J. Membrane Biol. 10, 237 – 246 (1972)

Phospholipid Bilayers as Biological Membrane Models: The Effect of N,N′-*Bis*(Dichloroacetyl)-1,12-Diaminododecane

Daniel Alkaitis, A. John Merola, and Albert L. Lehninger

Department of Physiological Chemistry,
The Johns Hopkins University School of Medicine,
725 North Wolfe Street, Baltimore, Maryland 21205 and
Department of Physiological Chemistry, Ohio State University,
370 West 9th Avenue, Columbus, Ohio 43210

Received 6 November 1972

Summary. N,N′-*bis*(dichloroacetyl)-1,12-diaminododecane is a potent inhibitor of microsomal drug metabolism and also uncouples succinate-linked mitochondrial oxidative phosphorylation, apparently by promoting a transient permeability to anions. When added in very small concentrations to synthetic phospholipid bilayers made from a 1:4:4 mixture of purified cardiolipin, phosphatidylcholine, and phosphatidylethanolamine, the drug causes a rapid, transient decrease in electrical resistance with a return to an end-resistance somewhat lower than the initial one. The magnitude of the decrease was related to drug concentration. However, the drug produced a nontransitory, i.e. permanent decrease in resistance of bilayers made from pure phosphatidylcholine or cardiolipin. The 1:4:4 mixture of lipids, which closely resembles the lipid composition of the inner mitochondrial membrane yielded drug effects most closely resembling those observed in intact mitochondria. Transference number measurements on the 1:4:4 bilayer with an impressed KCl gradient revealed that the drug-induced decrease in electrical resistance was caused by an increase in the fraction of current carried by the anions in the system. The 1:4:4 phospholipid bilayer thus mimics the mitochondrial inner membrane in its response to this drug and indicates that the lipid composition of the lipid bilayer is a major determinant of at least some of its physical characteristics. The effect of varying the structure of the drug was also examined.

N,N′-*bis*(dichloroacetyl)-1,12-diaminododecane is a potent inhibitor of microsomal drug metabolism [11] and of mitochondrial pyridine nucleotide-linked electron transport [8, 10]. It is also a moderately strong uncoupler of succinate respiration of an unusual nature, since it induces a stimulation of state 4 respiration which is transitory in nature and is followed by a return to nearly normal state 4 respiration and an orthodox response to a subsequent addition of ADP [9]. In an earlier paper, evidence was presented that this compound uncouples by a mechanism differing from the action of dinitro-

phenol (DNP) and *m*-chlorocarbonylcyanidephenylhydrazone (CCP), which conduct protons, or the trialkyltins, which promote Cl^-/OH^- exchange [13]. It was tentatively concluded that this drug, hereafter abbreviated Cl_2C_{12}, uncouples by inducing a transient permeability to anions or possibly a transient Cl^-/OH^- exchange, with a concomitant collapse of the transmembrane electrochemical gradient. The return to the coupled state is accompanied by regeneration of the transmembrane electrochemical gradient by respiration-driven mechanisms.

The unique behavior of this agent prompted us to investigate its behavior in synthetic phospholipid bilayers, which have been shown to undergo large decreases in electrical resistance in the presence of proton-conducting uncoupling agents [2]. In this paper we report that N,N′-*bis*(dichloroacetyl)-1,12-diaminododecane also induces a reduction in the electrical resistance of phospholipid bilayers. Furthermore, when the bilayer is prepared from a mixture of purified phospholipids resembling in composition those found in the mitochondrial inner membrane, the drug-induced decrease in resistance is transitory, resembling the transitory effect of the drug on intact mitochondria. These observations are in agreement with the views of Hopfer, Lehninger and Lennarz [5, 6], who emphasized the relationship between the lipid composition and the physico-chemical properties of bilayers.

Materials and Methods

Doubly distilled water was used for all aqueous solutions. Analytical grade KCl was recrystallized from 1 mM ethylenediaminetetraacetate (EDTA). The *n*-decane was distilled and passed through an alumina column before use. Bovine cardiolipin (DPG), egg phosphatidylcholine (PC), and bovine phosphatidylethanolamine (PE) were purchased from Supelco, Inc., Bellefonte, Pa. These phospholipids were 98% pure and were used directly. The N,N′-*bis*(dichloroacetyl)-1,12-diaminododecane (Cl_2C_{12}), N,N′-*bis*(trichloroacetyl)-1,12-diaminododecane (Cl_3C_{12}), N,N′-*bis*(chloroacetyl)-1,12-diaminododecane (Cl_1C_{12}), N,N′-*bis*(acetyl)-1,12-diaminododecane (Cl_0C_{12}), N,N′-*bis*(dichloroacetyl)-1,8-diaminooctane (Cl_2C_8), and the N,N′-*bis*(dichloroacetyl)-1,10-diaminodecane (Cl_2C_{10}) were synthesized and crystallized as reported previously [10].

The techniques and apparatus for the formation of the bilayers and for making the electrical measurements were described in detail by Hopfer *et al.* [5]. The aperture employed had an area of 0.03 cm^2. The bilayer-forming solution consisted of a 1% solution of phospholipid in *n*-decane. When a mixture of phospholipids was desired for the bilayer-forming solution, 1% solutions of each phospholipid were mixed in appropriate ratios. Lipid solutions were stored under nitrogen in the deep-freeze when not in use. The two chambers holding the bathing aqueous medium were open to the atmosphere; they were stirred with Teflon-coated magnetic bars. The standard bathing solution consisted of 10 mM KCl, 10 μM EDTA, and 1 mM Tris chloride, pH 7.4. The bilayers were formed by the brush technique. The crucial step in forming mechanically stable bilayers of reproducibly high resistance was the prepainting procedure. This consists of

brushing the standard lipid solution on the dry septum with subsequent evaporation of the solvent under high vacuum. Both sides of the septum were prepainted twice in this manner. The pretreated chamber was then connected to the saturated KCl-agar electrodes, buffer added to the chambers, and the lipid solution brushed on the submerged pretreated surfaces and allowed to thin out to form the bilayer.

The resistance measurements were made after impression of a 20 mV potential difference across the membrane. Additions of the uncoupling agents, dissolved in ethanol, were made with microsyringes. The volume of the ethanol solution added to a 10 ml volume of buffer did not exceed 10 μliters. Ordinarily, the uncoupling agents were added to both chambers to ensure that the concentration was equal on both sides of the membrane. Some experiments were carried out in an apparatus with one closed chamber, which yielded more stable membranes. Although addition of uncoupling agent to the single open chamber yielded the same results as in systems with two open chambers, the latter technique was normally used to avoid possibly spurious effects resulting from unilateral additions.

Calculation of the transference number of K^+ (t_{K+}) was carried out from measurements of the transmembrane emf generated by a concentration gradient of KCl, by the equation

$$V_m = (1 - 2t_{K+}) \frac{RT}{F} \ln \frac{a}{a_{ref}}$$

where V_m is the measured emf across the bilayer, R is the gas constant, T is the absolute temperature, F is the Faraday, a is the activity of K^+ in one chamber and a_{ref} the activity in the reference chamber. Generally, several activities of KCl were tested and from a plot of V_m *vs.* $\ln \frac{a}{a_{ref}}$, t_{K+} was calculated. To measure the time course of the change in t_{K+} on addition of Cl_2C_{12} a concentration gradient of KCl was imposed and the emf monitored before and after addition of the drug to both chambers. The t_{K+} was calculated from the above equation; the change in emf directly reflects the change in t_{K+}.

Results

Fig. 1 shows the change with time of the electrical resistance of a mixed phospholipid bilayer (molar ratio, 1:4:4 DPG:PC:PE, approximately the ratio in the inner membrane of rat-liver mitochondria) after the addition of 5 μM Cl_2C_{12}. Immediately after the addition of the drug there was a large and rapid decrease in the resistance of the bilayer. The resistance then rose to a new stable level which was always somewhat lower than the initial membrane resistance. The entire cycle was complete in about 20 min. Fig. 2 shows the effect of the concentration of the drug on the bilayer. The resistance-lowering effect was transient at all concentrations tested, from 0.5 to 5.0 μM; these concentrations are considerably lower than the 12.5 μM concentration used to achieve maximal release of state 4 respiration in mitochondria. The time required to complete a cycle, i.e. to attain a stable end-resistance, was shortest ($\sim$5 min) at the lowest concentration (0.5 μM) and longest (20 to 30 min) at the highest concentration (5 μM) of the drug. In

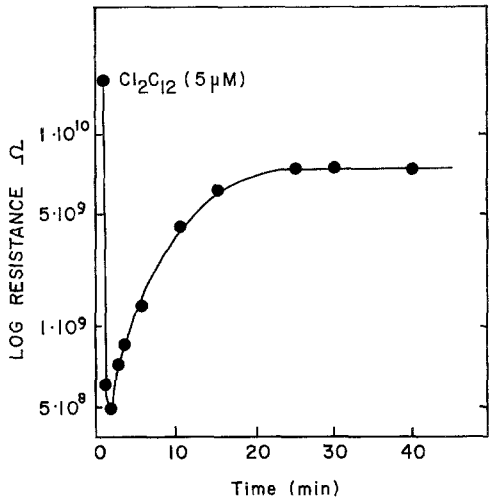

Fig. 1. Effect of 5 μM Cl_2C_{12} on the electrical resistance of a 1:4:4 mixed-lipid bilayer with time. Details are given in the text

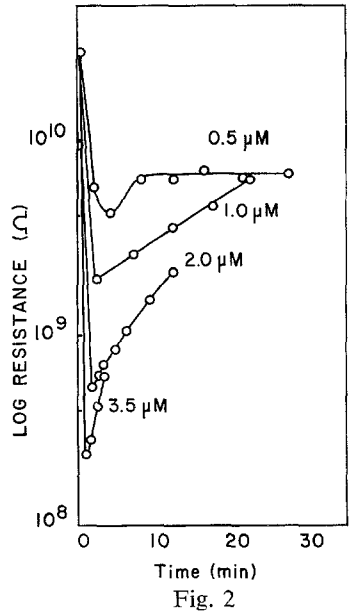

Fig. 2

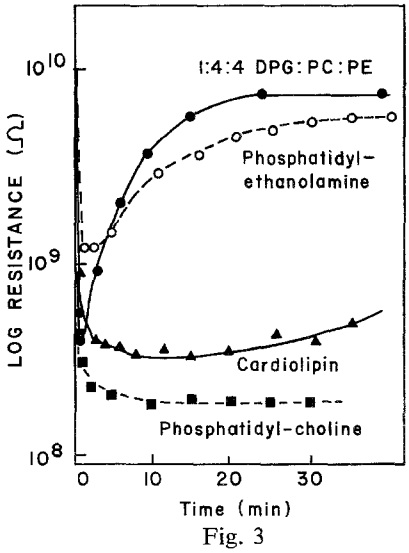

Fig. 3

Fig. 2. Effect of the concentration of Cl_2C_{12} on resistance changes of 1:4:4 mixed-lipid bilayers

Fig. 3. Effect of the lipid composition on the resistance changes of phospholipid bilayers induced by Cl_2C_{12}

intact heart mitochondria the cyclic response to the drug varies but is usually complete in about 2 min.

Fig. 3 contrasts the effects of Cl_2C_{12} on bilayers formed from single pure phospholipids and from the usual 1:4:4 mixture. First, it is noteworthy

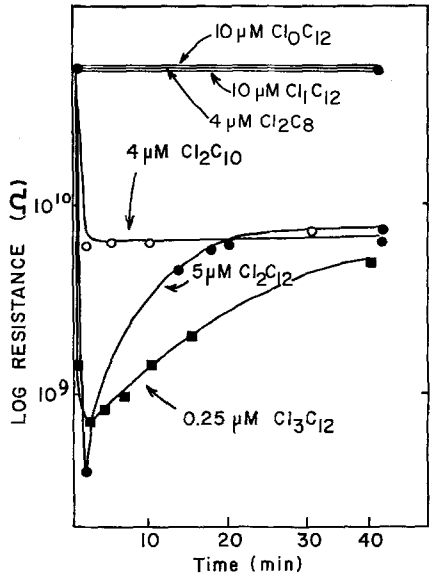

Fig. 4. Effect of alterations in the molecular structure of the drug on bilayer resistance. The abbreviations of drug names are given in the text

that the resistance of the freshly formed bilayers of single lipids in the absence of drug was lower than for bilayers formed from the 1:4:4 mixture of DPG:PC:PE, suggesting that the electrical resistance of a bilayer formed from this mixture of lipids is a nonadditive function. The bilayer formed from phosphatidylethanolamine alone gave the same type of transitory resistance drop following addition of Cl_2C_{12} as is shown by the bilayer formed from the mixture of phospholipids, but the drop was less marked and the return somewhat slower. The bilayers formed from phosphatidylcholine alone and from cardiolipin alone behaved rather differently. Although both underwent small initial drops in resistance on addition of the drug, the phosphatidylcholine bilayer showed no rise in resistance after the initial drop and that formed from cardiolipin showed only a very slow and limited rise. Thus, the transitory nature of the resistance decrease induced by Cl_2C_{12} depends on the lipid composition of the bilayer, a mixture of lipids similar to that found in the mitochondrion [3], giving an effect most closely resembling the transitory uncoupling effect of this agent on intact mitochondria. In general, the higher the initial electrical resistance of the lipid bilayer, the more pronounced is the return of the resistance to a high end-value.

The effects of various analogues of N,N′-*bis*(dichloroacetyl)-1,12-diaminododecane on the bilayer were, in general, similar to their effects on mitochondria (Fig. 4). Parallel with their lack of uncoupling activity on

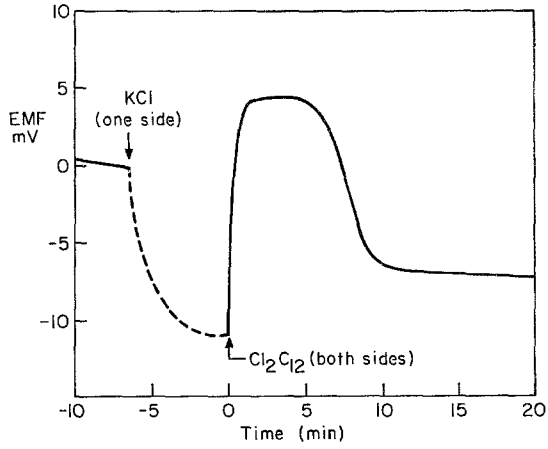

Fig. 5. Effect of Cl_2C_{12} on the transmembrane potential and transference number across the mixed-lipid bilayer. The standard 1:4:4 mixed-lipid bilayer was bathed on both sides by a medium of 10 mM KCl, 10 μM EDTA, and 1.0 mM Tris chloride, pH 7.4. At the point shown, additional KCl was added to one side of the membrane to yield a concentration of 22 mM KCl. At zero time, 4 μM Cl_2C_{12} were added to both sides of the membrane. The trace shows the changes in membrane emf; the changes in transference number of K^+ are given in the text

mitochondria [10], the drugs Cl_0C_{12} and Cl_1C_{12} had no depressing effect on the resistance of the bilayer in concentrations up to 10 μM. The only compound of this series whose action on the bilayer did not parallel its action on mitochondria was the Cl_3C_{12} analogue, which has only a very weak uncoupling activity on mitochondria [8] but which produced a pronounced decrease in the resistance of the bilayer. However, the time relationship in the resistance change induced by Cl_3C_{12} is markedly different from that induced by Cl_2C_{12}.

The effect of the carbon-chain length of the drug in relation to its activity was also investigated. The 8-carbon analogue of Cl_2C_{12} produced no decrease in resistance at concentrations up to 10 μM; at 100 μM it gave only a slight lowering of the resistance of the bilayer, from 1.8×10^9 Ω cm^2 to 1×10^9 Ω cm^2. The 10-carbon analogue Cl_2C_{10} (tested at 4 and 8 μM) produced a permanent lowering of the resistance of the bilayer. Thus, an intermediate chain of 12 carbon atoms is required to yield a transitory decrease in electrical resistance.

The effect of Cl_2C_{12} on the transference number of K^+ (t_{K^+}) is shown in Fig. 5. In this experiment, a 2.2-fold concentration gradient of KCl was established across the membrane, which yielded an initial transmembrane potential of -11 mV from which t_{K^+} is calculated to be 0.62, the expected

value. On addition of 4 μM Cl_2C_{12} the emf immediately rose to +7 mV ($t_{K^+}=0.42$) and then declined slowly over 15 min to −7 mV ($t_{K^+}=0.57$). It is therefore clear from this type of experiment that the fraction of the total diffusion current carried by K^+ is decreased substantially but transitorily by the drug; correspondingly, of course, the fraction carried by chloride is transitorily increased.

Discussion

The results in this paper provide another example of a case in which a characteristic action of a drug on a biological membrane can be mimicked using a synthetic phospholipid bilayer, others being the induction of K^+ permeability by low levels of the macrocylic antibiotic valinomycin [1, 7, 12], the induction of cation permeability by polyoxyethylene ether detergents [16], and the induction of proton permeability by some uncoupling agents [2]. In the case of the uncoupling drug studied here, the analogy is especially striking, since the synthetic bilayer not only shows a decrease in electrical resistance characteristic of uncoupling agents, but also results in a transitory response much like that given by this drug in intact mitochondria. These findings clearly indicate that the characteristic transitory effect of Cl_2C_{12} on mitochondria may be caused by a direct action on the phospholipid phase of the membrane rather than its enzyme components. In support of this view we have found no evidence that this drug is metabolized or transformed by mitochondria. The transitory effect of the drug may thus be intrinsic in the physical interaction between the drug and the phospholipid bilayer; it is unlikely that the synthetic bilayer induces a chemical change in the drug.

The experiments on the effect of alterations in structure of the drug revealed some important features. One is the length of the hydrocarbon chain separating the two amino groups acylated with dichloroacetyl functions; the 12-carbon chain gives a maximal effect while the 10-carbon and 8-carbon homologues are nearly inactive. This suggests a possible relationship between the length of the hydrocarbon chain in the drug and the thickness of the nonpolar zone of the bilayer. Or it may be a reflection of a sharply optimal partition coefficient of the drug between water and the lipid phase of the bilayer [15]. The other significant feature is the number of chlorine atoms on the methyl group; the maximal decrease in resistance with the shortest return time was given by the dichloro derivative. This observation suggests that the effectiveness of the drug may be related to the electron-withdrawing capacity of the chlorine atoms and, therefore, the

degree of polarity of the carbonyl oxygen atoms, which may influence their hydrogen-bonding capacity and the tendency of the amino hydrogen atoms to dissociate. This is an especially attractive idea since we have reported that tertiary amides are inactive as uncouplers [8].

Our observations also provide further evidence that the nature of the phospholipids in a synthetic bilayer determine its permeability and electrical resistance. For example, earlier work from the Baltimore laboratory has demonstrated the dependence of cation *vs.* anion permeability on the type of phospholipids present in the bilayer and the contributions they make to its surface electrical charge [5]. In the present study it is clear that the transient increase in conductance induced by the drug is dependent on the presence of phosphatidylethanolamine in the bilayer and that the maximal and most rapid transitory effects are given by a 1:4:4 DPG:PC:PE mixture. This observation suggests that some physical properties of mixed-lipid bilayers are not merely additive functions of the properties contributed by the individual phospholipids. It also suggests that its characteristic and constant proportion of different phospholipids endows each type of biological membrane with some special property by virtue of nonlinear, possibly cooperative interactions among the lipid components.

The transitory changes of resistance of the synthetic phospholipid bilayers induced by the drug raise fundamental questions regarding their origin. There have been earlier reports of transient decreases in resistance of lipid bilayers. In 1965, Seufert [14] observed that nonionic detergents produced transient decreases in the resistance of bilayers formed in histidine buffers containing KCl. He suggested that the detergents modified the bilayer structure. Recently, Van Zutphen, Merola, Brierley and Cornwell [16] re-investigated the interaction of nonionic detergents with bilayers and concluded that the polyoxyethylene ether detergents induce cation permeability by acting as ionophores. The decrease in resistance was transient when the detergent was present on one side of the bilayer but was permanent when detergent was present on both sides of the phospholipid film. However, we have found that Cl_2C_{12} induces transient changes in resistance when added to both sides of the bilayer, as well as when added to one chamber alone, so that it appears more likely that the transitory effects are caused by time-dependent change in the physical properties of the phosphatidylethanolamine-containing bilayer. A trivial possibility is that the drug equilibrates at unequal rates with the bilayer and with the torus of lipid mixture surrounding the aperture, so that it equilibrates first with the bilayer, causing a decrease in its resistance, followed by diffusion of the drug from the bilayer into the torus, thus leading to an increase in resistance. If this

were the case, a similar phenomenon should have been seen with other uncoupling agents and ionophores and with closely related analogues of Cl_2C_{12}, but it has not been reported. If one assumes, as does Hansch [4], that the drug molecule makes a random walk after entering a system containing several microscopic compartments or phases, it can be expected that a highly water-insoluble drug such as Cl_2C_{12} might elicit a response in one sensitive compartment or phase before finally migrating to another, less sensitive compartment with a higher affinity for the drug. The former site could very well be a zone occupied largely by phosphatidylethamine, particularly its polar head groups, whereas the less sensitive, high-affinity compartment may be the hydrophobic fatty acyl portion of the bilayer. Once such a diffusive relocation of the drug occurred the resistance of the bilayer would increase again. Against such an explanation is the relatively slow movement of the transitory change, which seems incompatible with the very short diffusion times expected between adjacent compartments of molecular dimensions.

The evidence reported in this paper supports the view developed from earlier studies on mitochondria that Cl_2C_{12} acts by inducing anion permeability. The experiments summarized in Fig. 5 show that the drug induces an immediate but transitory decrease in the transference number of K^+, corresponding to a transitory increase in the fraction of the total current carried by anions in the system. This result could be brought about if the drug brings about one of the following changes: (*a*) increases the permeability of the membrane to chloride, the major anion in the system, (*b*) increases the permeability to hydroxyl ions, which are present at low concentrations, (*c*) promotes a transmembrane chloride-hydroxyl exchange, or (*d*) decreases the permeability to K^+ (a most unlikely event).

This work was supported by grants from the National Science Foundation (GB-20519) and the National Institutes of Health (GM-05919) to Dr. A. Lehninger. Dr. D. Alkaitis was the recipient of a National Science Foundation Fellowship (1970-71).

References

1. Andreoli, T. E., Tieffenberg, M., Tosteson, D. C. 1967. The effect of valinomycin on the ionic permeability of thin lipid membranes. *J. Gen. Physiol.* **50**:2527.
2. Bielawski, J., Thompson, T. E., Lehninger, A. L. 1967. The effect of 2,4-dinitrophenol on the electrical resistance of phospholipid bilayer membranes. *In:* Mitochondrial Structure and Compartmentation. E. Quagliariello, S. Papa, E. C. Slater and J. M. Tager, editors. p. 181. Adriatica Editrice, Bari, Italy.
3. Fleischer, S., Rouser, G., Fleischer, B., Casu, A., Kritchevsky, G. 1967. Lipid composition of mitochondria from bovine heart, liver, and kidney. *J. Lipid Res.* **8**:170.

4. Hansch, C. 1968. Use of substituent constants in drug metabolism. *Farmaco Ed. Sc.* **23**:293.
5. Hopfer, U., Lehninger, A. L., Lennarz, W. J. 1970. The effect of the polar moiety of lipids on the ion permeability of bilayer membranes. *J. Membrane Biol.* **2**:41.
6. Hopfer, U., Lehninger, A. L., Lennarz, W. J. 1970. The effect of the polar moiety of lipids on bilayer conductance induced by uncouplers of oxidative phosphorylation. *J. Membrane Biol.* **3**:142.
7. Lev, A. A., Buzhinski, E. P. 1967. Cation specificity of model biomolecular phospholipid membranes with exposure to valinomycin. *Tsitologiya* (Russian). **9**:102.
8. Merola, A. J., Brierley, G. P. 1970. Inhibition of mitochondrial oxidation and uncoupling of phosphorylation by antispermatogenic bis-dichloroacetamides. *Biochem. Pharmacol.* **19**:1429.
9. Merola, A. J., Brierley, G. P. 1970. Respiratory control associated with cyclic pH transitions induced by N,N′-bis(dichloroacetyl)-1,12-diaminododecane. *Biochem. Biophys. Res. Commun.* **41**:628.
10. Merola, A. J., Hwang, K. M., Jurkowitz, M., Brierley, G. P. 1971. Structural requirements in the uncoupling of oxidative phosphorylation by N,N′-bis(dichloroacetyl)-diamines. *Biochem. Pharmacol.* **20**:1393.
11. Merola, A. J., Turnbull, J. D. 1967. The inhibition of drug metabolism by antispermatogenic N,N′-bis(dichloroacetyl) diamines. *Biochem. Pharmacol.* **16**:211.
12. Mueller, P., Rudin, D. O. 1967. Development of K^+-Na^+ discrimination in experimental biomolecular lipid membranes by macrocyclic antibiotics. *Biochem. Biophys. Res. Commun.* **26**:398.
13. Selwyn, M. J., Dawson, A. P., Stockdale, M., Gains, N. 1970. Chloride-hydroxide exchange across mitochondrial, erythrocyte and artificial lipid membranes mediated by trialkyl- and triphenyltin compounds. *Europ. J. Biochem.* **14**:120.
14. Seufert, W. D. 1965. Induced permeability changes in reconstituted cell membrane structure. *Nature* **207**:174.
15. Turnbull, J. D., Biagi, G. L., Merola, A. J., Cornwell, D. G. 1971. Structure-activity relationships for N,N′-bis(dichloroacetyl) diamines and substituted naphthoquinones in the inhibition of mitochondrial electron transport. *Biochem. Pharmacol.* **20**:1383.
16. Van Zutphen, H., Merola, A. J., Brierley, G. P., Cornwell, D. G. 1972. The interaction of non-ionic detergents with lipid bilayer membranes. *Arch. Biochem. Biophys.* (*In Press*).