MODIFICATION OF ION TRANSPORT IN LIPID BILAYER MEMBRANES IN THE PRESENCE OF 2,4-DICHLOROPHENOXYACETIC ACID

II. Suppression of Tetraphenylborate Conductance and Changes of Interfacial Potentials

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ABSTRACT It has been shown that the blocking of negatively charged tetraphenylborate ion transport in phosphatidylcholine (PC)-cholesterol membranes by the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is dominated by the suppression of $TPhB^-$ diffusion across the membrane interior, rather than by the decrease of adsorption of $TPhB^-$ ions at the membrane surface. The blocking effect can be associated with the decrease of electric potential inside the membrane with respect to that of the aqueous medium, this decrease being proportional to the concentration of 2,4-D in the aqueous solution. It has been estimated that 25–30% of the total 2,4-D-induced change of the potential difference is between the plane of adsorption of $TPhB^-$ and the aqueous solution, and the remaining fraction is between the membrane interior and the adsorption plane. The results of this study support the dipolar hypothesis of 2,4-D action in lipid membranes. These conclusions are further supported by measurements of changes of electric potential difference across air/water and air/lipid monolayer/water interfaces. It has been found that the electric potential of the nonpolar side of the interface decreases in the presence of neutral molecules of 2,4-D and that this effect becomes more prominent in the presence of electrolyte. We have confirmed that PC-cholesterol monolayer cannot be considered as a model for half of the bilayer membrane because of the disagreement between the changes of the interfacial potential difference of PC-cholesterol monolayers and those determined from studies of transport of positive and negative ions across bilayer membranes. In contrast, we have found close agreement between the 2,4-D-induced changes of electric potential of the lipid hydrocarbon region in glycerolmonooleate (GMO) membranes and GMO monolayers. We suggest that the action of 2,4-D in lipid membranes is not associated with the changes of orientation of dipoles of lipids constituting the membrane, but rather with a layer of 2,4-D molecules adsorbed at the nonpolar/polar membrane boundary.

INTRODUCTION

We have previously reported that pesticide 2,4-dichlorophenoxyacetic acid (2,4-D) modifies ionic selectivity of lipid bilayer membranes. It was found that it facilitates transport of lipid soluble cations, such as tetraphenylarsonium ($TPhAs^+$) and nonactin-K^+ complex, but inhibits transport of negatively charged tetraphenylborate ($TPhB^-$) ions (1).

From the studies of the voltage dependence of nonactin-K^+ conductance it was possible to

conclude that 2,4-D increases the ratio of the translocation to the dissociation rate constant of nonactin-K^+ complex. It was observed that the action of 2,4-D on ion transport in membranes is similar to that of phloretin (2, 3). It is very likely caused by a layer of 2,4-D molecules adsorbed within the membrane interfacial region. The postulated charge distribution and orientation of adsorbed 2,4-D molecules are such that the electric potential of the membrane interior decreases (1).

In this paper we present the results of further studies on the interference of 2,4-D with transport of negatively charged $TPhB^-$ ions. The usefulness of $TPhB^-$ as a membrane probe originates from the high coefficient of distribution of $TPhB^-$ ions between the membrane surface and water, and from the experimentally favorable redistribution time of $TPhB^-$ ions in membranes (10^{-2}–10^{-3} s). Current relaxation experiments with $TPhB^-$ permit one to measure separately both the changes of the density of $TPhB^-$ adsorbed at the membrane surface in the presence of 2,4-D, as well as the changes of the translocation rate constant (4–9). From studies of membrane properties with the $TPhB^-$ probe one can make some conclusions about the location of the adsorbed layer of 2,4-D within the membrane, and specifically, about its position with respect to the adsorption plane of $TPhB^-$. We also report on the measurements of electric potential difference across the air/water and air/lipid monolayer/water interfaces when 2,4-D is present in the aqueous subphase, and compare the results with data on electric potential distribution changes extracted from the results of studies of ion transport in bilayer membranes.

Transport of Tetraphenylborate Ions across Lipid Membranes

The exponentially decaying transient conduction current observed immediately after the application of a constant potential difference across the membrane is due to the flow of $TPhB^-$ ions across the membrane interior from ion potential energy well at one interface to that at the other (4). The diffusion of $TPhB^-$ ions toward and away from the membrane is insignificant due to the fact that the $TPhB^-$ permeability of membranes is much greater than that of the aqueous solution. Thus on the time scale of current decay, the amount of $TPhB^-$ ions at the membrane surface is conserved.

Andersen and Fuchs (5) have shown that a quasistationary solution of electrodiffusion of $TPhB^-$ ions across the membrane represented by an image-potential barrier satisfactorily describes the transient membrane current. The initial membrane conductance $G(V)|_{t=0}$, (computed from the initial current that is obtained by extrapolating the conduction current to the zero time), is equal to

$$G^{ON}(V)|_{t=0} = \frac{2kT}{e\beta V}\frac{\sinh(e\beta V/2kT)}{\exp[\omega(eV/kT)^2]}G^{ON}(0)|_{t=0}, \tag{1}$$

where $G^{ON}(0)|_{t=0}$ is the zero voltage initial conductance given by

$$G^{ON}(0)|_{t=0} = \frac{e^2\beta D_m N_{eq}/\gamma}{kT\int_{\eta}^{d-\eta}\exp[W(x)/kT]\,dx}. \tag{2}$$

The superscript ON refers to the transient process observed after the steplike increase of membrane bias from zero to V. The membrane is assumed to extend from $x = 0$ to $x = d$; η is the distance between the $TPhB^-$ adsorption plane and the membrane surface on either side of

membrane. $W(x)$ is the difference of ion potential energy at a distance x with respect to that at $x = \eta$, N_{eq} is the equilibrium surface density of adsorbed $TPhB^-$, γ is the effective width of the adsorbed $TPhB^-$ layer, ω is a coefficient taking into account the deformation of the ion potential energy barrier due to the applied bias, D_m is the ion diffusion coefficient in the membrane interior, and βV is the effective potential difference driving the ion transport.

After the application of bias voltage, V, the net ion flow in the membrane decreases exponentially with time because of the depletion of ions on the negatively biased side of membrane and their accumulation on the positive side. The characteristic time of this redistribution process is

$$\tau^{ON}(V) = \frac{\exp\left[\omega(eV/kT)^2\right]}{\cosh\left(e\beta V/2kT\right)}\tau^{ON}(0), \tag{3}$$

where

$$\tau^{ON}(0) = (\gamma/2D_m) \cdot \int_{\eta}^{d-\eta} \exp\left[W(x)/kT\right] dx. \tag{4}$$

In computations involving Eqs. 1 and 3 we set $\omega = 0.005$ corresponding to membrane thickness of approximately 3.2 nm (references 1 and 5).

The total amount of charge transferred across the membrane during the current relaxation process is equal to

$$\begin{aligned} Q^{ON}(V) &= Q_{ads} \cdot \tanh\left(e\beta V/2kT\right) \\ &= \int_0^{\infty} J^{ON}(V)|_{t=0} \cdot \exp\left[-t/\tau^{ON}(V)\right] dt \\ &= J^{ON}(V)|_{t=0} \cdot \tau^{ON}(V), \end{aligned} \tag{5}$$

where $J(V)|_{t=0}$ is the initial membrane conduction current density, and $Q_{ads} = eN_{eq}$ is the equilibrium density of membrane surface charge due to adsorbed $TPhB^-$ ions.

Transient conduction currents can also be observed when the external bias voltage is switched off. The OFF-currents are caused by the return flow of $TPhB^-$ ions that have been displaced from its equilibrium distribution under the previously applied bias. The initial membrane conductance associated with the membrane conduction when the bias voltage is turned off can be defined in a similar way,

$$G^{OFF}(V)|_{t=0} = (2kT/e\beta V) \cdot \tanh\left(e\beta V/2kT\right) \cdot G^{OFF}(0)|_{t=0}. \tag{6}$$

It can be shown that $G^{OFF}(0)|_{t=0}$, the zero voltage initial membrane conductance of the switch-OFF process is equal to the initial zero voltage conductance observed under the switch-ON conditions.

The time constant of the switch-OFF relaxation current is equal to

$$\tau^{OFF} = (\gamma/2D_m) \cdot \int_{\eta}^{d-\eta} \exp\left[W(x)/kT\right] dx. \tag{7}$$

In contrast to the time constant of the switch-ON process, the time constant τ^{OFF} is voltage independent, and is equal to the zero voltage time constant of the switch-ON process, i.e., $\tau^{OFF} = \tau^{ON}(0)$.

The total amount of electric charge transferred across the membrane during the OFF-pulse charge redistribution is

$$Q^{OFF}(V) = \int_0^\infty J^{OFF}(V)|_{t=0} \cdot \exp(-t/\tau^{OFF})\, dt = J^{OFF}(V)|_{t=0} \cdot \tau^{OFF}. \quad (8)$$

If there is no exchange of ions between the membrane and the aqueous solution, then $Q^{OFF}(V) = Q^{ON}(V)$.

The adopted model (5), which has been outlined above, has been recently extended by the development of the "three capacitor model" (9), and by the introduction of dielectric saturation of water at the membrane surface (10). These extensions take into account the existence of large boundary potentials at $TPhB^-$ concentrations exceeding 10^{-6} M. In the present study of the effect of 2,4-D on $TPhB^-$ transport, we do not take explicitly into account any of these refinements. We prefer the transport model (5) outlined above for its simplicity because the $TPhB^-$ concentration employed in our experiments was only 1×10^{-7} M.

Electric Potential Difference across Air/Water and Air/Lipid Monolayer/Water Interfaces

Results of earlier studies of electrical conductivity of lipid membranes in the presence of 2,4-D have been found compatible with the hypothesis that 2,4-D decreases electric potential of the interior of membrane (1). Thus it is of interest to measure directly the changes of electrical potential difference between the hydrocarbon region of lipid monolayer spread at the water surface and the aqueous subphase as a function of various conditions (2,4-D concentration, pH, ionic strength) that have been found to affect ion flow in membranes.

Assuming that a layer of electrically neutral 2,4-D molecules at the interface can be represented by two oppositely charged sheets embedded in a medium of dielectric constant ϵ, the electric potential difference across such a double layer is equal to

$$\Delta V = N p_\perp / \epsilon \epsilon_0, \quad (9)$$

where $p_\perp$ is the normal component of the dipole moment, and N the surface density of 2,4-D molecules. For N corresponding to 1 molecule/50 Å^2 (which is approximately the area occupied by one lipid molecule), $p_\perp = 1$ Debye and $\epsilon = 10$, the expected potential difference is approximately 75 mV. ΔV of this magnitude can be easily measured by conventional methods (11, 12).

Surface potential measurements have not only a supportive role in confirming the conclusions drawn from the membrane conductivity measurements, but are also helpful in elucidating the problem as to whether a lipid monolayer can be regarded as a half of the bilayer membrane. Some of the earlier studies (11, 12) gave affirmative answers to this question, but Andersen et al. (2) have provided evidence that the similarity between lipid monolayers and bilayer membranes may be superficial.

PROCEDURES AND MATERIALS

The black lipid membranes were formed by the brush technique on a 2-mm diameter hole in a TFE (tetrafluoroethylene resin) cell. (Teflon, DuPont Co., Wilmington, Del.). The membrane-forming solution was lecithin and cholesterol (PC-chol) in decane, the mole fraction of cholesterol was 0.76 and the total lipid content (i.e., lecithin + cholesterol) was 11.5 mg/ml.

The aqueous electrolyte solution contained NaCl, buffer (phosphate, citrate, borate; ratios 0.002/0.002/0.0005 M), and 2,4-D. Sodium tetraphenylborate ($TPhB^-$) was dissolved in ethanol and then the ethanolic stock solution was added to the aqueous solution, which was prepared fresh every day. The volume of ethanol in the final solution was about 0.2%.

The procedure used in the preparation of the TFE cell for making the membranes was described in the previous paper (1). The current-voltage data were taken about 30 min after the membrane became thin. Each data point given in the figures represents an averaged value obtained on four membranes and the error bars denote 1 SD.

The transient current measurements were performed by using a two-electrode "voltage clamp" arrangement. The electrodes used were sintered Ag/AgCl of Annex Instruments, type 140 H (Santa Ana, Calif.). A known voltage pulse was applied across the membrane by a pulse generator (Hewlett-Packard, Loveland, Col.). The membrane current signal was converted first into a voltage signal. The current-voltage converter, based on operational amplifier LH00602 (National Semiconductor Corp., Santa Clara, Calif.) was similar to that described by Sargent (13). The amplified transient current was first stored in the Biomation transient recorder (model 802; Biomation Corp., Cupertino, Calif.), and then transferred to a x-y plotter (model 2000; Houston Instruments, Bausch & Lomb, Inc., Austin, Tex.). From the plot, quantities of interest, such as the initial membrane conductance $G(V)|_{t=0}$ and the membrane current relaxation time constant $\tau(V)$ were obtained. The membrane current relaxation time constant $\tau(V)$ and $I|_{t=0}$ were obtained from the fit of $I(t) = I|_{t=0} \cdot \exp[-t/\tau(V)]$ to the experimental data. The initial membrane conductance was calculated from the initial current density $J|_{t=0} = I|_{t=0}/A$, where $I|_{t=0}$ is the initial membrane conduction current and A the area of the hole, and the applied potential difference V, $G(V)|_{t=0} = J(V)|_{t=0}/V$. The zero voltage conductance $G(0)$ and zero voltage time constant $\tau(0)$ were obtained by extrapolating low voltage data (up to 80 mV in most cases) by using a polynomial of the second degree. The amount of charge per unit membrane area, $Q(V)$, transferred across the membrane during the transient conduction process, was calculated according to Eq. 5 or 8, and the area of the hole. The total charge density due to $TPhB^-$ ions adsorbed at the membrane surface, Q_{ads}, was assumed to be the average of $Q(V)$ measured at high membrane bias: 160, 180, 200, and 220 mV, because tanh at those voltages approaches unity.

The method of measurements of interfacial potential difference was similar to that described in references 11 and 12. It was measured by means of a polonium electrode (Nuclear Products Co., El Monte, Calif.) and PAR electrometer, model 135 (Princeton Applied Research Corp., Princeton, N.J.). The aqueous solution on which the monolayer was spread was contained in a 100-mm crystallizing dish, and was electricially connected with a reference calomel electrode by means of a KCl bridge. The polonium electrode was placed several millimeters above the aqueous surface. To stabilize the potential of the measuring electrode, it was kept for about half a day above the clean water surface before the start of the experiment. If the system were clean, the interfacial potential difference across the air/deionized water (Millipore Q2 system, Millipore Corp., Bedford, Mass.) surface was -440 ± 10 mV. The subphase aqueous solution contained KCl, 2,4-D, and buffer; LiCl was used for the adjustments of ionic strength. The experimental conditions are described in detail in the figure legends. The monolayer-forming solution was prepared by dissolving lipids in hexadecane; 1% of methanol was also added to the lipid solution to improve lipid solubility. The cholesterol mole fraction was the same as in the membrane-forming solution (i.e., 0.76). For PC-cholesterol monolayers, the forming solution contained 29 mg of lecithin and cholesterol in 1 ml of hexadecane, for glycerolmonooleate (GMO) monolayers, the concentration of GMO in hexadecane was 30 mg/ml.

The air/aqueous solution interfacial potential difference was recorded 15–20 min after the radioactive electrode was placed above the aqueous surface. Then about 3 μl of lipid solution in hexadecane were injected slowly along the surface of the crystallizing dish, and the new value of the potential difference recorded after it stabilized. In each case we have made about four measurements for each monolayer because of the surface potential fluctuations. We have also noticed that the surface potential of GMO monolayers fluctuated less than that of the PC-cholesterol. After the measurement, the dish was rinsed thoroughly with methanol and deionized water, and the surface potential of clean water measured again to check the cleanliness of the equipment. Each data point represents an average for four monolayers and the error bar one SD.

RESULTS AND DISCUSSION

Membrane Conductance, Relaxation Time Constant, and Net Transfer of Charge

Our objective was to determine the effect of 2,4-D on the membrane conductance due to negatively charged tetraphenylborate ions, the relaxation time constant of membrane conduction current, and the amount of charge transported across the membrane during the relaxation process. At the same time we were interested in the applicability of the simple transport model formulated by Andersen and Fuchs (5) to the membrane perturbed by the presence of 2,4-D.

Fig. 1 shows the changes of the initial membrane conductance as a function of 2,4-D concentration for both the switch-ON and switch-OFF conditions. Two interesting properties were observed: the decrease of the magnitude of the initial membrane conductance with the increasing concentration of 2,4-D, while the changes of the voltage dependence of the conductance with 2,4-D concentration were very small. When comparing our results with those given in reference 5, we find that for untreated membranes the zero voltage conductance of PC-chol and bacterial phosphatidylethanolamine (BPE) are very similar, whereas the relaxation time constant of PC-chol membranes is greater than that of BPE membranes. For example, for $c_{TPhB} = 1 \times 10^{-7}$ M, the zero voltage conductances are about 3×10^{-4} S/cm^2 for PC-chol membranes and 4×10^{-4} S/cm^2 for BPE membranes. The relaxation time constant of PC-chol membranes was found to be (5–6) $\times 10^{-3}$ s, as compared with (1–2) $\times 10^{-3}$ s for BPE membranes (5, 9).

The suppression of the conductance by 2,4-D is associated with the increase of the relaxation time constant, which is the measure of the ion redistribution time in the membrane interior (Fig. 2). For 2,4-D concentration change from 0 to 7.5×10^{-4} M, the membrane conductance of PC-chol membranes decreased by a factor of about 100, and the relaxation time constant increased by about 30-fold.

The applicability of the above model to our membranes modified by 2,4-D can be judged from curves shown in Figs. 1 and 2. They represent a fit of Eqs. 1, 3, 6, and 7 to the experimental results. The value of β was obtained from the least-square fit of Eqs. 1, 3, 6, and 7 to the experimental data. Parameter β as a function of 2,4-D concentration is shown in Fig. 3. The values of parameter β for switch-ON and switch-OFF transients have been found different, β^{ON}, β^{OFF}, and, furthermore, β^{ON} monotonically increased with 2,4-D, whereas β^{OFF} remained unchanged. At high 2,4-D concentration (5×10^{-4} M and above), the value of β cannot be accurately determined because of the poor signal-to-noise ratio of the transient currents, especially at low bias voltages. The dependence of β^{ON} on 2,4-D concentration can be understood in terms of the changes of adsorption of $TPhB^-$.

The net charge per unit membrane area, translocated across the membrane as a function of applied potential difference and 2,4-D concentration, is given in Fig. 4. For the purpose of illustration of the model, the curves in this figure represent the voltage dependence of transferred charge as given by Eq. 5. The limiting value of $Q(V)$ at high voltages is equal to the surface density due to adsorbed $TPhB^-$ ions, Q_{ads}. The results indicate that 2,4-D inhibits adsorption of $TPhB^-$ because Q_{ads} decreases with increasing 2,4-D concentration. In the absence of 2,4-D, we find that the surface charge density of PC-chol membranes in the presence of $TPhB^-$ of 1×10^{-7} M is about 1×10^{-7} C/cm^2, which is comparable to that found for BPE membranes (5, 9), and is about three times greater than that for GMO

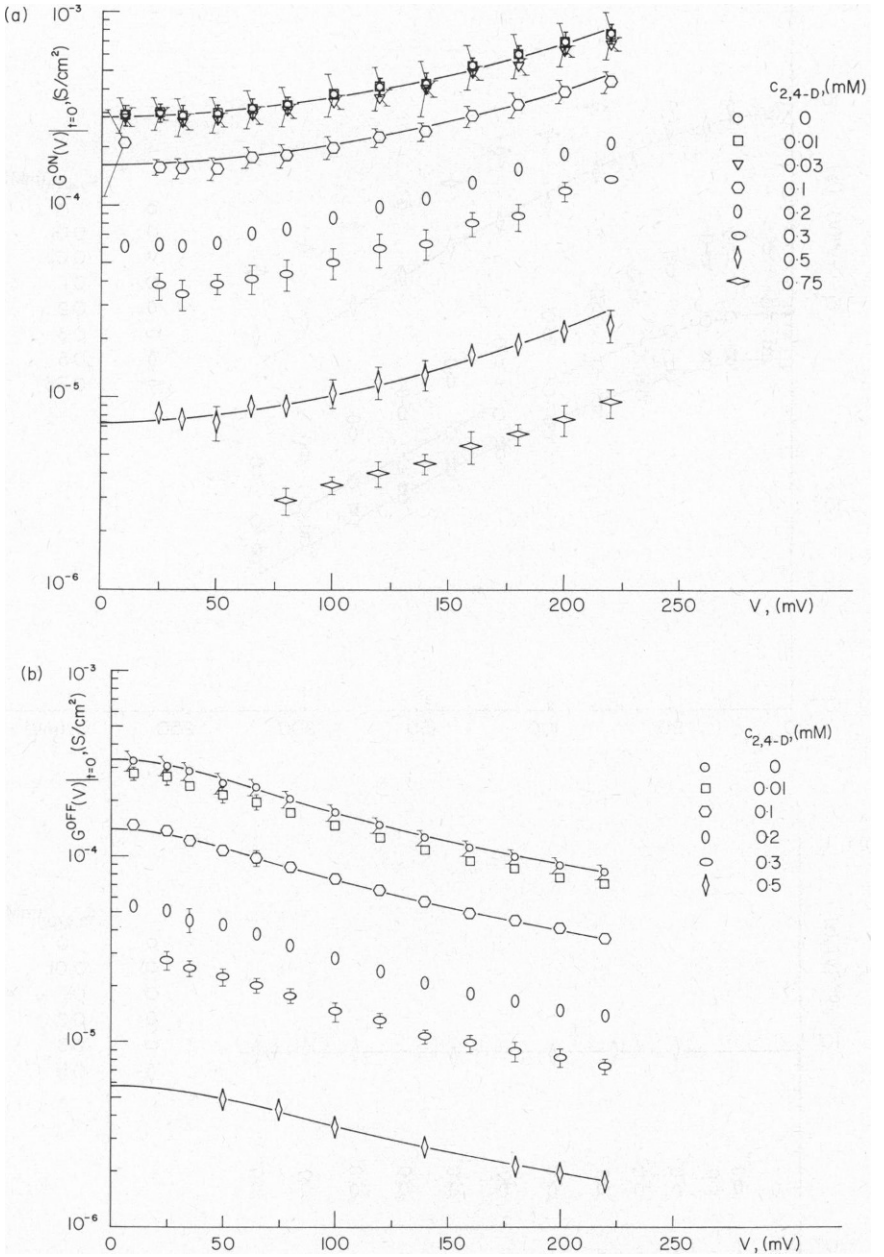
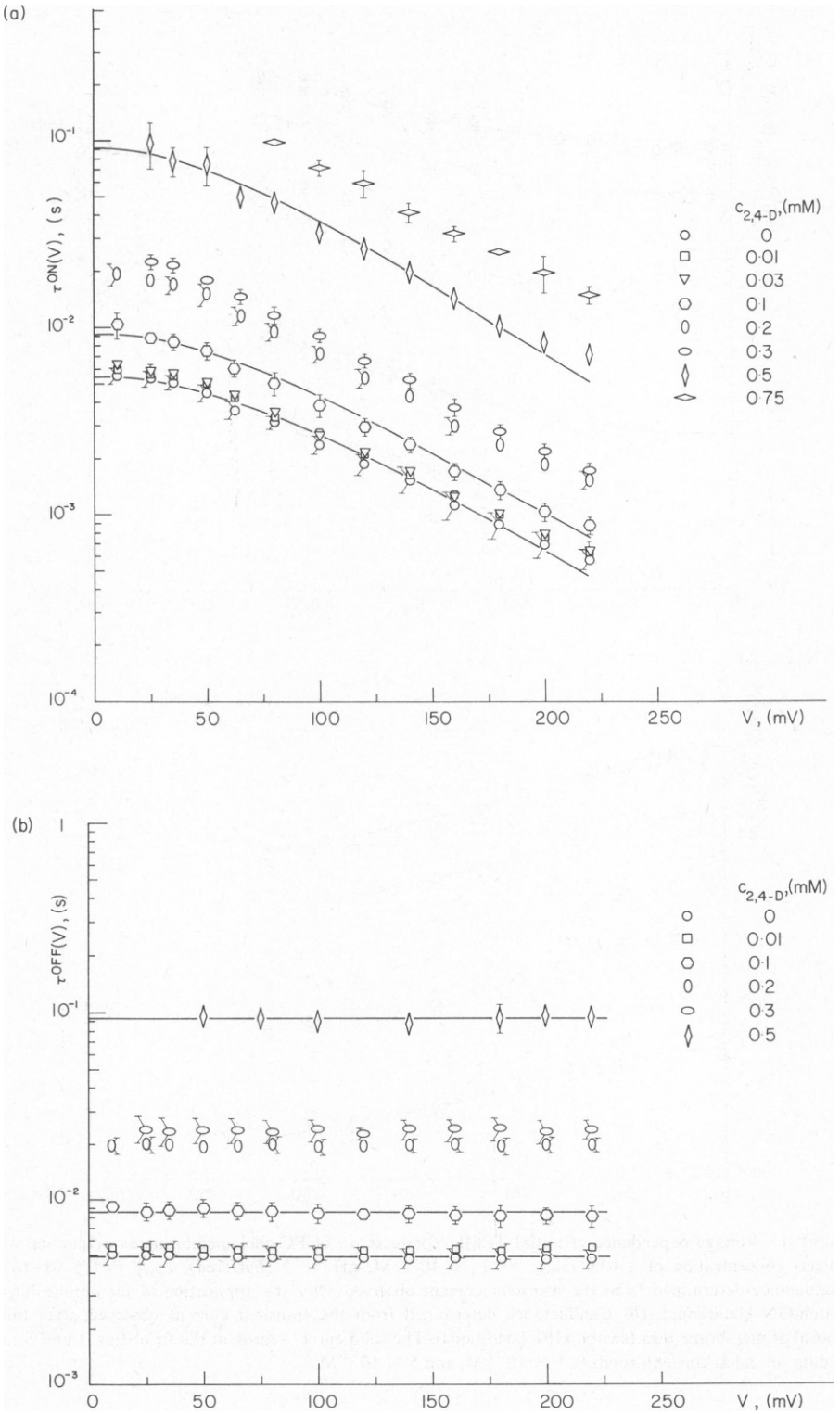


FIGURE 1 Voltage dependence of initial $TPhB^-$ conductance of PC-chol membrane as a function of aqueous concentration of 2,4-D. $c_{TPhB^-} = 1 \times 10^{-7}$ M, pH = 2 (buffered), $c_{NaCl} = 0.5$ M. (*a*) Conductance determined from the transient current observed after the application of membrane bias (switch-ON conditions). (*b*) Conductance determined from the transient current observed after the removal of membrane bias (switch-OFF conditions). The solid curves represent the fit of Eqs. 1 and 6 to the data for 2,4-D concentrations 0, 1×10^{-4} M, and 5×10^{-4} M.


(a)
$\tau^{ON}(V)$, (s)
10^{-1}
10^{-2}
10^{-3}
10^{-4}
0
50
100
150
200
250
V, (mV)
$c_{2,4\text{-}D}$, (mM)
0
0·01
0·03
0·1
0·2
0·3
0·5
0·75
(b)
1
$\tau^{OFF}(V)$, (s)
10^{-1}
10^{-2}
10^{-3}
0
50
100
150
200
250
V, (mV)
$c_{2,4\text{-}D}$, (mM)
0
0·01
0·1
0·2
0·3
0·5

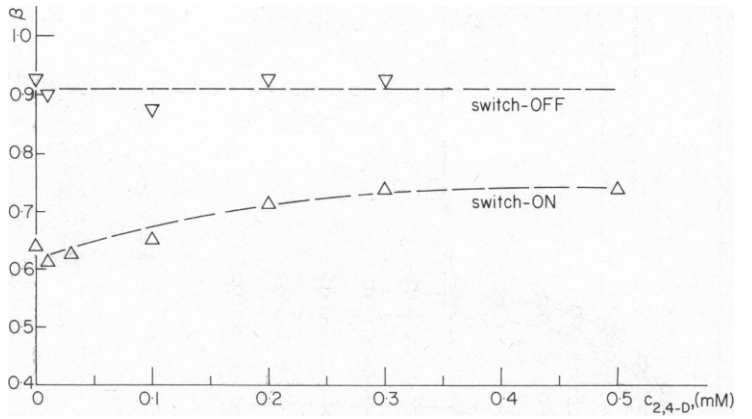


FIGURE 3 Dependence of parameter β on 2,4-D concentration for the switch-ON and switch-OFF transient processes. The value of β has been obtained from the best fit of the model equations to the voltage dependence of the initial membrane conductance and the relaxation time constant.

membranes (9). The values of parameter β for PC-chol and BPE membranes are also similar: β^{ON}(PC-chol) $\approx$ 0.6 as compared to β(BPE) = 0.71 (9) for comparable $TPhB^-$ concentrations [(1–3) $\times$ 10^{-7} M]. We interpret the increase of β^{ON} in the presence of 2,4-D as a consequence of inhibition of $TPhB^-$ adsorption by 2,4-D. In terms of the "three capacitor model," as demonstrated by Andersen et al. (9), the effective potential difference driving the ion diffusion across the membrane decreases with the increasing surface charge density of the membrane permeable ions. The observed increase of β^{ON} and the associated decrease of the surface charge density, Q_{ads}, with the increasing concentration of 2,4-D (Fig. 4) are consistent with the conclusions derived from the three capacitor model (9). The observed monotonic change of β^{ON} with the decrease of $TPhB^-$ surface charge density indicates that the assumption of negligible potential difference between the $TPhB^-$ adsorption plane and the aqueous medium (as compared to kT/e), implied in the adopted model (5), is not strictly satisfied. From this standpoint it would be desirable to do similar study at $TPhB^-$ concentration well below 10^{-7} M. This, however, would limit the 2,4-D concentration range because of smaller signal-to-noise ratio of the transient currents. We consider β^{OFF} to be a better parameter for $TPhB^-$ transport in PC-chol membranes. The value of β^{OFF} (PC-chol) = 0.91, obtained in the present work is close to β(BPE) = 0.86 determined at low $TPhB^-$ concentration, $c_{TPhB^-} = 1 \times 10^{-8}$ M (9) and to β, (dioleylphosphatidylethanolamine) = 0.92 (5). The origin of the difference between β^{ON} and β^{OFF} is not understood.

At higher 2,4-D concentration, the redistribution time of $TPhB^-$ ions in the membrane increases (Fig. 2). There is a possibility that during the measurement of the relaxation current, the condition of isolation of $TPhB^-$ ions trapped in the membrane from the aqueous

FIGURE 2 Voltage dependence of membrane current relaxation time constant as a function of aqueous 2,4-D concentration. Experimental conditions are identical to those given in Fig. 1. (*a*) The time constant determined from the transient current observed after the application of bias voltage across the membrane (switch-ON conditions). (*b*) The time constant determined from the transient current observed after the removal of bias voltage (switch-OFF conditions). The solid curves in *a* represent fit of Eq. 3 to the data. The straight lines in *b* represent the average time constant. For the purpose of illustration we have chosen 2,4-D concentrations 0, 1×10^{-4} M, and 5×10^{-4} M.

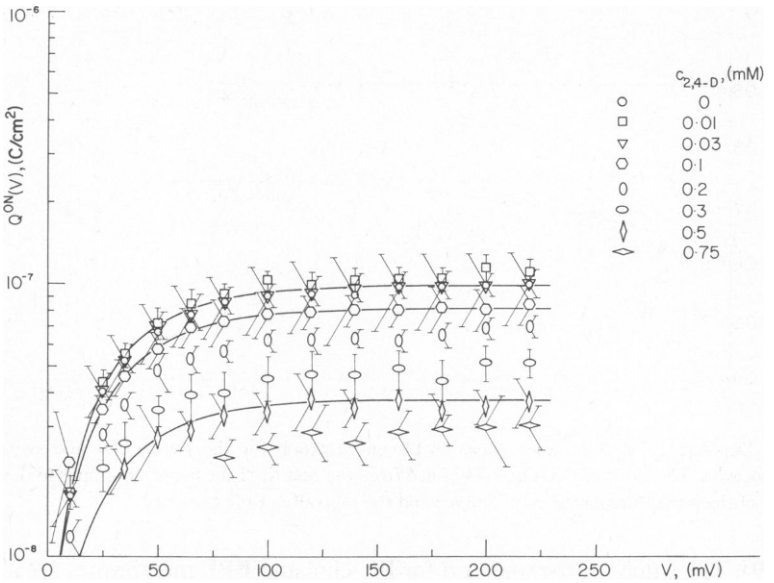


FIGURE 4 The effect of 2,4-D on the transfer of electric charge associated with $TPhB^-$ ions across PC-chol membranes. The solid curves have been drawn according to Eq. 5 for $\beta = 0.91$.

solution, as implied in the adopted model, is violated, because of the outflow of ions from the positively biased membrane side. We have checked this possibility by comparing the amount of charge transported during the switch-ON and switch-OFF transient conduction. It was found that the exchange of $TPhB^-$ between the membrane and the aqueous solution was at most 20%, and thus the assumption of complete trapping of $TPhB^-$ ions in the membrane remains approximately valid.

The 2,4-D-induced changes of membrane conductance characteristics, as illustrated in Figs. 1, 2, and 4, provide more detailed information on membrane modification than the steady-state studies with positive ions because of the possibility to determine separately the effect of 2,4-D on the density of adsorbed $TPhB^-$ ions and on their translocation across the membrane. The decrease of membrane permeability to $TPhB^-$, as evidenced by the decrease of membrane conductance, is in part due to the smaller adsorption coefficient of $TPhB^-$ ions at the membrane surface, and in part due to the change of the kinetics of ion translocation. The kinetic aspect of blocking the ion transport must be the dominating one, as can be clearly seen in Fig. 5, where we compare the decrease of membrane conductance with the changes of $TPhB^-$ adsorption. For example, an increase of 2,4-D concentration from 0 to 4.5×10^{-4} M corresponds to a decrease of the density of surface charge due to adsorbed $TPhB^-$ by a factor of about 3, whereas the conductance decreases about 38-fold.

The changes of the kinetics of $TPhB^-$ transport are directly reflected in the changes of the relaxation time constant. A semilog plot of the dependence of the relaxation time constant on 2,4-D concentration (Fig. 6) indicates that the redistribution time of $TPhB^-$ ions in the membrane increases exponentially in the presence of 2,4-D.

In our earlier study (1) we have shown that 2,4-D increases membrane conductance due to the positively charged $TPhAs^+$ ion and carrier complex nonactin-K^+, and that both the rate

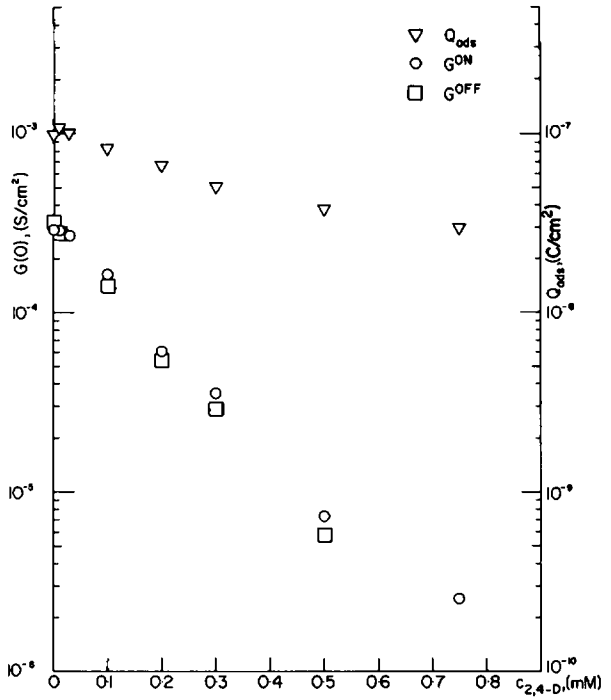


FIGURE 5 A comparison of 2,4-D concentration dependence of switch-ON and switch-OFF $TPhB^-$ membrane conductance with 2,4-D concentration dependence of the density of surface charge due to $TPhB^-$ ions adsorbed at the membrane surface.

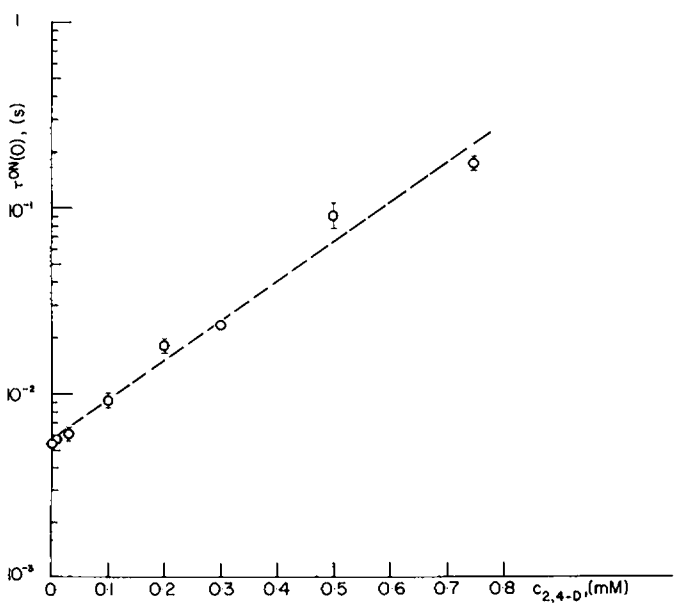


FIGURE 6 A plot of the dependence of zero voltage relaxation time constant on 2,4-D concentration.

constant of translocation of nonactin-K^+ and the $TPhAs^+$ conductance increase with ionic strength. Because 2,4-D suppresses the $TPhB^-$ conductance, an opposite dependence on ionic strength was expected in the case of $TPhB^-$. This possibility has been tested at two concentration levels of LiCl: 0.1 and 2 M, and 2,4-D concentration of 7.5×10^{-4} M (data not shown). We have found that under these conditions the conductance change was insignificant. Although the conductance effect was absent, an increase of the density of adsorbed $TPhB^-$ (2.1-fold), and an increase of the relaxation time constant (2.2-fold) were observed at higher ionic strength. Because the membrane initial conductance is proportional to the surface density of adsorbed $TPhB^-$, but inversely proportional to the relaxation time constant, the changes in $TPhB^-_{ads}$ and τ compensate each other. In contrast, in the absence of 2,4-D, the $TPhB^-$ membrane conductance increased with the ionic strength (1.7-fold). This change is associated with increased adsorption of $TPhB^-$ (2.5-fold) and a small increase (1.2-fold) in the relaxation time constant. The latter is a consequence of greater adsorption of $TPhB^-$ (9). Thus the change of membrane conductance, and specifically the increase of the relaxation time constant with the ionic strength, indicate the enhancement of 2,4-D adsorption at the membrane, because the effect of ionic strength is qualitatively similar to the increase of aqueous concentration of 2,4-D.

The plots of the dependence of membrane conductance, density of surface charge due to adsorbed $TPhB^-$ ions, and the relaxation time constant versus 2,4-D concentration (Figs. 5 and 6) suggest that these quantities change exponentially with 2,4-D concentration. On the basis of the observed charge asymmetry of the effect of 2,4-D on ion transport in membranes (1), we further assume that the changes of membrane conductance characteristics associated with $TPhB^-$ transport are of electric origin, and can be taken into account by a Boltzmann factor. The change of membrane conductance can be associated with net change of the height of membrane barrier, $e\Delta\psi$, defined as the change in the ion potential energy difference between the central plane of the membrane and the aqueous solution,

$$G(0) = G^{ref}(0) \cdot \exp(b_G c_{2,4\text{-D}}) = G^{ref}(0) \cdot \exp(e\Delta\psi/kT). \quad (9)$$

The change in the redistribution time of ions across the membrane can be related to the change of the ion potential energy difference between the central membrane plane and the adsorption plane, $e\Delta\phi$,

$$\tau(0) = \tau^{ref}(0) \cdot \exp(b_\tau c_{2,4\text{-D}}) = \tau^{ref}(0)/\exp(e\Delta\phi/kT). \quad (10)$$

Finally, the change of the density of adsorbed $TPhB^-$ ions depends on the depth of the ion potential energy well at the adsorption plane, $e\Delta\theta$,

$$Q_{ads} = Q^{ref}_{ads} \cdot \exp(b_Q c_{2,4\text{-D}}) = Q^{ref}_{ads} \cdot \exp(e\Delta\theta/kT). \quad (11)$$

The changes of electric potential differences with 2,4-D concentration, as determined from the changes of the three independently measured quantities: conductance, relaxation time constant, and surface charge density due to the adsorbed $TPhB^-$ ions, are shown in Fig. 7. Thus in the presence of 2,4-D, both the potential difference between the $TPhB^-$ adsorption plane and the aqueous medium, θ, and that between the membrane interior and the adsorption plane, ϕ, become more negative. It is of interest to compare $\Delta\psi$ deduced from the membrane conductance changes with the sum $\Delta\theta + \Delta\phi$ obtained from the separate measurements of the

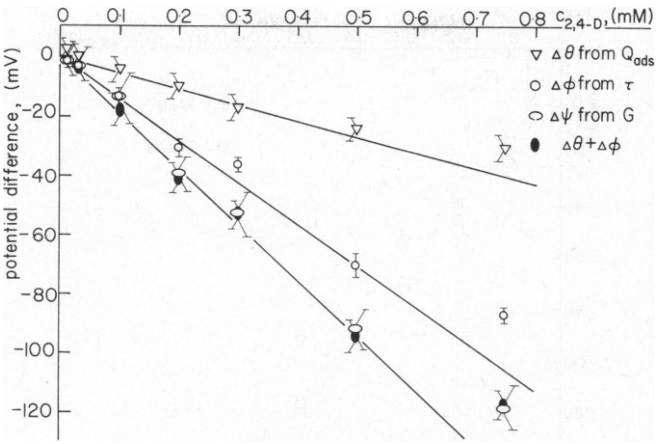


FIGURE 7 Changes of various electric potential differences as a function of aqueous 2,4-D concentration determined from the studies of relaxation of $TPhB^-$ conduction current in PC-chol membranes. $\Delta\theta$ is the change of the potential difference between the $TPhB^-$ adsorption plane and the aqueous solution, $\Delta\phi$ is the change of the potential difference between the central membrane plane and the adsorption plane. $\Delta\psi$ is the change of the potential difference between the central membrane plane and the aqueous solution.

surface charge density, ($\Delta\theta$), and the relaxation time constant, ($\Delta\phi$), because in terms of the adopted barrier model, the barrier height $e\psi = e\theta + e\phi$. If the transport model takes properly into account the changes induced by the presence of 2,4-D, it is to be expected that $\Delta\psi = \Delta\theta + \Delta\phi$. As follows from the comparisons given in Fig. 7, this expectation is fulfilled; $\Delta\psi$ (open symbols) agrees very closely with the sum $\Delta\theta + \Delta\phi$ (filled symbols).

Fig. 7 also makes clear that the changes of the potential difference between the central plane of the membrane and the $TPhB^-$ adsorption plane, $\Delta\phi$, are considerably greater than those between the adsorption plane and the aqueous solution, $\Delta\theta$. In terms of the dipolar hypothesis (1, 2), the relationship between $\Delta\phi$ and $\Delta\theta$ indicates that the layer of oriented 2,4-D molecules is predominantly located below the $TPhB^-$ adsorption plane. The present result, namely, that 2,4-D molecules are inserted into the membrane rather than adsorbed on the aqueous side of the membrane surface, agrees with the conclusion drawn from the studies of the effect of 2,4-D on nonactin-mediated transport of K^+ (1).

Finally, we can compare the results obtained on positively charged probes with those derived from $TPhB^-$ data. The net changes of the potential difference between the membrane interior and the aqueous solution, $\Delta\psi$, obtained from the $TPhAs^+$ and $TPhB^-$ conductance data are compared in Fig. 8 *a*. The 2,4-D-induced electric potential difference, as determined by positive ions, is by about 30–40% greater than that derived from the negative ions. For reasons that are not understood, the changes of conductance due to various membrane modifiers as detected by positively charged probes are often greater than those observed with negative probes[1] (9). In the case of phloretin, this discrepancy has been associated with the presence of cholesterol in the membrane (2). In the present work we have noted that $\Delta\theta$, $\Delta\phi$, and $\Delta\psi$, determined from independent measurements, are selfconsistent in that $\Delta\psi = \Delta\theta + \Delta\phi$. Changes of $TPhB^-$ conductance can be accounted for by the changes of $TPhB^-$

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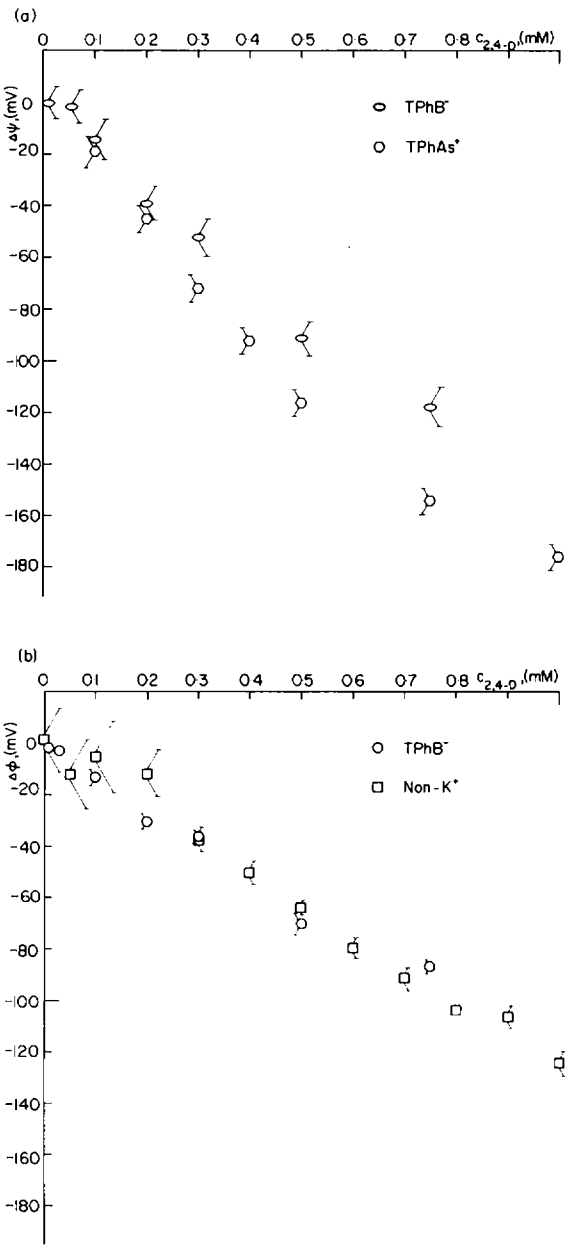

FIGURE 8 (*a*) A comparison of the changes of the electric potential difference between the central plane of the membrane and the aqueous solution as determined from the changes of membrane conductance due to $TPhAs^+$ an $TPhB^-$ ions. Conditions of the $TPhAs^+$ experiment (1): $c_{TPhAs^+} = 2 \times 10^{-3}$ M, pH = 2 (buffered), $c_{LiCl} = 0.5$ M. (*b*) A comparison of the changes of the electric potential difference between the central plane of the membrane and the adsorption-reaction plane as determined from the changes of kinetics of nonactin-K^+ transport, $\Delta\phi$ (nonactin-K^+), and the relaxation time constant of $TPhB^-$ transport, $\Delta\phi$ ($TPhB^-$). Conditions of nonactin-K^+ experiment (1): $c_{nonactin}$(aqueous solution) = 1.1×10^{-7} M, $c_{nonactin}$(membrane solution) = 3.2×10^{-5} M, $c_{K^+} = 0.06$ M, pH = 2 (buffered), ionic strength (LiCl + KCl) = 1 M.

adsorption and the kinetics of translocation. It is possible that in addition to electrostatic effects, as discussed above, 2,4-D facilitates adsorption of positively charged ions at the interface, which would account for the greater enhancement of cationic conductance as compared with the anionic conductance. It is interesting to note that the changes of the potential difference between the membrane interior and the adsorption/reaction plane, as determined from the changes of kinetic parameter A of nonactin-K^+ transport (1), and from the changes of the relaxation time constant of $TPhB^-$ conductance, are similar (Fig. 8 *b*). This experimental result suggests several conclusions. First, both the increase of the nonactin-K^+ translocation rate constant (1) and the increase of the $TPhB^-$ relaxation time constant can be accounted for by the change of the height of ion potential energy barrier. Second, the location of the layer of oriented 2,4-D molecules relative to the adsorption plane of $TPhB^-$, and the recombination plane of nonactin with K^+, is about the same.

Electric Potential Difference across Air/Water and Air/Lipid Monolayer/Water Interfaces

The measurements of surface potentials have been done under conditions similar to those for which a modified electrical conductivity of membranes has been observed; that is, we have used as the subphase the same electrolytic solution as in the membrane studies, and the same lipid compostion of solutions for monolyers as for membranes. Because 2,4-D changes the electric potential difference across air/water interface alone, even in the absence of a lipid monolayer, we present separately the experimental results for interfaces of both types, rather than subtract the surface potential difference of the aqueous solution from that of the monolayer. The data represent the electric potential difference between the ionizing electrode (air), and the reference electrode that is in contact with the subphase (aqueous solution).

The effect of 2,4-D on the boundary potential difference is shown in Fig. 9. The lower set of

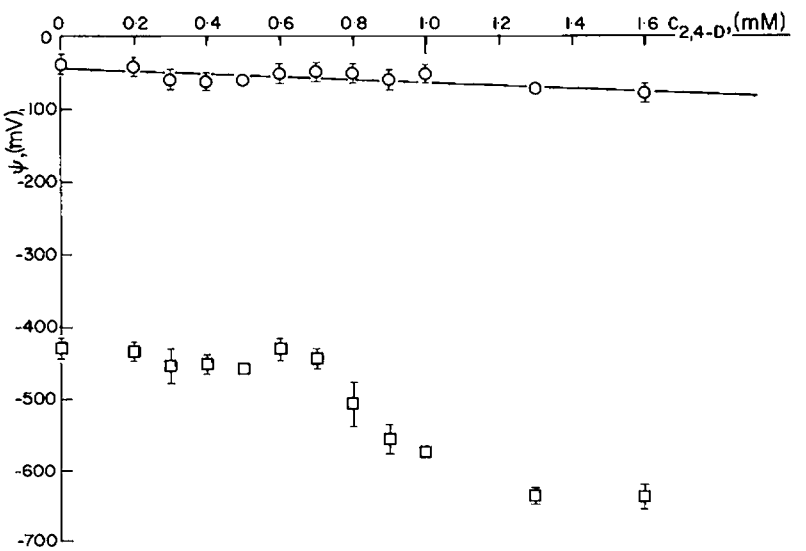

FIGURE 9 Changes of interfacial potential difference across the PC-chol monolayer (top) and the air/aqueous solution interface (bottom) as a function of aqueous concentration of 2,4-D. pH = 2 (buffered), c_{KCl} = 0.5 M.

data represents the air/water (i.e., air/aqueous electrolyte) interface in the absence of lipid monolayer, and the top set of data the potential difference across the air/lipid monolayer/water interfaces. In the absence of 2,4-D, the monolayer potential difference, defined as $\psi_{PC\text{-}chol} = \Delta V$(air/PC-chol monolayer/water) $- \Delta V$(air/water), is 406 ± 7 mV, which agrees closely with 420 mV reported for PC-chol monolayers by Hladky and Haydon (14) and 400 mV by Anderson et al. (5), and 425 mV for BPE monolayers (5). The potential difference across the air/water interface, i.e., the potential of the air side of the interface, becomes more negative by about 200 mV at 2,4-D concentrations above 1×10^{-3} M, which suggests that at sufficiently high aqueous concentration the 2,4-D molecules become stacked at the aqueous surface. In contrast, the potential difference across the PC-chol monolayer decreases in the presence of 2,4-D much less, only by about 35–40 mV. Because the decrease of the surface potential can be also due to the presence of 2,4-D anions at the interface, we have studied the dependence of interfacial potential difference on pH. If the adsorption of negative 2,4-D ions were the major factor determining the surface potential difference, it would become more negative at higher pH because the concentration of 2,4-D anions in the aqueous medium, and subsequently their density at the interface, would increase. The experimental results (Fig. 10) do not confirm this expectation; on the contrary, the interfacial potential difference becomes more positive at higher pH. The half-change occurs at pH comparable to the pK_a of 2,4-D, which is about 2.6–2.8 (15, 16). This indicates that the observed changes of the interfacial potential difference are related to 2,4-D dissociation equilibrium, and that the observation of more negative interfacial potential difference is related to the presence of neutral 2,4-D molecules.

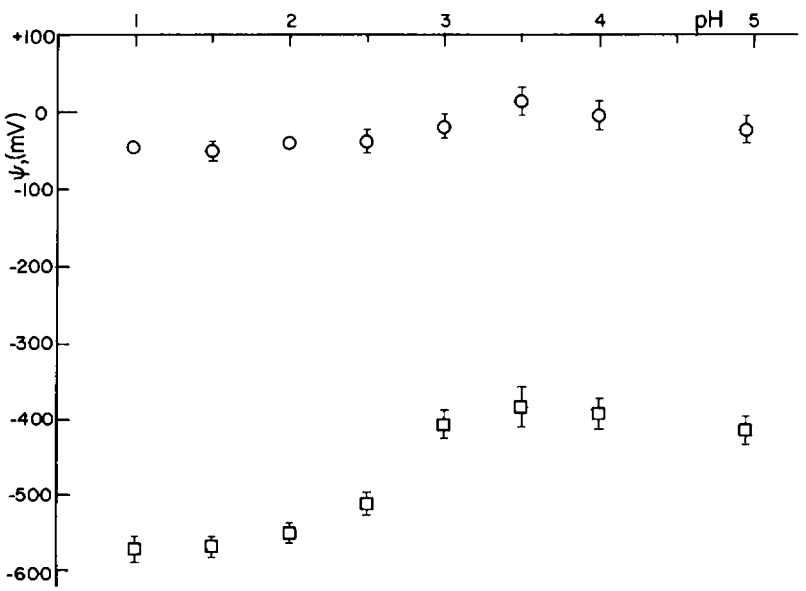

FIGURE 10 Changes of interfacial potential difference across the PC-chol monolayer (top) and the air/aqueous solution interface (bottom) as a function of pH (buffered). $c_{KCl} = 0.5$ M, $c_{2,4\text{-}D} = 1 \times 10^{-3}$ M.

In addition to the 2,4-D concentration and pH dependence, we have studied the effect of salt concentration on the interfacial potential difference, because the presence of electrolyte enhances the effect of 2,4-D on membrane conductance (increase of conductance of positive ions, and increase of relaxation time constant of $TPhB^-$ conductance). If the increase of ionic strength of the aqueous medium can be associated with the decrease of the electric potential of membrane interior, as suggested by the membrane conductivity studies, one may expect that the interfacial potential difference would change in a similar way. This was indeed observed, as can be seen from Fig. 11. Because the effect of ionic strength is rather small, we have compared two sets of measurements of $c_{2,4\text{-D}} = 1 \times 10^{-3}$ M: one at low ionic strength (0.05–0.2 M) and one at high ionic strength (1.0–2.5 M). For PC-chol monolayers the interfacial potential differences are as follows: -71 ± 3 mV at low, and -98 ± 14 mV at high, ionic strength. For air/water interface the effect is qualitatively similar: -514 ± 52 mV at low ionic strength and -622 ± 13 mV at high ionic strength. Thus in either case the less polar side of the interface becomes more negative at high ionic strength. This observation is consistent with the conclusion drawn from the conductance studies (1), namely, that the partition coefficient of 2,4-D between the nonpolar and aqueous medium increases with the electrolyte concentration.

The results of surface potential measurements provide additional and rather direct support for the dipolar hypothesis of 2,4-D action in lipid membranes: (*a*) 2,4-D decreases the electric potential of the nonpolar medium; (*b*) this effect is largest at pH $<$ pK_a of 2,4-D, i.e., it can be associated with the presence of electrically neutral 2,4-D molecules; and (*c*) the decrease of electric potential of the nonpolar side of the interface is further lowered in the presence of

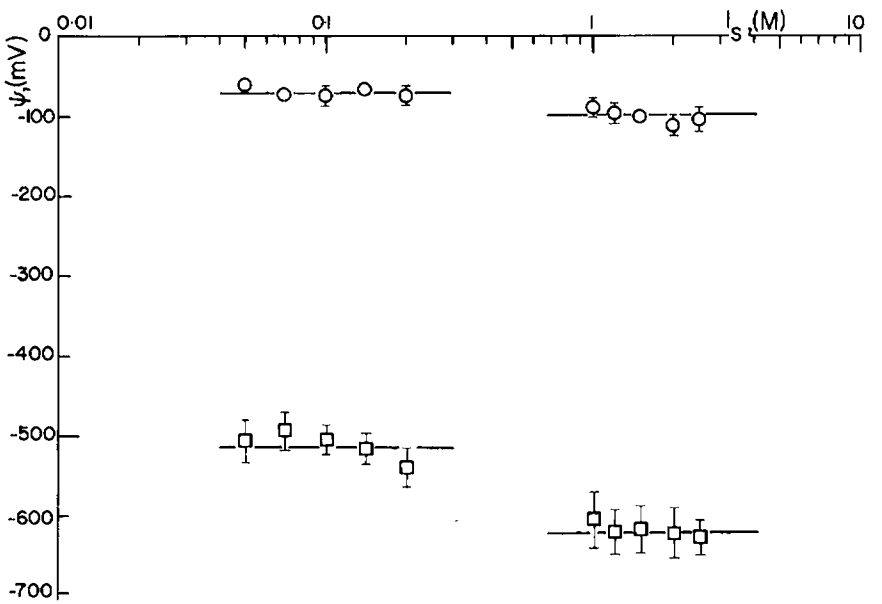

FIGURE 11 A comparison of interfacial potential differences across the PC-chol monolayer (top) and the air/aqueous solution interface (bottom) at high and low ionic strength. pH = 2 (buffered), $c_{2,4\text{-D}} = 1 \times 10^{-3}$ M. Ionic strength was adjusted by KCl + LiCl, ratio $c_{KCl}/c_{LiCl} = 1$.

electrolyte. Thus at the interface the 2,4-D molecules are oriented so that their dipole moment is directed toward the aqueous medium. However, the qualitative agreement between the changes of electric potential difference between the membrane interior and the adsorption/reaction plane or the aqueous solution, as determined from ionic probes for PC-chol membranes and those measured for PC-chol monolayers, is rather poor. First of all, the effect of 2,4-D on the potential difference across the PC-chol monolayer is weak. The same kind of inconsistency has been observed for the action of phloretin in BPE-chol and PC-chol membranes and in monolayers, and has been associated with the presence of cholesterol in the monolayer (2).

We have found earlier that 2,4-D is also active in GMO membranes and that the changes of the potential difference between the nonactin-K^+ recombination plane and the membrane interior in GMO and PC-chol membranes are similar (1). Because Haydon and Myers (12) found an excellent agreement between the monolayer and the bilayer potential changes for several ionic and zwitterionic surfactants for cholesterol-free GMO membranes and monolayers, we have also studied the action of 2,4-D on GMO monolayers. Our results are given in Fig. 12. In the absence of 2,4-D the potential difference across the GMO monolayer was found to be 318 ± 9 mV, which agrees with 319–321 mV reported by Hladky and Haydon (14). In contrast to PC-chol monolayers, the electric potential of the hydrocarbon side of the GMO monolayer becomes significantly more negative in the presence of 2,4-D. The straight line in Fig. 12 indicates the linear relationship between the change of the monolayer surface potential difference, $\Delta\psi$, and the concentration of 2,4-D. From the least-square fit of $\Delta\psi = b_m \cdot c_{2,4\text{-D}}$ to the data, we obtain b_m(GMO) = 1.4×10^5 mV/M for GMO monolayers as compared with b_m(PC-chol) = 0.2×10^5 mV/M (poor correlation) for PC-chol monolayers.

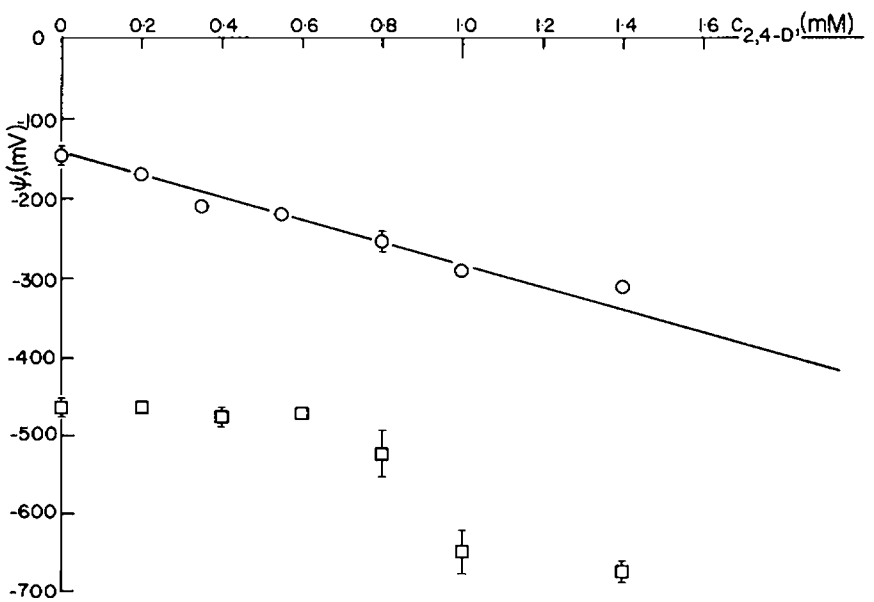

FIGURE 12 2,4-D concentration dependence of interfacial potential difference across the cholesterol-free GMO monolayer (top) and the air/aqueous interface (bottom). pH = 2 (buffered), c_{KCl} = 0.5 M.

For the bilayers, the change of the potential difference between the membrane core and the adsorption/reaction plane $\Delta\phi = b_b \cdot c_{2,4\text{-D}}$. From the concentration dependence of $\Delta\phi$ vs. $c_{2,4\text{-D}}$ (Fig. 9 of reference 1) we find b_b(GMO) = 0.99 × 10^5mV/M and b_b(PC-chol) = 1.2 × 10^5mV/M. Because $|\Delta\psi| \geq |\Delta\phi|$, it is to be expected that $|b_m| \geq |b_b|$. Experimental results for GMO confirm such relationship. In contrast, there is no such correspondence between the PC-chol monolayers and bilayers. Its absence is not understood. It is not clear whether the discrepancy is caused by lower partition coefficient of 2,4-D between PC-chol monolayer and the aqueous medium as compared with that of the bilayer, or whether it is due to the difference in the location and orientation of 2,4-D molecules with respect to the aqueous surface.

CONCLUSIONS

We have investigated the mechanism of permeatoxicity of 2,4-D in lipid membranes using negative tetraphenylborate ions. The primary goal was to understand the phenomenon of blocking $TPhB^-$ transport by 2,4-D. This has been achieved by studying the changes of membrane conductance, membrane current relaxation time constant, and membrane surface charge due to adsorbed $TPhB^-$ ions as a function of membrane bias voltage, 2,4-D concentration, and ionic strength. Furthermore, we have investigated the possibility that 2,4-D-induced changes of ion transport in membranes are directly related to the changes of electric potential difference across the membrane boundary. We have shown that the changes of kinectics of transport of $TPhB^-$ ions and of nonactin-K^+ complex support this hypothesis. Finally, we have measured the 2,4-D-induced changes of electric potential difference across air/water and air/lipid monolayer/water interfaces and have shown that the electric potential of the nonpolar side of the interface becomes more negative in the presence of 2,4-D, as suggested by the results of studies of membrane conductivity. Several of the more important conclusions are listed below:

(*a*) Suppression of transport of negative $TPhB^-$ ions in PC-cholesterol membranes by 2,4-D is dominated by the decrease of the probability of ion translocation across the membrane, as indicated by the increase of the current relaxation time constant. The effect of 2,4-D on $TPhB^-$ adsorption at the membrane/water interface is rather small.

(*b*) The results of $TPhB^-$ current relaxation studies indicate that 2,4-D-induced change of electric potential of the $TPhB^-$ adsorption plane constitutes about 25–30% of the total change of electric potential difference between the central plane of the membrane and the aqueous solution. The results support the dipolar hypothesis of action of 2,4-D; the layer of adsorbed 2,4-D molecules in PC-cholesterol membranes is located below the $TPhB^-$ adsorption plane, i.e., on the hydrocarbon side of the aqueous/membrane interface.

(*c*) Within the framework of the barrier model of lipophilic ion transport, the 2,4-D-induced changes of electric potential difference between the central plane of membrane and the adsorption-reaction plane for $TPhB^-$ and nonactin-K^+ ions have been found very similar. The changes of kinetics of transport of positive and negative ions also suggest that, except for the change of the electric potential, the ion transport properties in the membrane interior are not affected by the presence of 2,4-D. The effect of 2,4-D on ion transport across the membrane interior can be reduced to the jump of electric potential at the edge of membrane

hydrocarbon region. The 2,4-D-related electric potential difference is proportional to 2,4-D concentration in the aqueous solution.

(*d*) The results of measurements of interfacial potential difference across the air/water and air/lipid monolayer/water boundaries as a function of 2,4-D concentration, pH, and ionic strength indicate that the electric potential of the nonpolar side of the interface becomes more negative in the presence of neutral 2,4-D molecules, and that the magnitude of this effect increases with increasing ionic strength of the electrolyte. Because these features are displayed by interfaces of both types, as well as by lipid bilayer membranes, the results of interfacial potential measurements suggest that the action of 2,4-D in lipid membranes is not associated with the change of orientation of lipid molecule adsorbed at the nonpolar/polar boundary region of the membrane.

(*e*) We have confirmed that, in general, the lipid monolayer cannot be necessarily regarded as a model for half of the bilayer lipid membrane. We have shown that for PC-cholesterol monolayers and bilayer membranes there is a significant quantitative discrepancy between the 2,4-D-induced changes of electric potential difference between the membrane interior and the aqueous solution as determined from conductivity measurements with positive and negative probes, and the electric potential difference measured across the lipid monolayer. The results support the earlier findings that the discrepancy between the monolayer and bilayer results can be associated with the presence of cholesterol in the lipid monolayer. For cholesterol-free GMO monolayers and membranes, we have found close agreement between the changes of the electric potential difference in the membrane boundary region as determined from the ion transport and the changes of electrical potential difference across the monolayer.

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REFERENCES

1. SMEJTEK, P., and M. PAULIS-ILLANGASEKARE. 1979. Modification of ion transport in lipid bilayer membranes in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D). 1. Enhancement of cationic conductance and changes of the kinetics of nonactin-mediated transport of potassium. *Biophys. J.* **26**:441.
2. ANDERSEN, O. S., A. FINKELSTEIN, I. KATZ, and A. CASS. 1976. Effect of phloretin on the permeability of thin lipid membranes. *J. Gen. Physiol.* **67**:749.
3. MELNIK, E., R. LATORRE, J. E. HALL, and D. TOSTESON. 1977. Phloretin-induced changes in ion transport across lipid bilayer membranes. *J. Gen. Physiol.* **69**:243.
4. KETTERER, B., B. NEUMCKE, and P. LÄUGER. 1971. Transport mechanism of hydrophobic ions through lipid bilayer membranes. *J. Membr. Biol.* **5**:225.
5. ANDERSEN, O. S., and M. FUCHS. 1975. Potential energy barriers to ion transport within lipid bilayers. *Biophys. J.* **15**:795.
6. BRUNER, L. J. 1975. The interaction of hydrophobic ions with lipid bilayer membranes. *J. Membr. Biol.* **22**:125.
7. GAVACH, C., and R. SANDEAUX. 1975. Non-mediated zero voltage conductance of hydrophobic ions through bilayer lipid membranes. *Biochim. Biophys. Acta.* **413**:33.
8. SZABO, G. 1977. Mechanism by which small molecules alter ionic permeability through lipid bilayer membranes. *In* Membrane Toxicity. Morton W. Miller and Adil E. Shamoo, editors. Plenum Publishing Corp., New York. 167.

9. ANDERSEN, O. S., S. FELDBERG, H. NAKADOMARI, S. LEVY, and S. MCLAUGHLIN. 1978. Electrostatic interactions among hydrophobic ions in lipid bilayer membranes. *Biophys. J.* **21**:35.
10. WANG, C. C., and L. BRUNER. 1978. Dielectric saturation of the aqueous boundary layers adjacent to charged bilayer membranes. *J. Membr. Biol.* **38**:311.
11. MACDONALD, R. C., and A. D. BAGHAM. 1972. Comparison of double layer potentials in lipid monolayers and lipid bilayer membranes. *J. Membr. Biol.* **7**:29.
12. HAYDON, D. A., and V. B. MYERS. 1973. Surface charge, surface dipoles and membrane conductance. *Biochim. Biophys. Acta.* **307**:429.
13. SARGENT, D. F. 1976. An apparatus for the measurement of very small membrane relaxation currents. *Anal. Biochem.* **70**:100.
14. HLADKY, S. B., and D. A. HAYDON. 1973. Membrane conductance and surface potential. *Biochim. Biophys. Acta.* **318**:464.
15. NELSON, N. H., and S. D. FAUST. 1969. Acid dissociation constants of selected aquatic herbicides. *Environ. Sci. Technol.* **3**:1186.
16. BAILY, G. W., and J. L. WHITE. 1965. Herbicides: a compilation of their chemical and biological properties. *In* Residue Reviews. Springer-Verlag New York, New York. Vol. 10. 97.