at a clinically useful concentration for a longer time. In the studied suspensions, the viscosity maintained an acceptable concentration in the urine for at least 2 hr longer than that obtained from an aqueous dispersion.

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Surface Properties of Membrane Systems: Interaction of Electrolyte and Lipid with Ca²⁺ Ionophores

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Abstract □ When spread from organic solvents onto electrolyte solutions, the Ca²⁺ ionophores A23187 (I) and X537A (II) formed films with relatively high surface pressures and surface potentials. Ionophore I had collapse pressures between 16 and 19 dynes/cm and nearly equal surface activity on distilled water and on 1000 mEq of either sodium chloride or calcium chloride. Film pressure did not reveal appreciable ion selectivity. However, the surface potential of I on calcium chloride solution was higher than that on sodium chloride, and the potential difference, $\Delta(\Delta V)$, of 40 mv was independent of the electrolyte concentration. In contrast, the ion selectivity of II was dependent on the electrolyte concentrations since the $\Delta(\Delta V)$ value between calcium chloride and sodium chloride was maximal (130 mv) on 1000 mEq and negligible on 500- and 2000-mEq salt solutions. The isotherms of phospholipid–ionophore films were markedly different from those of the individual components, although they revealed ionophore characteristics at low film pressures and phospholipid behavior at high film pressures. The magnitude of the surface potential indicated that dipalmitoyl phosphatidylcholine enhanced, whereas mitochondrial lipid and cardiolipin reduced, the preference of the two ionophores for Ca²⁺ over Na⁺. Since the ion selectivity was manifested most at both high electrolyte and high lecithin concentrations, the ionophore probably prefers the low dielectric constant of neutral lipid membranes to complex with the selected cation.

Keyphrases □ Membrane systems—surface properties, interaction of electrolytes and lipids with Ca²⁺ ionophores □ Surface pressure—films of Ca²⁺ ionophores on electrolyte solutions, effect of membrane lipids □ Surface potential—films of Ca²⁺ ionophores on electrolyte solutions, effect of membrane lipids □ Ionophores, Ca²⁺—surface properties of films on electrolyte solutions, effect of membrane lipids

The ubiquitous occurrence and importance of Ca²⁺ in biological processes (enzymatic as well as mechanical) prompt interest in the modes of its availability, translocation, and action. The transport of ions in and out of cells may be aided by certain organic molecules, referred to as ionophores (1). These molecules are expected to influence metabolic (2) and physical (3) properties of cells in culture as well as *in vivo*.

Questions have been raised as to whether and how Ca²⁺ may stimulate or inhibit specific membrane and intracellular enzymes (4) and the contractile activity of systems within both the plasma membrane and the cytoplasm. Although extensive work has been published on the physicochemical properties of numerous ionophores and their interactions with natural and artificial membranes (1), little is known about the surface behavior of the Ca²⁺ ionophores A23187 (mol. wt. 523, I) and X537A (mol. wt. 590, II); this information is important since at least some of the action of the ionophore develops at membranes, whose structure and function are controlled by surface phenomena.

As in the case of valinomycin (5, 6), surface activity and surface potential should reflect certain structural char-
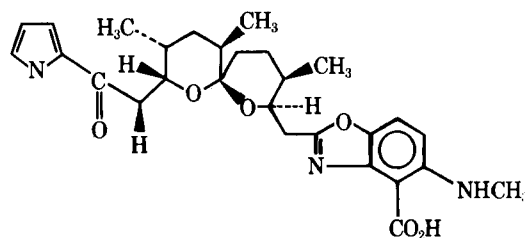
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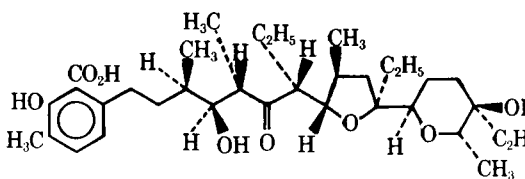
II

acteristics of the ionophore molecule and could be related to two phenomena: (a) the ability of the ionophore to complex with the various ions, and (b) the modifications brought about in the architecture of the membrane when the ionophore picks up or delivers the ion or when the ionophore finally lodges in or crosses the membrane.

This paper describes the surface pressure and surface potential characteristics of films of ionophores I and II on various concentrations of sodium chloride and calcium chloride solutions. To evaluate the influence of membrane lipids on these parameters, films of mixtures of the ionophore with dipalmitoyl phosphatidylcholine, total mitochondrial lipids, and cardiolipin also were studied.

EXPERIMENTAL

The sodium salts of ionophores I and II were used[1]. Routine TLC (2) showed that the dipalmitoyl phosphatidylcholine and beef heart cardiolipin[2] were homogeneous, and they were not purified further. Rat liver mitochondria[3] were prepared according to published methods (7); the total lipid was extracted by shaking the aqueous mitochondrial suspension once with four volumes of chloroform–methanol (2:1) and then with four volumes of chloroform.

TLC was performed with precoated silica gel G plates[4], chloroform–methanol–water–concentrated ammonium hydroxide (70:30:2:4) as the solvent, and the appropriate lipid standards. The lipid pattern was typical of mitochondria, with phosphatidylcholine > phosphatidylethanolamine > cardiolipin > sphingomyelin. For all practical purposes, this lipid composition of mitochondria is independent of either the organ or the animal species (8).

The lipids for the film preparation were dissolved to give 1 mg/ml in chloroform–methanol (85:15), and the solutions were stored at −20° for no longer than 2 weeks. During the experiments, the lipid solutions were kept in ice. The organic solvents were of the highest spectroscopic grade. Water was distilled twice (9). The electrolyte solutions, prepared with freshly distilled water and reagent grade salts, were foamed to remove surface-active contaminants (10) and were stored for no longer than 3 days at room temperature.

The techniques for the determinations of surface pressure and surface potential were described previously (10), except that a circular constant area trough was used (11) instead of a rectangular trough. Measured volumes of the lipid or ionophore solution in organic solvent were applied onto a 20-cm² area, and the values of surface pressure and surface potentials were related to the quantity of the material applied per square centimeter or to the apparent molecular area of the ionophore and other lipid.

RESULTS AND DISCUSSION

The surface pressure and surface potential values are averages from three experiments, with deviations of ±0.2 dyne/cm and ±5 mv, respectively. The π and ΔV effects of the ionophores revealed characteristics that should be related to their molecular structures.

Theoretical Considerations—Since the two monocarboxylic acid ionophores, I and II, are complex molecules, the effects of the electrolyte on surface pressure and surface potential may not be predicted at once. Such a prediction, however, will contain information or suggestions about several possible phenomena: (a) interaction of the electrolyte cation with the ionized carboxylic group of the ionophore; (b) discrimination between Na^+ and Ca^{2+} in terms of surface pressure and/or surface potential; (c) conformational changes, which are known to occur during such interactions in bulk (1); (d) rigidity or flexibility of the ionophore molecule; (e) electrolyte penetration into the interface (12); and (f) changes in the interfacial structures of the water of hydration (13) and in the distribution of dipoles (13, 14).

Each of these effects is expected to contribute to both surface pressure and surface potential but, unfortunately, cannot be evaluated individually in the present state of the art. Nevertheless, determinations of surface
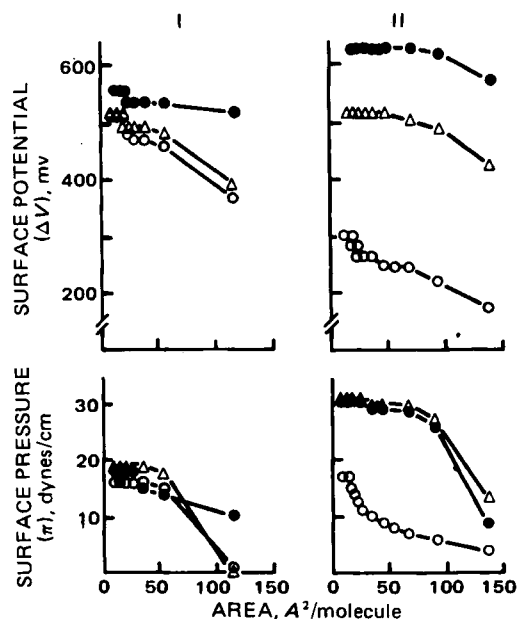
[1] These salts were supplied by Dr. Robert J. Hosley of Eli Lilly, Indianapolis, Ind., and Dr. W. Scott of Hoffmann–La Roche, Nutley, N.J., respectively.
[2] Applied Science Laboratories, State College, Pa.
[3] Gift of Dr. R. Rezek, Departments of Neurology and Biochemistry, Albert Einstein College of Medicine, New York, NY 10461.
[4] Analtech, Newark, N.J.

Figure 1—Surface pressure–area curves and surface potential–area curves of ionophores I and II spread on distilled water (O), 1 M NaCl (△), and 0.5 M CaCl₂ (●) at 25°.

pressure and surface potential of the Ca^{2+} ionophores can be used to establish empirical correlations between structure and function, correlations that are fundamental to future biological studies.

Effects of Electrolyte on Ionophore Surface Pressure–Area (π–A) and Surface Potential–Area (ΔV–A) Curves—Figures 1 and 2 describe experiments in which films of ionophores I and II were spread on distilled water and on aqueous solutions containing three concentrations of either sodium chloride or calcium chloride (150, 1000, and 2000 mEq). The film pressure, in dynes per centimeter, and the surface potential, in millivolts, are related to the apparent molecular area of each ionophore on water and on aqueous solutions of 1000 mEq of sodium chloride or calcium chloride in Fig. 1 and to the electrolyte concentration at the molecular area of 50 Å² for each ionophore in Fig. 2. The two ionophores were surface active with II more than I; according to their surface potentials, they discriminated differently between Na^+ and Ca^{2+}.

π–A Curves—Films of II were more expanded but had higher collapse pressures than those of I on the aqueous electrolytes; this difference could be ascribed to the greater flexibility of II, which requires constraints to build up pressure as compared to the more rigid I molecule, whose iso-
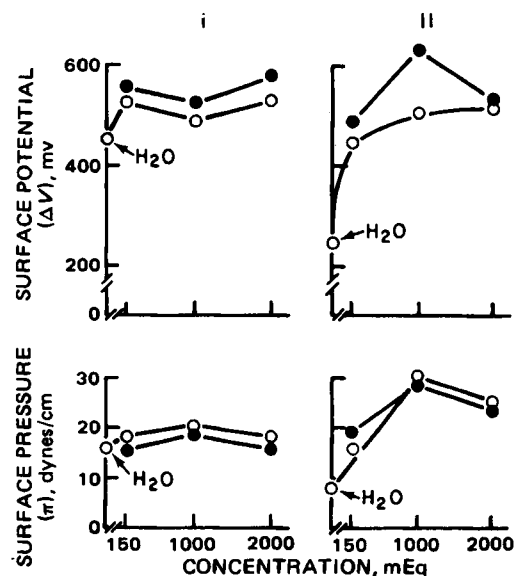

Figure 2—Influence of electrolyte concentration on the surface pressure and surface potential of ionophores I and II at the molecular area of 50 Å² at 25°. Key: O, sodium chloride; and ●, calcium chloride.
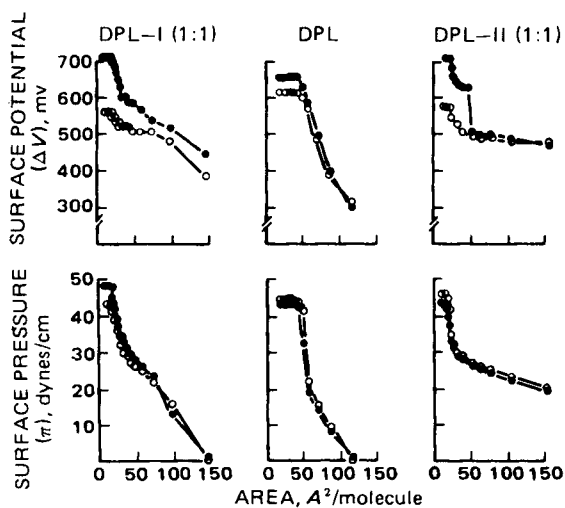
Figure 3—*Influence of ionophores I and II on the surface pressure-area and surface potential-area curves of dipalmitoyl lecithin in mixed films spread on 1.0 M NaCl (O) and 0.5 M CaCl₂ (●) at 25°. The apparent molecular areas are averages of the areas of lecithin and the ionophore.*

therm on distilled water did not differ much from that on the subphase containing various electrolyte concentrations. The slow rise of pressure with compression indicates that II must encounter difficulty in making a film on distilled water (Fig. 1); II was much more surface active than I on electrolyte solutions. The collapse pressures on 1000 mEq of sodium chloride or calcium chloride were 30 dynes/cm for II and 18 dynes/cm for I.

The virtual identity of the isotherms of II on solutions of 1000 mEq of sodium chloride or calcium chloride means that, at this electrolyte concentration, the ionophore cannot distinguish between Na⁺ and Ca²⁺ on the basis of surface tension or surface pressure. Ionophore II did distinguish between the cations at lower concentration, where its isotherm on 150 mEq of calcium chloride (Fig. 2) was markedly more expanded (higher) than that on sodium chloride. At present, no explanation is available for this effect, which is the opposite of the film contraction expected from the interaction of Ca²⁺ with negatively charged lipids (15). The complexity of the ionophore molecule may be responsible for numerous deviations from the surface behavior of a straight-chain lipid.

The two ionophores were similar in that, on both electrolytes at all concentrations studied, they reached the different collapse or saturation pressures at an apparent molecular area of 50 Å², below which the π and ΔV values were quite stable. For reasons given earlier (6), interpretation of the apparent molecular area must be postponed until an exact determination is made of the effective number of ionophore molecules per square centimeter and their orientation in the film, parameters that are not accessible at present.

ΔV-A Curves—Unlike surface pressure, the surface potential revealed marked ion selectivities for both ionophores.

On distilled water, the surface potential of I was 200 mv higher than that of II. At all areas, I distinguished sodium chloride from water by 20 mv in favor of sodium chloride and sodium chloride from calcium chloride by 40 mv in favor of calcium chloride. Unlike I, II showed a marked dependence of π and ΔV on the electrolyte concentration (Fig. 2) and distinguished markedly well between Na⁺ and Ca²⁺ at one electrolyte concentration (1000 mEq), although by a much greater difference (130 mv) as compared to 40 mv for I.

The π-A curves of II on 1000 mEq of sodium chloride and calcium chloride were nearly superimposable. The ΔV-A curve of II on sodium chloride was 300 mv higher than that on water; on calcium chloride, it was 130 mv higher than that on sodium chloride (Fig. 1). In contrast, the selectivity of I for Ca²⁺ was about the same (40 mv) at all electrolyte concentrations (Fig. 2).

These differences in surface behavior between the two ionophores are consistent with a rigid structure for I and a flexible structure for II. At present, it is not possible to relate these observations to the conformational changes that the ionophores undergo in bulk (1), primarily because of a lack of spectroscopic methods capable of viewing the interface directly *in situ.*

Lipid Influence on Ion Selectivity and Surface Behavior of the Ionophore—The foregoing data show that ionophores I and II are surface active and distinguish between Na⁺ and Ca²⁺ in the absence of membrane

lipids. Since the transport of Ca²⁺ by the ionophore across the membrane could imply some interaction of the ionophore with the membrane structures that negotiate Ca²⁺ with the ionophore, information about the membrane behavior of the ionophore may be attained by a study of the interaction of the electrolyte with films of ionophore and phospholipid mixtures.

Three mixtures were studied: dipalmitoyl phosphatidylcholine–ionophore, mitochondrial lipid–ionophore, and cardiolipin–ionophore. The lipid mixtures in chloroform-methanol (85:15) were spread on the aqueous subphase containing 1000 mEq of sodium chloride or calcium chloride. This electrolyte concentration was chosen for convenience, since the surface potential effects were largest; they were small at either lower or higher salt concentrations.

Dipalmitoyl Phosphatidylcholine—Figure 3 shows the π-A and ΔV-A curves of dipalmitoyl lecithin (DPL) and equal weight mixtures of it with I and II on 1000 mEq of sodium chloride or calcium chloride.

With this salt concentration, the dipalmitoyl lecithin films were slightly more expanded, and the ΔV values were appreciably higher than on more dilute solutions [*e.g.*, 100 mEq (12)]; dipalmitoyl lecithin did not significantly discriminate between Na⁺ and Ca²⁺. However, both ionophores influenced appreciably the π-A and ΔV-A curves of the phospholipid in the dipalmitoyl lecithin–ionophore film, in which dipalmitoyl lecithin seemed to enhance markedly the ion selectivity of the ionophore (Ca²⁺ over Na⁺), especially in terms of surface potential.

The π-A and ΔV-A curves of the mixtures were quite different from those of individual components. The pressure–area curves of the mixed films had an ionophore character (expanded) at low pressure. At the average molecular area of 150 Å², the pressure was near zero for the dipalmitoyl lecithin–I system and near 20 dynes/cm for dipalmitoyl lecithin–II. This observation strengthens the suggestion that I is rigid whereas II is flexible and thus is more compressible than I.

Although the disappearance of the ionophore character from the mixed ionophore–lecithin film in terms of film pressure could suggest that, at high pressure, the ionophore may be squeezed out of the mixed film, which then resumes the lecithin character, this mechanism may not be valid because the surface potential increase at high pressure is that of a unique lecithin–ionophore mixture, far above the ΔV of lecithin alone. The mode of the ionophore–lecithin interaction thus remains to be investigated.

Whereas the dipalmitoyl lecithin–ionophore mixtures did not distinguish between Na⁺ and Ca²⁺ by the surface pressure, they did distinguish between the cations markedly well by the surface potential, especially at high pressure where the Δ(ΔV) difference between calcium chloride and sodium chloride reached ~150 mv for both ionophores as opposed to 40 mv for I and 130 mv for II in the absence of dipalmitoyl lecithin. The fact that the selectivity between Na⁺ and Ca²⁺ was manifested at high film pressure and high electrolyte concentration is reminiscent of the Na⁺-K⁺ discrimination of valinomycin films, which required 700–1000 mEq of electrolyte and film pressures above 20 dynes/cm to show a dramatic effect of K⁺ on the surface potential (5, 6, 16). Indeed, the presence of lipid brings about a low dielectric constant (favorable to ionic inter-
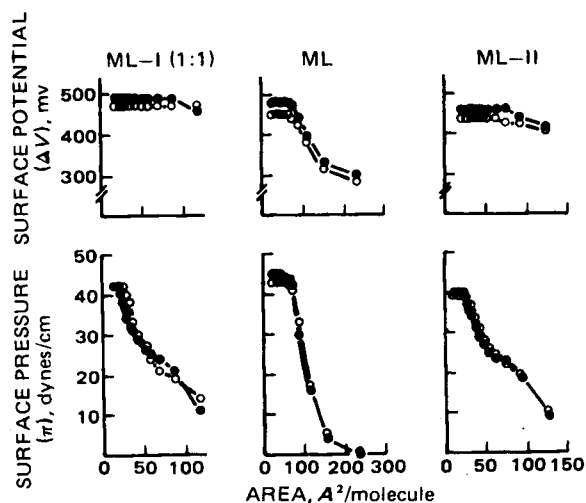

Figure 4—*Influence of ionophores I and II on the surface pressure-area and surface potential-area curves of mitochondrial lipid in mixed films spread on 1.0 M NaCl (O) and 0.5 M CaCl₂ (●) at 25°. The apparent molecular areas are calculated averages of the areas of ionophore and mitochondrial lipid.*
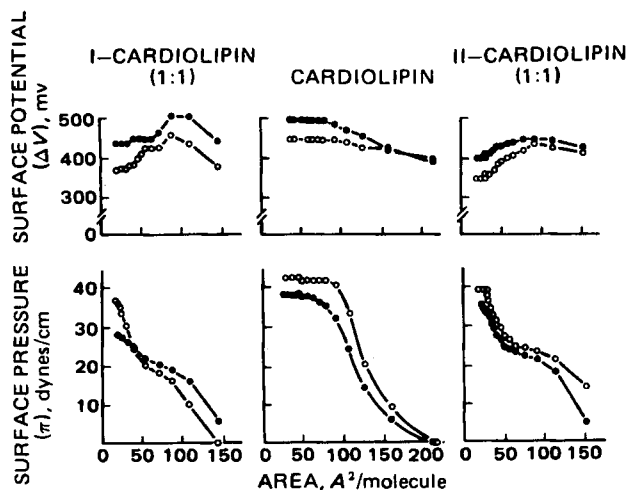
Figure 5—*Influence of ionophores I and II on the surface pressure–area and surface potential–area curves of cardiolipin on 1.0 M NaCl* (O) *and 0.5 M CaCl₂* (●) *at 25°. The apparent molecular areas are averages of the areas of cardiolipin and the ionophore.*

actions) and high surface potentials (6, 13). The fact that this effect was not seen at higher electrolyte concentrations is interesting, has no explanation as yet, and clearly reflects a specificity that calls for optimal conditions of dose response in the interaction of Ca^{2+} with the ionophore.

Mitochondrial Lipid—The film was made by spreading the total lipid extract of rat liver mitochondria as a model of a membrane containing negatively charged groups, namely, the two phosphate groups of cardiolipin (diphosphatidylglycerol). Unlike dipalmitoyl lecithin, the mitochondrial lipid (ML) conferred little ion-discriminating ability to either ionophore (Fig. 4).

Although a high collapse pressure of the mitochondrial lipid was revealed in the isotherm of mixed films of the ionophore with this lipid, the surface potential of the mixture reflected an ionophore character in the sense that its ΔV–A curve was lifted to a constant saturation value that extended over the entire area range. However, the ΔV effect of the electrolyte in the two mixtures was not different from that of the mitochondrial lipid alone.

Cardiolipin—Since the major ionic component of mitochondrial lipid is cardiolipin, the behavior of the cardiolipin–ionophore mixed film was evaluated to see if the behavior of the mitochondrial lipid could be ascribed to cardiolipin. The results (Fig. 5) confirm that possibility.

More evident than with the total mitochondrial lipid was the contraction in the molecular area of cardiolipin in the presence of Ca^{2+} as compared to Na^+ (Fig. 5), a discrimination that is known for acidic phospholipids (15, 17) but was not so apparent and was modified partially with the mixed ionophore–cardiolipin films (Fig. 5). As with the mitochondrial lipid and with cardiolipin alone, the surface potential of the cardiolipin–ionophore mixed film showed a modest separation in ΔV between Na^+ and Ca^{2+}, with the value for Ca^{2+} higher as expected.

An unusual feature of the ionophore–cardiolipin mixed film was the maximum in surface potential, between pressures of 20 and 22 dynes/cm, above which, although the pressure continued to rise until saturation, the surface potential decreased appreciably on sodium chloride and on calcium chloride solutions. This unusual behavior is reminiscent of the ΔV–A curve of gangliosides (18). Since the film pressure continued to rise, one can argue that the film was not collapsing and that the fall in surface potential could be accounted for by reorganization of molecules and/or dipoles so as to produce a film with a higher dielectric constant, an excess of $\mp$-oriented ionic dipoles (including water), or both (6, 13, 19–21).

CONCLUSIONS

In the surface chemistry literature, there has been a misleading tendency to speculate or even make conclusions about the orientation of molecular groups and dipoles from surface potential data. That approach has been challenged; but for a variety of reasons that are given elsewhere (6, 12, 13, 19–22), quantitative models must be postponed until the molecular correlates of surface potentials are understood. Such models may be derived only after information is obtained about the detailed molecular topography of the films at the air–water interface; such experiments involving radiolabels and direct spectroscopy of the interface cannot be

done in the present state of the art (12). Meanwhile, empirical comparisons and correlations are valuable.

The comparisons between Na^+ and Ca^{2+} were based on equivalent concentrations instead of uncomparable ionic strength (0.225 for calcium chloride and 0.150 for sodium chloride). This choice was made to avoid blurring of the data by electrostatic effects due to ion binding competition in such different equivalent concentrations. For similar reasons, in working with ionophore–phospholipid mixtures, the empirical π–A relationships were preferred to graphs of mean molecular area *versus* mole fraction.

Unlike prostaglandins (23) [carboxylic acids that produced films with very small surface pressures (0–9 dynes/cm) and surface potentials (−10 to +250 mv)], I and II were quite surface active and had fairly large surface potentials. Both the surface pressure and especially the surface potential values were influenced by certain electrolyte concentrations. As observed with acidic phospholipids (15, 17) and fatty acids (24) at pH 5.7, the ΔV values on calcium chloride were higher than those on sodium chloride, indicating preferential involvement or binding of Ca^{2+}. However, at certain low and high electrolyte concentrations, the two ionophores, especially II, could not distinguish between Na^+ and Ca^{2+}. Discrimination between Na^+ and Ca^{2+} in terms of surface potential was enhanced markedly when the ionophore was mixed with dipalmitoyl lecithin; it was diminished when the ionophore was mixed with acidic lipids, either mitochondrial lipids or cardiolipin, despite the fact that such lipids interacted with Ca^{2+} with a marked surface potential effect.

The monolayer experiments suggest that, in the presence of Ca^{2+}, both ionophores prefer the hydrophobic medium of membranes and are admitted more easily into membranes containing high doses of neutral or hydrophobic phospholipid. These concepts of surface science could be exploited in the interpretation of the behavior of ionophores in experiments that are aimed at differentiating between cell types on the basis of the ionophore's selectivity for Ca^{2+} and for the membrane lipid.

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