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Model Translocators for Divalent and Monovalent Ion Transport in Phospholipid Membranes

I. The Ion Permeability Induced in Lipid Bilayers by the Antibiotic X-537A*

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Summary. Adsorption of the antibiotic X-537A to lipid bilayers increases the membrane conductance to monovalent and divalent cations up to 10^4 times. From biionic potential measurements the following ionic selectivity sequences are derived: $H^+ \gg Cs^+ > Rb^+$, $K^+ > Na^+ > Li^+$ for monovalent cations and $Ba^{++} > Ca^{++} > Mn^{++} > Sr^{++} \gg Mg^{++}$ for divalent cations. The zero-current membrane potential in a gradient of H^+ or Ca^{++} exhibits Nernst behavior. The membrane conductance is proportional to approximately the second power of the antibiotic concentration for both H^+ and Ca^{++} conductances; it is linearly proportional to the H^+ and Ca^{++} concentration in the aqueous phase at constant X-537A concentration. The H^+-conductance exhibits a maximum at pH$\sim$3.7, which may tentatively be ascribed to the salicylate moiety of the ionophore. When equimolar solutions of HCl and a divalent chloride salt are placed on opposite sides of an X-537A-doped membrane, a discrimination of H^+ over the divalent cation is obtained. The dependence of the conductance on ionophore and ion concentration suggests that the permeant species for the H^+ conductance is the dimer $(HA_2)^-$, (where A indicates the anionic form of the ionophore) and for the Ca^{++} conductance the dimer $(CaHA_2)^+$.

Compound X-537A is a carboxylic polyether antibiotic (Berger, Rachlin, Scott, Sternbach & Goldberg, 1951; Westley, Evans, Williams & Stempel, 1970) which forms lipophilic complexes with monovalent and divalent cations in water-lipid bulk-phase systems (Pressman, 1968, 1972, 1973; Pressman & Heeb, 1970), in phospholipid vesicles as well as in membranes from erythrocytes (Henderson, McGivan & Chappell, 1969; Pressman &

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

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Heeb, 1970), mitochondria (Lardy, Graven & Estrada-O., 1967), chloroplasts (Shavit, Degani & San Pietro, 1970; Degani & Shavit, 1972), sarcoplasmic reticulum (Caswell & Pressman, 1972; Entman, Gillette, Wallick, Pressman & Schwartz, 1972; Scarpa & Inesi, 1972) and mast cells (Foreman, Gomperts & Mongar, 1973).

The antibiotic has been reported to catalyze an alkali ion/H^+ exchange through water-lipid bulk interphases exhibiting the selectivity sequence: $Cs^+ > Rb^+ > K^+$, Li^+, Na^+ (Pressman, 1968) and in phospholipid vesicles and erythrocyte membranes with the sequence K^+, $Rb^+ > Na^+ > Cs^+ > Li^+$ (Henderson *et al.*, 1969; Pressman & Heeb, 1970).

The ionophore X-537A has also been found to induce a passive efflux of Ca^{2+} previously accumulated through energy-linked ATP hydrolysis in isolated sarcoplasmic reticulum vesicles (SR) (Caswell & Pressman, 1972; Scarpa, Baldassare & Inesi, 1972; Scarpa & Inesi, 1972) in red cells, intact mitochondria and plasma membranes of isolated myoblasts, as well as a passive efflux of Mg^{2+} from mitochondrial membranes (Lin & Kun, 1973). It induces Ca^{2+} uptake and histamine secretion in mast cells (Foreman *et al.*, 1973). The effects of X-537A on divalent cation movements has been ascribed to its ability to form lipophilic clathrates with Ca^{2+}, Mg^{2+} and Ba^{2+} (Pressman, 1968, 1973; Johnson, Herrin, Liu & Paul, 1970; Westley, Evans, Williams & Stempel, 1970; Degani, Friedman, Navon & Kossower, 1973).

Similar effects to those described for X-537A with respect to Ca^{2+} and Mg^{2+} movements in mitochondria and SR vesicles have also been reported by Reed and Lardy (1972) and Caswell and Pressman (1972) for compound A-23187.

We begin this series of papers describing the ionic selectivity properties of X-537A for monovalent and divalent cations in lipid bilayers. The results of this study will be used in presenting possible mechanisms of transport which account for the results on lipid bilayers herewith reported and for those described in the second paper (Estrada-O., Célis, Calderón, Gallo & Montal, 1974) on the transport properties induced by X-537A in mitochondrial membranes.

After this work was completed, an abstract by Mar and Pressman (1972) reporting the effect of carboxylic ionophores on lipid bilayers, came to our attention.

Materials and Methods

Lipid bilayers were formed and their electrical properties studied by the method of Mueller and Rudin (*cf.* Mueller & Rudin, 1969) as previously described in detail (Montal, 1972). The membranes were formed in a 1-mm aperture in a Teflon vessel. Both aqueous

compartments were continuously stirred by means of miniature magnetic spinbars. The membranes were formed from a solution of 4% egg phosphatidylcholine (Applied Science Laboratories, State College, Pa.) in *n*-octane (A.R. grade reagent from Mathenson, Coleman and Bell, Norwood, Ohio).

For the single salt case, the aqueous phase consisted of 1 mM of the adequate salt adjusted to the pH indicated in Figure and Table legends with the appropriate hydroxide. After the optically black configuration was attained, X-537A (in ethanol) was then added to the aqueous phase. The membranes attained conductances between 10^{-6} and 10^{-5} Ω^{-1} cm^{-2}. Concentration differences were established across the membrane by addition of small amounts of concentrated salt solution to one side, and the resultant membrane potential recorded. For the biionic case, the membranes were formed in unbuffered water brought to the pH indicated in Figure and Table legends by careful addition of NaOH; when the black membrane state was reached, one salt was added inside and the other one outside to the concentrations indicated in the Table; thereafter, X-537A was added to one compartment to give 1.7×10^{-5} M. The resultant potential difference across the membrane was recorded. The results remain essentially the same if the ionophore is added to both compartments. The selectivity sequence for monovalent cations derived from biionic potential measurements is analogous to that obtained from conductance measurements (Eisenman, Szabo, Ciani, McLaughlin & Krasne, 1973).

Being aware of the fact that biionic potentials for counter-ions of different valences are complicated phenomena critically dependent on concentration, stirring and membrane thickness (*cf.* Scatchard & Helfferich, 1956; Helfferich, 1962), no attempt will be made to obtain precise selectivity coefficients; however, they are instructive as far as they provide approximate selectivity sequences.

The ability of X-537A to extract acridine orange from an aqueous medium into a hexane phase was determined by the procedure of Pedersen (1968), equilibrating an aqueous solution at known pH containing 1.44×10^{-3} M acridine orange in a volume of 2.0 ml with an equal volume of *n*-hexane containing 1.63×10^{-3} M X-537A. The two-phase system was shaken in a vortex mixer for 3 min and phase separation was allowed to occur. A 0.1-ml aliquot of the aqueous phase was diluted 50 times and the absorbance at 500 nm, corresponding to the maximum absorption of acridine orange, determined. All data presented are representative of three to five experiments. All the experiments were performed at room temperature (22 ± 2 °C) unless otherwise noted.

Results

The Ionic Selectivity of X-537A in Lipid Bilayers

The ionic selectivity of X-537A in lipid bilayers as derived from biionic potential measurements is illustrated in Table 1. The sequence obtained for monovalent cations is the following: $H^+ \gg Cs^+ > Rb^+$, $K^+ > Na^+ > Li^+$ and for divalent cations is as follows: $Ba^{++} > Ca^{++} > Mn^{++} > Sr^{++} \gg Mg^{++}$. These sequences derived from electrochemical experiments in lipid bilayers are in close agreement with those obtained from partition measurements (Pressman, 1968) and with spectroscopic techniques (Degani *et al.*, 1973). Pressman and Mar (*cf.* Pressman, 1973) reported that $Ba^{++} < Ca^{++}$; the reason for the discrepancy cannot be resolved until a detailed experimental information is provided. It is apparent that no significant discrimination

Table 1. Biionic potentials (E_{AB}) for lipid bilayers in the presence of X-537A

Electrolyte A(in):B(out)	Concentration ($\times 10^{-3}$ mole $\times$ liter^{-1})	E_{AB} (mV)
Na^+ :Li^+	12	− 6
K^+ :Li^+	12	−20
Rb^+ :Li^+	12	−21
Cs^+ :Li^+	12	−23
H^+ :Li^+	1.2	−43
Ca^{++} :Mg^{++}	1.2	−94
Ca^{++} :Sr^{++}	1.0	− 9
Ca^{++} :Mn^{++}	1.0	− 7.5
Ba^{++} :Ca^{++}	1.0	−15
Ca^{++} :Li^+	12	−38
Mg^{++}:Li^+	12	−14.5
H^+ :Ca^{++}	1.2	−37
H^+ :Mg^{++}	1.2	−97

Membranes were formed in water (pH 5.5). Indicated cations in the form of chloride salts were added to opposite compartments at the concentration indicated so that $(A)_{in} = (B)_{out}$. X-537A was then added to inner compartment to a concentration of 1.7×10^{-5} M. The resulting membrane potential was measured.

between the alkali monovalent cations is obtained whereas a large affinity for the divalent cations is exhibited (third column-Table 1). However, the biionic potentials between monovalent and divalent cations indicate that H^+ is the species which is preferentially transported over any other one (fourth column-Table 1); hence, the position of H^+ in the sequence deserves particular attention. Considering the biological importance of both H^+ and Ca^{++} their conductances in lipid bilayers will be presented in detail.

The H^+ Conductance of Lipid Bilayers in the Presence of X-537A

The addition of X-537A to lipid bilayers in the presence of H^+ results in a conductance increment; if under this condition a H^+ gradient is set up across the membrane, a potential difference can be measured. The sign of the potential indicates that H^+ is transported across the membrane. The membrane potential follows the logarithm of the H^+ activity ratio across the film as illustrated in Fig. 1*A*. The line through the experimental points departs from the theoretical Nernst slope of 59 mV/decade having a value of 47 mV/decade. The transference number for H^+ as derived from this experiment gives a value of 0.88, indicating that X-537A has conferred to the BLM the property of H^+ selectivity, although it is not ideal. No sig-

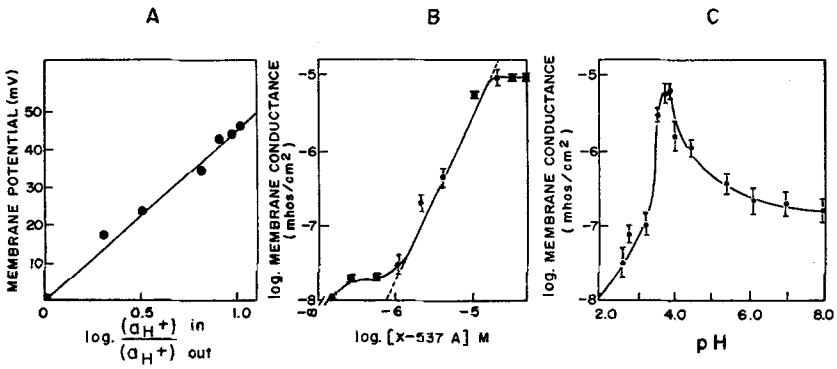


Fig. 1. Effects of X-537A on the membrane conductance properties of lipid bilayers in the presence of H^+. (*A*) Potential difference across lipid bilayers treated with X-537A as a function of the ratio of concentrations of HCl in the two aqueous phases. Membranes were formed in 10^{-4} M HCl and X-537A was then added to the inner compartment to a concentration of 1.7×10^{-5} M. The bilayers attained a conductance of approximately $5 \times 10^{-6}\ \Omega^{-1}\,cm^{-2}$. Concentration differences were established across the membrane by addition of small amounts of concentrated HCl solution to the inner compartment, and the resulting membrane potential measured. The slope of the line is 47 mV/decade. (*B*) Relation between membrane conductance and concentration of X-537A in the aqueous phase at constant HCl concentration (pH 3.0) and constant voltage (50 mV). From the slope of the linear portion of the trace we have $G \propto C^2_{X\text{-}537A}$. (*C*) Relation between membrane conductance and concentration of HCl in the aqueous phase, at constant X-537A concentration (2.1×10^{-5} M) and constant voltage (50 mV)

nificant differences in the transference number were detected when anions other than Cl^- were used.

The dependencies of the membrane conductance on ionophore and ion concentration are illustrated in Fig. 1 *B* and *C*, respectively. The log conductance *vs.* log X-537A concentration plot (Fig. 1 *B*) is sigmoidal, exhibiting linear behavior between 10^{-6} and 10^{-5} M, the point at which it levels off. The slope of the linear portion of this plot is 2. The log conductance *vs.* pH plot (Fig. 1 *C*) exhibits a maximum at pH 3.7.

The Ca^{++}-Conductance of Lipid Bilayers in the Presence of X-537A

The characteristics of the Ca^{++} conductance of X-537A-doped lipid bilayers, are presented in Fig. 2. The dependence of the membrane potential on the log of Ca^{++}-activity ratio across the membrane, is illustrated in Fig. 2 *A*, indicating that the system follows the Nernst behavior. The transference number for Ca^{++} as calculated from membrane potential measurements (Montal, 1972) is 0.95, manifesting that in the presence of X-537A

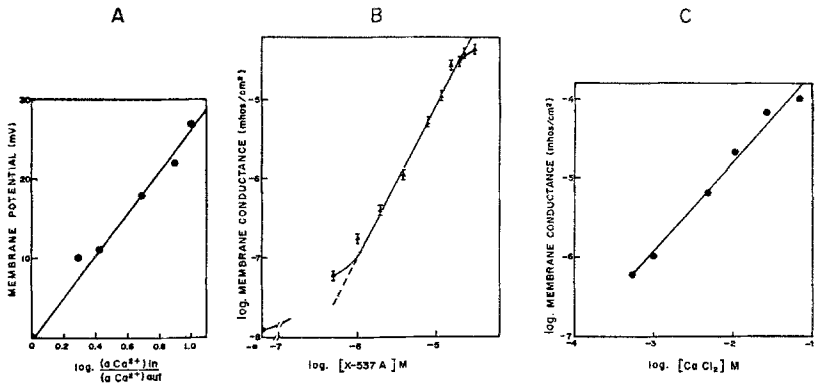


Fig. 2. Effects of X-537A on the membrane conductance properties of lipid bilayers in the presence of Ca^{2+}. (*A*) Potential difference across lipid bilayers treated with X-537A as a function of the ratio of concentrations of $CaCl_2$ in the aqueous phases. Membranes were formed in 10^{-3} M $CaCl_2$ at pH 8.0 (adjusted with NaOH) and X-537A was then added to the inner compartment to a concentration of 1.7×10^{-5} M. The bilayers attained a conductance of approximately $8 \times 10^{-6}\ \Omega^{-1}\,cm^{-2}$. Concentration differences were established across the membrane by addition of small amounts of concentrated $CaCl_2$ solution to the inner compartment, and the resulting membrane potential measured. The slope of the line is 27 mV/decade. (*B*). Relation between membrane conductance and concentration of X-537A in the aqueous phase at constant $CaCl_2$ concentration (50 mM) at pH 8.0 (adjusted with NaOH) and constant voltage (50 mV). From the slope of the linear portion of the trace we have $G \propto C^2_{X\text{-}537A}$. (*C*). Relation between membrane conductance and concentration of $CaCl_2$ in the aqueous phase (pH 8.0), at constant X-537A concentration (2.1×10^{-5} M) and constant voltage (50 mV). From the slope of the line one obtaines $G \propto C_{CaCl_2}$

the membrane behaves as a quasi-Ca^{++}-selective system. The dependence of the membrane conductance on ionophore concentration is presented in Fig. 2*B*. At 50 mM Ca^{++}, a sigmoidal behavior is apparent; a line with a slope of 2.0 can be drawn through most of the experimental points. In agreement with this result, independent measurements by Dr. Stuart McLaughlin (*personal communication*) indicate a slope of 2 at 50 mM Ca^{++} in the presence of citrate. On the other hand, the membrane conductance is linearly dependent on Ca^{++} concentration presenting a slope of 1, as shown in Fig. 2*C*.

The Equilibrium Extraction of Acridine Orange into n-Hexane by X-537A

To clarify the mechanism by which the ionophore X-537A makes BLM selectively permeable to cations and specifically the pH-dependent behavior

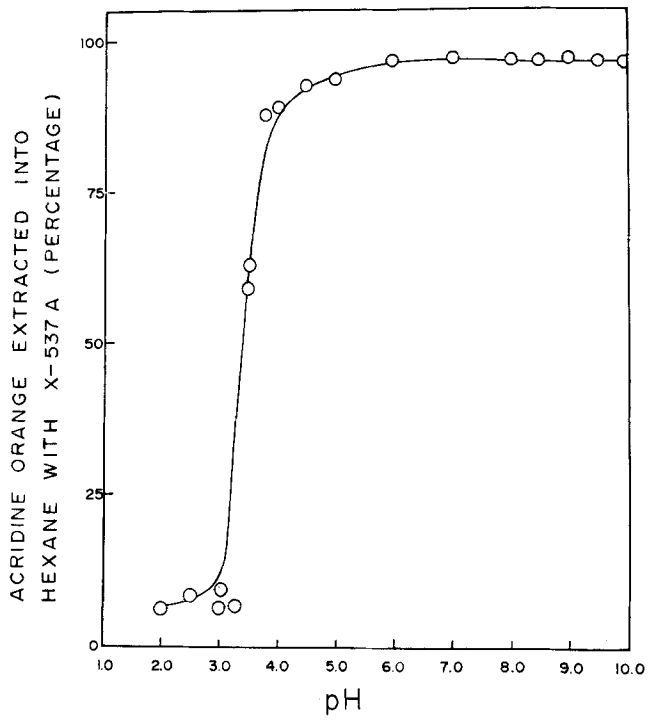


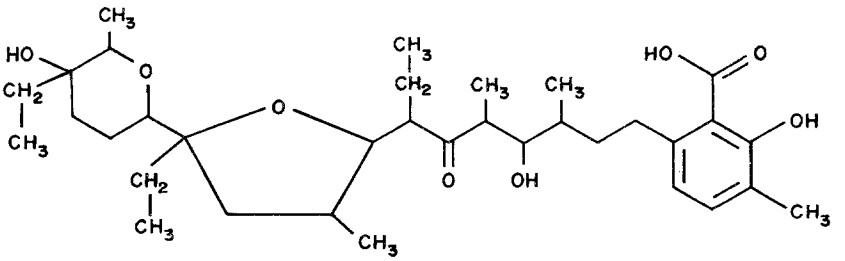
Fig. 3. Equilibrium extraction of acridine orange into *n*-hexane with X-537A

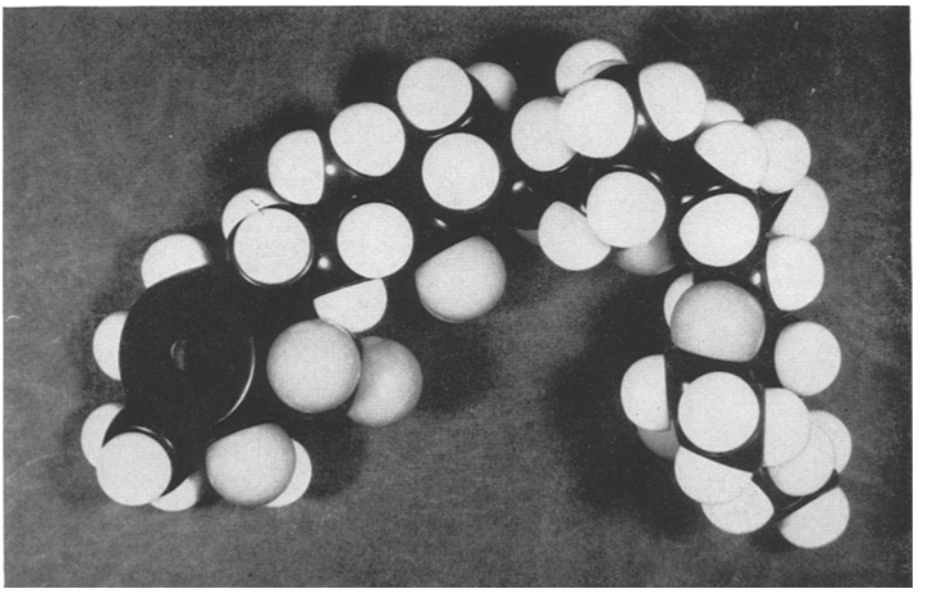
obtained (Fig. 1*C*), the extraction of a lipophilic colored cation such as acridine orange into hexane, a solvent which mimics the properties of the liquid hydrocarbon interior of a lipid bilayer, was studied. As illustrated in Fig. 3, the ability of X-537A to complex with acridine orange and partition it into the low dielectric constant phase is markedly pH-dependent. Fig. 3 resembles a pH titration of a weak acid, with a characteristic sigmoidal shape. The apparent pK_a is approximately 3.5. This value is in close agreement with the pH for maximum H^+-conductance (Fig. 1*C*). As a first approximation, it is possible to ascribe this pK_a to the salicylate moiety of this carboxylic ionophore (pK_a of salicylate$\cong$3.7).

Discussion

The results hitherto reported establish that the carboxylic antibiotic X-537A allows the diffusion of monovalent and divalent cations across the

hydrocarbon region of lipid bilayers. The structure of X-537A is as follows (Westley *et al.*, 1970):





The potential sites for coordination with the metal ions are two ether oxygens, two hydroxyls, a ketonic carboxyl and a carboxyl oxygen. The association of H^+ is at the carboxylate group of the salicylate moiety considering the pH-dependence of membrane conductance (G_m) (Fig. 1*C*) and the pK_a of the antibiotic as derived from the extraction of the acridine cation from aqueous to hexane phases (Fig. 3); i.e. pK_a X-537A $\cong pK_a$ salicylate $\cong 3.7$; this may also explain the high affinity of the ionophore for H^+ over any other ion. The crystal structure of the barium salt of X-537A (Johnson *et al.*, 1970) indicates the presence of a dimer in which the ion is approached by six oxygen atoms from one antibiotic, by two from the other one and another from the water molecule of crystallization. Thus, three distinct modes of ion-

ionophore association are possible: (a) H^+-with the salicylate anion, (b) alkali monovalent cations to the oxygen-bearing groups of the monomer, and (c) divalent cations to the oxygen-bearing groups of the dimer.

It is instructive to compare the large selectivity for alkali monovalent cations of amide and ether containing ionophores (e.g. valinomycin, actins; Eisenman *et al.*, 1973) with the poor selectivity exhibited by X-537A, a carboxyl-containing molecule. Apparently, the affinity of amide and ether oxygens for these cations is larger than that of carboxyl oxygens. On the other hand, the fact that X-537A exhibits greater affinity for divalent than for monovalent cations suggests that carboxyl oxygens interact with divalent ions to a greater extent than amide and ether oxygens. It is also worth noting that in the calcium-binding protein from carp muscle, whose three-dimensional structure has been obtained by X-ray analysis (Nockolds, Kretsinger, Coffee & Bradshaw, 1972), the Ca^{++} coordination site consists of one glutamic and three aspartic acid carboxyl groups in a tetrahedral arrangement, the Ca^{++}-O^- distance being 2.5 Å. This coordination distance is close to the Ba^{++}-O^- distances in the barium salt of X-537A, which range from 2.71 to 3.08 Å (Johnson *et al.*, 1970). Eisenman *et al.* (1973) have previously elaborated on the specificity of ion-ligand interactions with regard to the similarity of the selectivity patterns for valinomycin and cell membranes and the large differences between the ionic selectivities of macrotetralide actins and biological membranes. The wide selectivity of X-537A can also be accounted for by the conformational freedom allowed by its open structure, in contrast to the comparatively rigid cyclic peptides (Eigen & Winkler, 1970; Simon, Morf & Meier, 1973).

The fact that there is a second-power dependence of membrane conductance on X-537A concentration (Figs. 1*B*, 2*B*) together with the observation that G_m is proportional to the first power of permeant cation concentration (Figs. 1*C*, 2*C*) indicates the existence of a complex in which two antibiotic molecules are needed to solubilize the cation (H^+ or Ca^{++}) in the hydrocarbon region of the membrane.

The Ca^{++} conductance can be accounted for by the transfer of charge associated to the diffusion of a complex formed between one neutral, one negatively charged ionophore and $Ca^{++}(CaHA_2)^+$. This is consistent with the finding that a concentration gradient of Ca^{++} generates a membrane potential (Fig. 2*A*).

The H^+ conductance induced by X-537A in lipid bilayers is very similar to that reported for some weak acid uncouplers of oxidative phosphorylation (*cf.* Liberman & Topaly, 1968*a*, *b*; *cf.* Haydon & Hladky, 1972), i.e., the zero-current membrane potential in a pH gradient exhibits Nernst slopes;

an apparent second-order dependence of G_m on uncoupler concentration is obtained and the G_m versus pH curve has a pronounced maximum which is approximately the same as the pK. The membrane conductance reaches a maximum with increasing concentrations of antibiotic (i.e., exhibits saturation behavior), and in this respect, is analogous to that described for lipophilic anions such as tetraphenylborate or dipicrylamine anion (Ketterer, Neumcke & Lauger, 1971) and uncouplers (E. Bamberg, *personal communication*). This phenomenon has been ascribed to the limit in the number of ions which may be adsorbed at the interface as well as to the "blocking" effect (Bruner, 1970) consequent to the development of a negative surface potential (*see also* McLaughlin, 1972).

Liberman, Mokhova, Skulachev and Topaly (1968) explain their data on the basis of a 1–1 carrier model. Finkelstein (1970) (*see also* Lea & Croghan, 1969) has proposed a variation of the carrier model in which two uncoupler molecules and H^+ combine to form the permeant species. This model explains the fact that the pH for maximum conductance is close to the pK (Finkelstein, 1970) and also the quadratic dependence of G_m on ionophore concentration. McLaughlin (1972) has recently shown that if the negative surface potential induced by the adsorption of the uncoupler, in this case 2,4-dinitrophenol, on neutral lipid bilayers is taken into account, the model of Finkelstein (1970) and of Lea and Croghan (1969) is compatible with the available experimental evidence for this class of uncouplers. Le Blanc (1971) presented a detailed study of the conductance of neutral bilayers in the presence of another class of uncouplers, the carbonylcyanide phenylhydrazones (CCP's), where G_m is directly proportional to the uncoupler concentration; Le Blanc proposed a model in which the permeant species are the weak acid uncoupler (HA) and its anion (A^-) that was capable of describing the effects of chloro-CCP on lipid bilayers. The model of Finkelstein (1970) gives an appropriate description of the H^+ conductance of bilayer membranes in the presence of X-537A, i.e. a 2:1 complex formed by two X-537A molecules and $H^+(HA_2)^-$. The analogy to uncouplers of oxidative phosphorylation can be extended by the fact that X-537A is also an uncoupler in mitochondria (*see* accompanying paper, Estrada-O. *et al.*, 1974).

We arrive at the conclusion that the properties exhibited by X-537A-doped lipid bilayers can be accounted for by the carrier model of ion transport across membranes (Eisenman, Ciani & Szabo, 1968; Markin, Krishtalik, Liberman & Topaly, 1969; Stark, Ketterer, Benz & Läuger, 1971; *cf.* Haydon & Hladky, 1972). Preliminary electrical relaxation experiments (R. Benz & M. Montal, *unpublished observations*) have demonstrated a slow relaxation, thus supporting the carrier model. On the other hand, attempts to apply the

single channel technique as a test for the channel-model (Bean, Shepherd, Chan & Eichner, 1969; Ehrenstein, Lecar & Nossal, 1970; Gordon & Haydon, 1972; *cf.* Haydon & Hladky, 1972) have so far been negative (E. Bamberg & M. Montal, *unpublished observations*).

It is important to emphasize that the information hitherto obtained is derived from steady-state measurements. It is to be expected that important clues for the elucidation of the mechanism of action of X-537A will be achieved by a thorough kinetic analysis (Stark *et al.*, 1971).

The authors are indebted to Dr. Stuart McLaughlin for providing data analogous to that presented in Fig. 2*B* prior to publication, and to Drs. E. Bamberg, R. Benz, C. Gómez-Lojero, J. Korenbrot, P. Läuger, S. McLaughlin and G. Stark for helpful criticism and comments.

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