+ on the inhibition by X-537A of the oxidation of glutamate stimulated by monazomycin in mitochondria. The medium was similar to that of Fig. 1, except for the indicated concentrations of KCl. Valinomycin and X-537A were added at a concentration of 5×10^{-7} M and 13×10^{-6} M, respectively

concentration of cation (20 mM), leads also to a complete reversal of the inhibited respiration at pH 8.2.

The inhibition by X-537A of ATPase or respiration previously stimulated by either monazomycin or beauvericin, is not reversed by the addition of concentrations of the chloride salts of Ca^{2+} or Mg^{2+} as high as 10 and 25 mM, respectively. Thus, it is clear that this inhibitory effect of X-537A is primarily associated with the exchange of protons and K^+ induced by the carboxylic ionophore across the mitochondrial membrane.

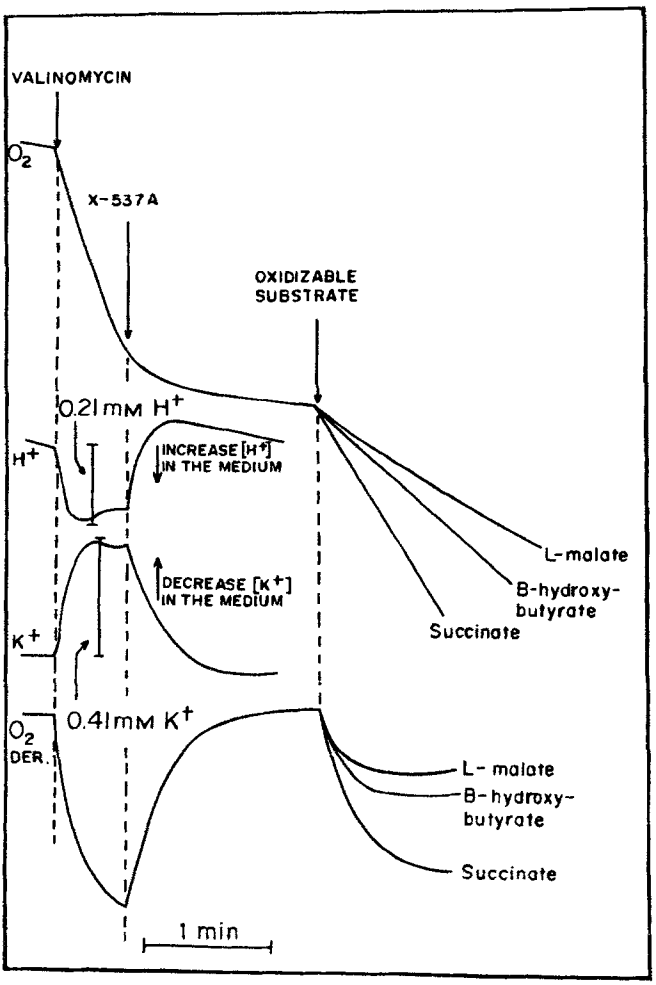


Fig. 4. Effects of X-537A on the oxidation of different substrates and the K^+ and H^+ movements stimulated by valinomycin in mitochondria. Except for the addition of 30 mM KCl, conditions were similar to those of Fig. 3. Substrates were added as the triethanolamine salts at 10 mM

Substrate selectivity for the respiratory inhibition mediated by X-537A in mitochondria. As illustrated in Fig. 4, 2.5×10^{-7} M or lower concentrations of compound X-537A inhibit the valinomycin-stimulated oxidation of glutamate parallel to an induced influx of protons associated to an exchange for internal K^+ (Fig. 5). Similar effects are obtained when valinomycin is replaced by ADP and phosphate. The oxidation of pyruvate, L-malate, α-ketoglutarate or citrate oxidation is also blocked simultaneously with the cation/H^+ exchange mediated by X-537A, whereas that of succinate or β-hydroxybutyrate is significantly stimulated by the combination of valinomy-

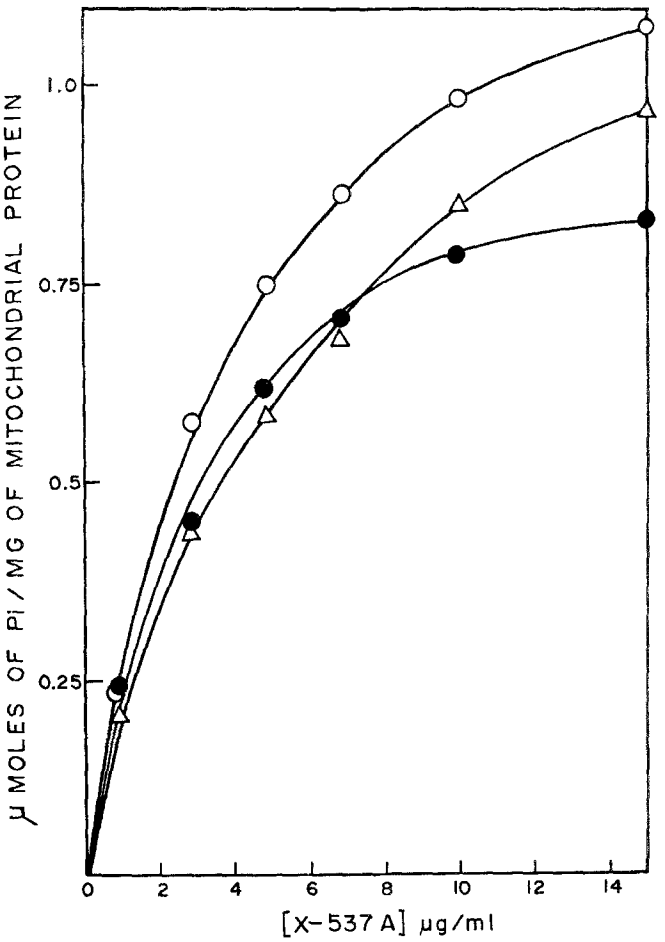


Fig. 5. Effect of X-537A on the hydrolysis of ATP of intact mitochondria and mitochondria depleted of endogenous K^+. The reaction mixture contained 6 mM tris ATP (pH 7.4), 10 mM acetate-triethanolamine (pH 7.4), 200 mM sucrose, 8 mg mitochondrial protein and, where indicated, 30 mM KCl. (○) Intact mitochondria containing 40 mEquiv internal K^+/mg protein incubated in medium free of added K^+; (●) mitochondria depleted of endogenous K^+ containing 3.5 mEquiv internal K^+/mg protein incubated in medium free of added K^+; (△) mitochondria depleted of endogenous K^+ (3.5 mEquiv K^+/mg protein) incubated in 30 mM KCl

cin and X-537A; moreover, glutamate reverses the inhibition of L-malate oxidation mediated by X-537A (Fig. 4; *see also* Ferguson, Estrada-O. & Lardy, 1971). These effects are similar to other nigericin-like antibiotics such as the monensins, dianemycin or Lilly A-217 (Graven *et al.*, 1966; Lardy *et al.*, 1967; Henderson *et al.*, 1967; Estrada-O. & Calderón, 1970; Ferguson *et al.*, 1971). Thus, compound X-537A shares with other nigericin-like ionophores similar characteristics to inhibit substrate oxidation.

The Ionic Selectivity of X-537A to Uncouple Oxidative Phosphorylation in Intact Mitochondria

Preliminary results (Estrada-O. *et al.*, 1967; Henderson, 1969) suggested that compound X-537A uncoupled oxidative phosphorylation in intact mitochondria at concentrations higher than 10^{-6} M. Fig. 5 indicates that the antibiotic does not require alkali metal cations to stimulate ATP hydrolysis in mitochondria. X-537A stimulates comparable rates of ATP hydrolysis in mitochondria depleted or undepleted of its endogenous K^+ content irrespective of the presence of K^+ in the medium. This clearly indicates that alkali ions are not involved in the uncoupling action of the antibiotic. ATPase activity is not overstimulated by X-537A in intact or K^+-depleted mitochondria upon the addition of increasing concentrations of Ca^{2+} or Mg^{2+} (up to 10 mM). In contrast, the antibiotic inhibits the hydrolysis of ATP stimulated by Ca^{2+} in intact mitochondria without affecting the ATPase stimulated by Mg^{2+} in aged particles (Estrada-O. *et al.*, 1972). Moreover, X-537A maximally stimulates ATPase activity in membrane preparations where the method used to deplete endogenous K^+, also removes a significant amount of mitochondrial Mg^{2+} –from 20 to 12 nmoles per mg protein (*cf.* Settlemire *et al.*, 1968). Thus, since X-537A translocates protons more efficiently than Ca^{2+}, Mg^{2+} or alkali metal cations across lipid bilayers (Célis *et al.*, 1974), the capacity of the antibiotic to uncouple oxidative phosphorylation can be accounted for by its ability to mediate the net translocation of protons across the intact membrane.

The Selectivity of X-537A to Transport Ions Across Submitochondrial Membrane Particles

Fig. 6 shows the release of the respiratory control of submitochondrial sonic particles (SMSP) caused by X-537A as a function of its ability to transport alkali ions, protons and Ca^{2+}. It is clear that in the presence of alkali ions, the independent addition of 5.1×10^{-6} M X-537A releases the oligomycin-induced coupled respiration of the vesicles (upper tracing, Fig. 6*A*) with the following selectivity: $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$. The antibiotic also uncouples respiratory control as a function of its concentration in media free of added cations other than choline chloride (lower tracing, Fig. 6*A*). Thus, in agreement with the results obtained under similar conditions in lipid bilayers (Célis *et al.*, 1974) and intact mitochondrial membranes (Fig. 5, this paper), it is possible to attribute this latter effect to the proton-conducting ability of the antibiotic.

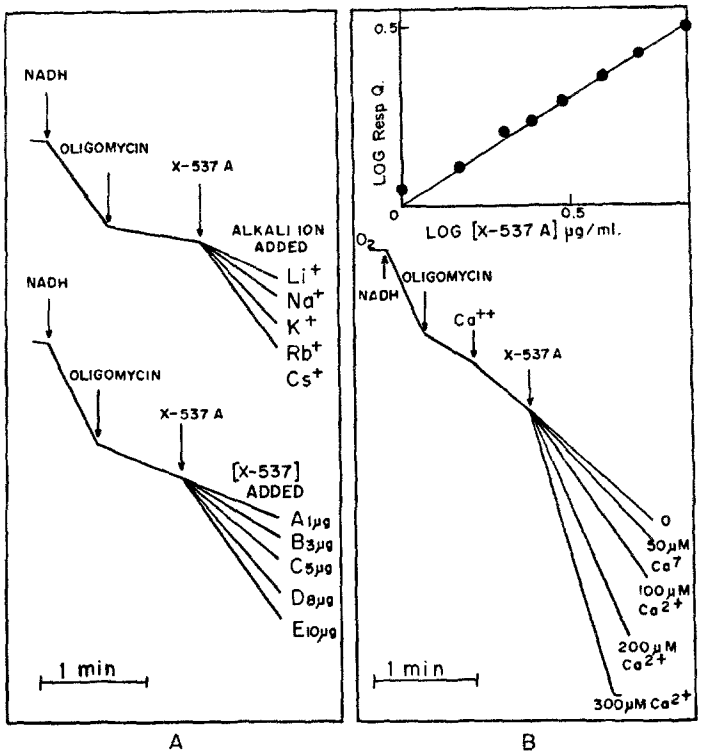


Fig. 6. Effect of X-537A and different ions on the respiratory control induced by oligomycin in beef heart submitochondrial sonic particles. The reaction mixture contained 10 mM acetate triethanolamine, pH 7.4, 0.5 μmole NADH, 180 mM sucrose and 50 mM of the chloride salts of either Li^+, Na^+, K^+, Rb^+ or Cs^+ plus 1×10^{-6} M X-537A (upper left tracings), 20 mM choline chloride in the presence of the indicated concentrations of X-537A (lower left tracings) and the indicated concentrations of $CaCl_2$ plus 3.5×10^{-6} M X-537A (lower tracings, panel *B*)

Compound X-537A causes the release of accumulated Ca^{2+} from intact mitochondria (Scarpa & Inesi, 1972; Lin & Kuhn, 1973). On the other hand, as illustrated in Fig. 7*A*, the reversal of the mitochondrial membrane sidedness or polarity, allows X-537A to induce Ca^{2+} uptake in submitochondrial sonic particles as a function of translocator concentration. The antibiotic-mediated Ca^{2+} uptake is insensitive to the addition of NADH, succinate or ATP as well as that of rotenone, antimycin, or oligomycin. These data suggest that X-537A promotes Ca^{2+} accumulation into submitochondrial particles not through an electrogenic or energy-dependent process but through an ion exchange translocation. Moreover, the antibiotic also releases the respiratory control of SMSP as a function of the concentration of Ca^{2+} added (lower tracing, Fig. 6*B*). At concentrations below 2.2×10^{-6} M the rates of oxygen uptake mediated by X-537A and 300 μM Ca^{2+} vary with

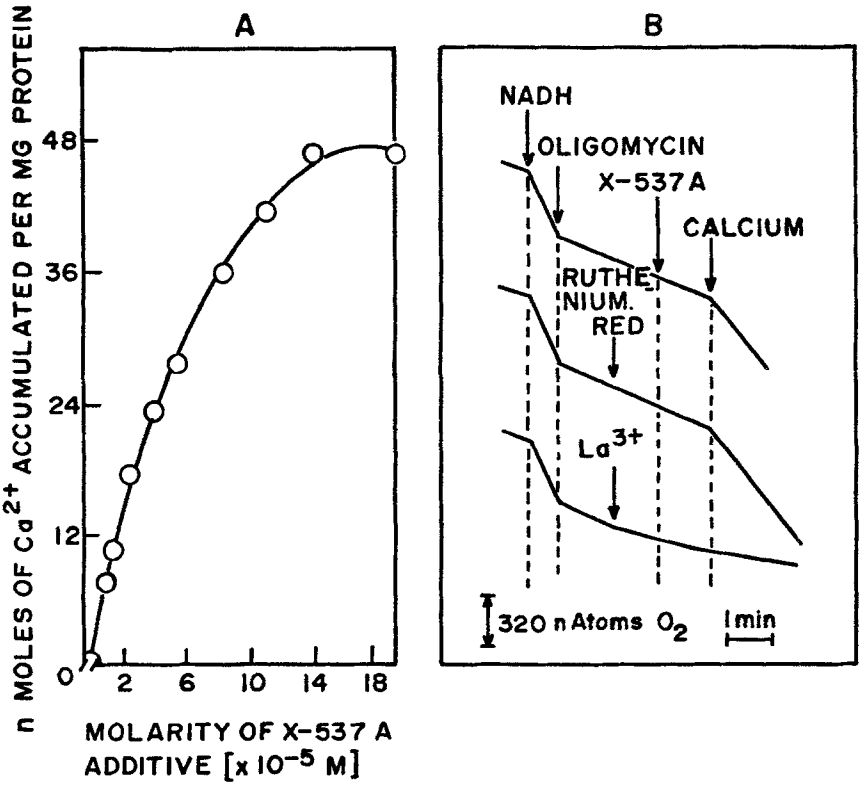


Fig. 7. Ca^{2+} uptake stimulated by X-537A in submitochondrial sonic particles (*A*) and effect of ruthenium red and lanthanum chloride on the respiratory control of submitochondrial sonic particles uncoupled by X-537A and Ca^{2+} (*B*). The reaction mixture of panel *A* contained: 10 mM triethanolamine-HCl (pH 7.4), 180 mM sucrose, 100 μM $CaCl_2$, 50 μM murexide, 2.5 mg mitochondrial protein and the indicated concentrations of antibiotic X-537A. The reaction mixture of panel *B* was identical to that of Fig. 6*B* except for the addition of 50 μM ruthenium red and 25 μM lanthanum chloride where indicated

approximately 0.6 to 0.8 the power of antibiotic concentration (insert, Fig. 6*B*) whereas at those higher than 2.2×10^{-6} M (not shown) the ratio is slightly higher than 1.5. These values are roughly similar to those obtained in lipid bilayers (Célis *et al.*, 1974) for the hyperbolic dependence of Ca^{2+} conductance on translocator concentration. In addition, respiratory control is released by the antibiotic in the presence of 500 μM divalent cations with the selectivity $Ca^{2+} > Sr^{+} > Mg^{2+}$. This latter sequence is identical to that observed for the antibiotic to transport divalent cations across lipid bilayers. As shown in Fig. 7*B*, 50 μM lanthanum inhibits the release of respiratory control caused by X-537A in the presence of 500 μM Ca^{2+}. In contrast, concentrations of ruthenium red as high as 100 μM do not affect it.

Discussion

The experiments described herein clearly show that the transport of ions catalyzed by the antibiotic X-537A in intact mitochondrial membranes and submitochondrial sonic vesicles exhibits common features with that observed in lipid bilayers of the Mueller-Rudin type (Célis *et al.*, 1974). Thus, the distinct ability of the antibiotic to mediate the net translocation of protons across lipid bilayers is properly correlated with its uncoupling effect (Mitchell, 1968) both in intact mitochondria and SMSP when suspended in alkali cation-free media (Figs. 5 and 6*A*). Its selectivity to inhibit glutamate oxidation and ATP hydrolysis in intact mitochondria, $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ (Fig. 1) as well as to uncouple NADH oxidation in SMSP (Fig. 6*A*) is identical to that observed for the antibiotic to transport alkali metal cations across lipid bilayers. Moreover, the selectivity of X-537A to mediate the translocation of divalent cations across SMSP membranes ($Ca^{2+} > Sr^{2+} > Mg^{2+}$) is analogous to that observed in lipid bilayers (Célis *et al.*, 1974). In addition, important clues to understand the molecular events involved in the mechanism of: (a) respiratory and ATPase inhibition, (b) uncoupling of oxidative phosphorylation, and (c) transport of divalent cations mediated by X-537A in intact mitochondria and SMSP are provided in the present work.

The Mechanism of Inhibition of Glutamate Oxidation and ATP Hydrolysis by X-537A in Mitochondrial Membranes

The inhibition of glutamate oxidation and ATP hydrolysis in intact mitochondria are probably caused by the H^+ influx mediated by X-537A, in exchange for endogenous K^+ (Fig. 4). The reversal of X-537A-mediated inhibition of glutamate oxidation or ATP hydrolysis caused by increasing the extramitochondrial K^+ concentration (Figs. 2*A* and 3) as well as by decreasing the extramitochondrial proton concentration (Fig. 2*B*) is in agreement with our proposal. Likewise, the requirement of $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$ to inhibit glutamate oxidation in the presence of X-537A (Fig. 1), as well as of low concentrations of added alkali ions to block the hydrolysis of ATP stimulated by monazomycin (Fig. 2*A*), suggests a role of monovalent cations in the antiport-type turnover of protons catalyzed by low concentrations of X-537A across the membrane.

The suggestions by Lin and Kuhn (1973) that X-537A inhibits glutamate oxidation by complexing a small fraction of Mg^{2+} bound to hydrophobic metal-high-energy coupling sites would require either that the antibiotic would simultaneously inhibit respiration at the three coupling sites of the respira-

tory chain or alternatively that the respiratory inhibition would be "coupling site-dependent". However, X-537A shows neither effect. In fact, the translocator induced a substrate-specific inhibition of respiration (Fig. 4) identical to that observed for other carboxylic ionophores such as nigericin, dianemycin or the monensins (Lardy, Johnson & McMurray, 1958; Graven *et al.*, 1966; Estrada-O., Rightmire & Lardy, 1967; Lardy *et al.*, 1967). The loss of internal phosphate caused by the H^+ influx mediated by carboxylic antibiotics has been established as the cause of such substrate-specific respiratory inhibition (Henderson *et al.*, 1969; Estrada-O. & Calderón, 1970; Ferguson *et al.*, 1971). Thus, it is clear that X-537A shares with other carboxylic ionophores the same mechanism for inhibiting substrate oxidation and ATP hydrolysis in mitochondria, namely an alkali ion-dependent change in ΔpH across the membrane leading to a reduced uptake of oxidizable substrate anions into mitochondria (Mitchell, 1968; Palmieri & Quagliariello, 1969). The additional collapse of membrane potential caused by monazomycin in parallel to the decrease of pH gradient mediated by X-537A (Figs. 1, 2*A*, *B*), may further contribute to prevent ATP accumulation in mitochondria. The above interpretation is also supported by the fact that X-537A shows higher affinity to transport protons than Mg^{2+} through lipid bilayers (Célis *et al.*, 1974). Nonetheless, further evidence is required to understand why the increase in translocator concentration overcomes the inhibited ATPase state.

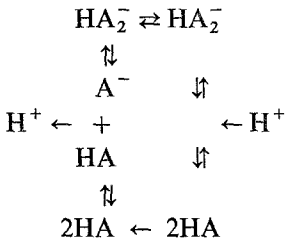
The fact that X-537A, the monensins and nigericin induce an identical substrate-specific respiratory inhibition coupled to a parallel H^+ influx indicates that the role of Mg^{2+} in X-537A mode of action to inhibit ATPase and respiration is negligible. Neither nigericin nor the monensins are able to form lipophilic complexes with divalent cations such as Mg^{2+} or Ca^{2+} as does X-537A (Pressman, 1973; Célis *et al.*, 1974).

X-537A clearly differs in the above respect from antibiotic A-23187, also a divalent cation-transporting antibiotic, which indeed inhibits ATPase and respiration apparently resulting from its ability to complex divalent ions (Reed & Lardy, 1972).

The Mechanism of Uncoupling of Electron Transport-linked Phosphorylation by X-537A in Energy-Conserving Membranes

Compound X-537A showed a considerable ability to transport H^+ over divalent ($Ca^{2+} > Sr^{2+} > Mg^{2+} > Mn^{2+}$) or monovalent cations ($Cs^+ > Rb^+$, $K^+ > Na^+ > Li^+$) across lipid bilayers (Célis *et al.*, 1974). This property is sufficient to explain its ability to uncouple oxidative phosphorylation in intact mitochondria or SMSP in the absence of added cations (Figs. 5 and

6*A*) as well as that to uncouple photophosphorylation in chloroplast membranes (Shavit *et al.*, 1967). It has been shown that X-537A transports net charge across bilayers apparently as a dimer complexing one proton $(HA_2)^-$, where A denotes one monomer of X-537A (Célis *et al.*, 1974). Thus, the uncoupling effect is the result of the net translocation of H^+ which collapses the membrane potential and abolishes the gradient of H^+ across the membrane (Mitchell, 1968), according to the following scheme (Neumcke & Bamberg, 1973):



The Mechanism of Uncoupling of Respiratory Control in Submitochondrial Particles by X-537A

The ionic selectivity of X-537A to uncouple the respiratory control of SMSP is properly correlated with its ion-translocating ability observed in lipid bilayers (Estrada-O. *et al.*, 1973; Célis *et al.*, 1974). Thus, its uncoupling action in SMSP suspended in media free of added alkali or divalent cations (Fig. 6*A*) can be accounted for by the net translocation of H^+ across the membrane. Likewise, its partial uncoupling effect dependent on the concentration of added alkali metal cations (Fig. 6*A*) is very similar to that promoted by nigericin in SMSP (Montal, Chance & Lee, 1969; Chance & Montal, 1971). Its further stimulation by valinomycin or tetraphenylboron supports this possibility (Montal *et al.*, 1969).

The Mechanism of Ca^{2+} Translocation Mediated by X-537A in Submitochondrial Particles

X-537A induces Ca^{2+} uptake in SMSP as a function of translocator concentration (Fig. 7*A*), uncoupling also the respiratory control of SMSP as a function of the concentration of Ca^{2+} added (Fig. 6*B*). This Ca^{2+}-dependent uncoupling effect is inhibited by lanthanides (Fig. 7*B*) under conditions where concentrations of ruthenium red as high as 100 μM do not affect it. The antibiotic is an ideal translocator for trivalent cations showing to be less capable for transporting Ca^{2+} than Pr^{3+} in lipid bilayers (Estrada-

O. *et al.*, 1973; Fernández *et al.*, 1973). Hence, the inhibitory action of lanthanides on Ca^{2+} translocation in SMSP (Fig. 7*B*) may be accounted for by a competition between Ca^{2+} and La^{3+} for complex formation with the antibiotic.

Thus, in contradistinction with the similar inhibitory effects of both lanthanides or ruthenium red on A-23187-induced Ca^{2+} movements in intact mitochondria (Reed & Lardy, 1972), which suggests an interaction of A-23187 in parallel with high affinity Ca^{2+} binding sites from the membrane, our data indicate that X-537A bypasses possible interactions with natural Ca^{2+} binding sites existent in SMSP membranes.

The fact that X-537A induces Ca^{2+} uptake and uncouples the respiratory control of SMSP as a function of the concentration of Ca^{2+} added, suggests that the antibiotic is promoting the translocation in Ca^{2+} in SMSP in a manner perhaps phenomenologically similar to that observed in inside-out mitochondrial membranes by Loyter, Christianse, Steensland, Saltzgaber and Racker (1969) and Pedersen and Coty (1972). As seen in the scheme, it is likely that a dimer complex of ionophore and Ca^{2+} (CaA_2) could facilitate in SMSP the antiport-type of electroneutral exchange diffusion of Ca^{2+} out/$2H^+$ in (Chance & Montal, 1971).

$$\begin{array}{ccccc} Ca^{2+} & \rightarrow & CaA_2 & \rightarrow & CaA_2 \rightarrow Ca^{2+} \\ & & \uparrow & & \downarrow \\ & & 2A^- & & 2A^- \\ & & \uparrow & & \downarrow \\ 2H^+ & \leftarrow & 2HA & \leftarrow & 2HA \leftarrow 2H^+ \end{array}$$

In such a case a complex of one neutral and one negatively charged antibiotic would be the true uncoupling species.

The authors are indebted to Drs. C. Gómez-Lojero, J. Korenbrot and S. McLaughlin for criticism.

References

Caswell, A. H., Pressman, B. C. 1972. Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biochem. Biophys. Res. Commun.* **49**:292

Célis, H., Estrada-O., S., Montal, M. 1974. Model translocators for divalent and monovalent ion transport in phospholipid membranes. I. The ion permeability induced in lipid bilayers by the antibiotic X-537A. *J. Membrane Biol.* **18**:187

Chance, B., Montal, M. 1971. Ion-translocation in energy-conserving membrane systems. *In:* Current Topics in Membranes and Transport. F. Bronner and A. Kleinzeller, editors. Vol. 2, pp. 99–156. Academic Press, New York

Degani, H., Shavit, N. 1972. Ion movements in isolated chloroplasts. III. Ionophore-induced ion uptake and its effect on photophosphorylation. *Arch. Biochem. Biophys.* **152**:339

Dorschner, E., Lardy, H. A. 1968. Effects of beauvericin in mitochondrial ion transport. *In:* Antimicrobial Agents and Chemotherapy. G. L. Hobby, editor. p. 11. American Society of Microbiology, Ann Arbor, Michigan

Eisenman, G., Rudin, D. O., Casby, J. V. 1957. Glass electrodes for measuring sodium ions. *Science* **126**:831

Entman, M. L., Gillete, P. C., Wallick, E. T., Pressman, B. C., Schwartz, A. 1972. A study of calcium binding and uptake by isolated cardiac sarcoplasmic reticulum: The use of new ionophore (X-537A). *Biochem. Biophys. Res. Commun.* **48**:847

Estrada-O., S., Calderón, E. 1970. Potassium and proton movements in relation to the energy-linked transport of phosphate in liver mitochondria. *Biochemistry* **9**:2092

Estrada-O., S., Célis, H., Fernández, M. S., Montal, M. 1973. The mode of action of compound X-537A: A model calcium translocator in natural and artificial membranes. *In:* Ca^+ Binding Proteins. W. Drabikoski, E. Carafoli, A. Schwartz, and J. Gergely, editors. Elsevier, Amsterdam (*In press*)

Estrada-O., S., De Céspedes, C., Calderón, E. 1972. Accumulation of calcium and phosphate stimulated by carboxylic antibiotics into mitochondria. *Bioenergetics* **3**:361

Estrada-O., S., Gómez-Lojero, C. 1971. Effects of monazomycin on ion transport and oxidative phosphorylation in liver mitochondria. *Biochemistry* **10**:1598

Estrada-O., S., Gómez-Lojero, C., Montal, M. 1972. Variability of the K^+, Na^+ discrimination of beauvericin in mitochondrial membranes. *Bioenergetics* **3**:417

Estrada-O., S., Graven, S. N., Lardy, H. A. 1967. Effects of nigericin on mitochondrial ion transport and oxidative phosphorylation. *Fed. Proc.* **26**:610

Estrada-O., S., Rightmire, B., Lardy, H. A. 1967. Antibiotics as tools for metabolic studies. XI. Specific inhibition of ion transport in mitochondria by the monensins. *In:* Antimicrobial agents and chemotherapy. G. L. Hobby, editor. p. 279. American Society for Microbiology. Ann Arbor, Michigan

Ferguson, S. M. F., Estrada-O., S., Lardy, H. A. 1971. Potassium-specific uncoupling by nigericin. *J. Biol. Chem.* **246**:5645

Fernández, M. S., Célis, H., Montal, M. 1973. PMR detection of ionophore mediated translocation of praseodymium ion across phospholipid vesicles. *Biochim. Biophys. Acta* **323**:600

Fessenden, J. M., Racker, E. 1967. A-particles and P-particles. *In:* Methods in Enzymology. R. W. Estabrook and M. E. Pullman, editors. Vol. 10, p. 194. Academic Press, New York-London

Graven, S. N., Estrada-O., S., Lardy, H. A. 1966. Alkali metal cation release and respiratory inhibition induced by nigericin in rat liver mitochondria. *Proc. Nat. Acad. Sci.* **56**:654

Gómez-Puyou, A., Sandoval, F., Tuena, M., Peña, A., Chávez, E. 1969. Induction of respiratory control by K^+ in mitochondria. *Biochem. Biophys. Res. Commun.* **36**:316

Henderson, P. J. F. 1971. Ion transport by energy-conserving biological membranes. *Annu. Rev. Microbiol.* **25**:393

Henderson, P. J. F., McGivan, J. D., Chappell, J. B. 1969. The action of certain antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes. The role of induced proton permeability. *Biochem. J.* **111**:52

Jacobs, E. E., Jacobs, M., Sanadi, D. R., Bradley, L. B. 1956. Uncoupling of oxidative phosphorylation by cadmium ions. *J. Biol. Chem.* **223**:147

Johnson, S. M., Herrin, J., Liu, S. J., Paul, I. C. 1970. Crystal structure of barium complex of antibiotic X-537A, $Ba(C_{34}H_{53}O_8)_2\,H_2O$. *J. Amer. Chem. Soc.* **92**:4428

Johnson, D., Lardy, H. A. 1967. Isolation of liver or kidney mitochondria. *In:* Methods in Enzymology. R. W. Estabrook and M. E. Pullman, editors. Vol. 10, p. 94. Academic Press, New York-London

Lardy, H. A., Graven, S. N., Estrada-O., S. 1967. Specific induction and inhibition of cation and anion transport in mitochondria. *Fed. Proc.* **26**:1355

Lardy, H. A., Johnson, D., McMurray, W. C. 1958. Antibiotics as tools for metabolic studies. I. A survey of toxic antibiotics in respiratory, phosphorylative and glycolitic systems. *Arch. Biochem. Biophys.* **78**:587

Lardy, H. A., Wellman, H. 1953. The catalytic effect of 2,4-dinitrophenol on adenosine triphosphate hydrolysis by cell particles and soluble enzymes. *J. Biol. Chem.* **201**:357

Lin, D. C., Kuhn, E. 1973. Mode of action of antibiotic X-537A on mitochondrial glutamate oxidation. *Biochem. Biophys. Res. Commun.* **50**:830

Lin, D. C., Kuhn, E. 1974. Inhibition of the oxidation of glutamate and isocitrate in liver mitochondria at a specific $NADP^+$-reducing site. *Proc. Nat. Acad. Sci.* **70**:3450

Lindberg, O., Ernster, L. 1956. Determination of inorganic orthophosphate. *Methods Biochem. Anal.* **3**:6

Loyter, A., Christiansen, R. O., Steensland, H., Saltzgaber, J., Racker, E. 1969. Energy-linked ion transport in submitochondrial particles. I. Ca^{++} accumulation in submitochondrial particles. *J. Biol. Chem.* **224**:4422

Mitchell, P. 1968. Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation. Glynn Research, Ltd. Bodmin., Cornwall, England

Montal, M., Chance, B., Lee, C. P. 1969. Uncoupling and charge transfer in submitochondrial particles. *Biochem. Biophys. Res. Commun.* **36**:428

Neumcke, B., Bamberg, E. 1973. The mechanisms of action of uncouplers in bilayer lipid membranes. *In:* Membranes: A Series of Advances. G. Eisenman, editor. Marcel Dekker, New York (*In press*)

Ohnishi, T., Ebashi, S. 1963. Spectrometrical measurements of instantaneous calcium binding of the relaxing factor of muscle. *J. Biochem.* **54**:506

Palmieri, F., Quagliariello, E. 1969. Correlation between anion uptake and the movement of K^+ and H^+ across the mitochondrial membrane. *Europ. J. Biochem.* **8**:473

Pedersen, P. L., Coty, W. A. 1972. Energy-dependent accumulation of calcium and phosphate by purified inner membrane vesicles of rat liver mitochondria. *J. Biol. Chem.* **247**:3107

Pressman, B. C. 1965. Induced active transport of ions in mitochondria. *Proc. Nat. Acad. Sci.* **53**:1076

Pressman, B. C. 1967. Biological applications of ion-specific glass electrodes. *In:* Methods in Enzymology. R. W. Estabrook and M. E. Pullman, editors. Vol. 10, p. 714. Academic Press, New York-London

Pressman, B. C. 1968. Ionophorous antibiotics as models for biological transport. *Fed. Proc.* **27**:1283

Pressman, B. C. 1970. Energy-linked ion transport in mitochondria. *In:* Membranes of Mitochondria and Chloroplasts. E. Racker, editor. Vol. 165, p. 213. Van Nostrand Reinhold Co., New York

Pressman, B. C. 1972. Carboxylic ionophores as mobile carriers of divalents ion. *In:* The Role of Membranes in Metabolic Regulation. M. A. Mehlman and R. W. Hanson, editors. p. 149. Academic Press, New York-London

Pressman, B. C. 1973. Cation complexing, cation transport and *in vivo* properties of the ionophore X-537A. *Fed. Proc.* **32**:1698

Reed, P. W., Lardy, H. A. 1972. A23187: A divalent cation ionophore. *J. Biol. Chem.* **247**:6970

Roeske, R. W., Isaac, S., Steinrauf, L. K., King, T. 1971. Synthesis and ion-transport properties of the peptide antibiotic beauvericin. *Fed. Proc.* **30**:1282 (Abstract)

Scarpa, A., Inesi, G. 1972. Ionophore mediated equilibration of calcium ion gradients in fragmented sarcoplasmic reticulum. *FEBS Letters* **22**:273

Settlemire, C. T., Hunter, G. R., Brierley, G. P. 1968. Ion transport in heart mitochondria. XVIII. The effect of ethylendiaminetetraacetate on monovalent ion uptake. *Biochim. Biophys. Acta* **162**:487

Shavit, N., Degani, H., San Pietro, A. 1970. Effects of ionophorous antibiotics in chloroplasts. *Biochim. Biophys. Acta* **216**:208