ELECTRICAL CONDUCTIVITY IN LIPID BILAYER MEMBRANES INDUCED BY PENTACHLOROPHENOL

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ABSTRACT Electrical conductivity induced in thin lipid bilayer membranes by pentachlorophenol has been studied. The membranes were formed from phosphatidyl choline, phosphatidyl ethanolamine, or phosphatidyl glycerol and various amounts of cholesterol. The position and the magnitude of the maximum of the conductivity vs. pH curve depend on the type of lipids and cholesterol content. At low pentachlorophenol concentrations and low pH the concentration dependence of conductivity is quadratic and becomes linear at higher pH. Above 10^{-5} M of pentachlorophenol the concentration dependence of the membrane conductivity tends to saturate. Presence of pentachlorophenol enhances membrane transport of nonactin-K^+ complex. Increase of cholesterol content increases pentachlorophenol induced conductivity in all membranes and shifts the conductivity toward lower pH. For phosphatidyl choline the largest rate of change of membrane conductivity with cholesterol occurs at 1:1 phospholipid to cholesterol molar ratio. Pentachlorophenol is found to be a class II uncoupler and the experimental results are consistent with the hypothesis that the membrane permeable species are dimers formed by combination of neutral and dissociated pentachlorophenol molecules. Several schemes of membrane conduction, including dimer formation in the aqueous phase as well as at the membrane-water interface have been considered. Arguments are given in favor of the formation of dimers within the membrane surface.

INTRODUCTION

Pentachlorophenol (PCP) is widely used as a contact herbicide and wood preservative; its major biological activity is associated with uncoupling of oxidative phosphorylation (1). PCP has been found to increase electrical conductivity of lipid membranes (2, 3) and to facilitate proton transport in mitochondria and phospholipid vesicles (4). Several attempts have been made to correlate the increase of electrical conductivity of lipid bilayers induced by uncouplers with their toxic activity in mitochondria (3–5). Foster and McLaughlin (6) have pointed out that more attention should be paid to the equivalence of the experimental conditions under which conductivity measurements and respiration or swelling experiments are done. In order to make the biological studies more meaningful it is necessary to have more detailed information on the action of uncouplers in simpler membranes.

The inherent complexity of correlating biological activity with physicochemical properties of membranes is due, among other factors, to pH dependence, saturation of

membrane conductivity at higher concentration of the membrane modifiers, presence of unstirred layers, and effects related to charge and orientation of polar head groups of phospholipids in the membrane. In the case of uncouplers the problem of correlation is aggravated by the existence of at least two classes of substances with different mechanism of charge transport (7). Uncouplers of class I exhibit a linear increase of membrane conductivity with uncoupler concentration; typical compounds are carbonylcyanide *m*-chlorophenylhydrazone (CCCP), (7), and 3-t-butyl,5-chloro,2'chloro, 4'-nitrosalicylanilide (S 13) (8, 9). Uncouplers of class II exhibit a quadratic dependence of conductivity on concentration. The most investigated compounds in this category are 5,6-dichloro-2-trifluoro-methyl benzimidazole (DTFB) (6), tetrachloro-2-trifluoromethyl benzimidazole (TTFB) (6, 9, 12), and 2,4-dinitrophenol (DNP) (10, 11). The increase of membrane conductivity in these cases is associated with a bimolecular process whose nature is only partially understood. Several such conductivity mechanisms have been considered: proton hopping (13), collisional exchange of protons between the neutral and ionized forms of uncouplers (12), and negative charge transfer via dimers formed by association of the neutral molecule with the uncoupler anion (14, 15). The latter mode of transport appears to be energetically the most feasible one, although experiments attempting to observe dimers directly have not been so far successful (6).

This paper reports on experiments intended to confirm or disprove the hypothesis that PCP is a class II uncoupler, and to gain insight into the changes of physical properties of lipid matrices of biological membranes taking place in the presence of PCP. The mechanism of PCP-induced membrane conduction is inferred from several basic experiments which we have done. These involved measurement of membrane conductivity as a function of pH, dependence of conductivity on the concentration of PCP, membrane potentials, and conductivity characteristics at different buffer strength of the aqueous solution. We have found it useful to supplement these experiments by the determination of the pK of pentachlorophenol and its partition coefficient between water and hydrocarbon, and by the study of the enhancement of membrane conduction due to nonactin-potassium complex in the presence of PCP. We have also investigated the dependence of PCP-induced membrane conductivity on the polar moiety of phospholipids and cholesterol content. The experimental results support the hypothesis that PCP is a class II uncoupler, and that the membrane permeable species are negatively charged dimers. We have considered several schemes of membrane transport and have concluded that the membrane permeable species are dimers which probably are formed within the membrane surface.

MATERIALS AND METHODS

Materials

Chromatographically pure lecithin was extracted from fresh chicken egg yolks and purified on an alumina column according to the method of Singleton et al. (16) with the following modifications developed in Chapman's Laboratory at the University of

Sheffield, England. After the last extraction with ethanol the filtrate was evaporated in a rotary evaporator to a dark syrup, to which equal volumes of ethanol and acetone were added. The mixture was filtered into a separatory funnel and water was added to the filtrate. The precipitate thus formed was separated to dryness. The repeated petroleum ether-acetone processes in Singleton's method were replaced by ether-acetone processes until the precipitate was almost white in color. The effluent from the alumina column containing the lecithin portion was filtered through a Millipore filter. The lipid was stored in chloroform solution under nitrogen atmosphere in sealed ampoules at −15°C until use. Phosphatidyl ethanolamine was purchased from Supelco, Inc., Bellefonte, Pa. Recrystallized cholesterol was a gift from Dr. David McClure of the Chemistry Department. Pentachlorophenol and *n*-decane were purchased from Aldrich Chemical Co., Milwaukee, Wis. Nonactin was a gift from Squibb Institute of Medical Research.

The membrane forming solution was prepared by dissolving weighed amounts of phospholipid and cholesterol in *n*-decane. The phospholipid concentration was kept at 5 μmol/ml to avoid formation of cholesterol islands in the membrane at high cholesterol mole fraction. The electrolytic solution used for control experiments contained 0.1 M KCl, 5×10^{-4} M KOH, and a buffer composed of 0.2 M potassium dibasic phosphate, 0.2 M potassium citrate, and 0.05 boric acid. It was prepared with stock solution of twice the concentration of each constituent, titrated with HCl, and adjusted to the desired pH and specified concentration mentioned above by addition of water. For experimental solution containing PCP, the PCP was first dissolved in 0.2 M KCl and 10^{-3} M KOH, mixed with stock buffer solution of twice the concentration, and titrated with HCl in the same manner. For nonactin experiments, ethanol solution of this compound was added to the PCP solution; the volume of ethanol was 0.5% of the total fluid. Deionized distilled water from a Millipore Q2 System with resistivity exceeding 10 MΩ-cm was used in the preparation.

Membrane Formation

The membrane was formed by the brush technique (17), using a sable brush (size 000 and 0000) to introduce the membrane forming solution on a 2 mm hole in the wall of a Teflon cell immersed in the electrolytic solution. In all conductivity measurements, membranes were formed in the electrolytic solution containing PCP or nonactin or both. In the measurements of membrane potential due to pH gradient, HCl was added into one of the compartments and equal volume of buffer solution was added to the other to compensate for the hydrostatic pressure difference. The pH gradient across the membrane was monitored by a pair of miniature glass and calomel electrodes. For PCP concentration gradients, PCP solution was added into one compartment and equal volume of buffer was added to the other.

For cleaning, we routinely boiled the cell in 95% ethanol containing sodium hydroxide pellets, and rinsed several times with deionized water. Several coatings of membrane forming solution were applied to the dry surface around the orifice as well

as on the edge, until an even reflecting surface was formed. Between successive coatings the solution was blown dry with nitrogen. When the surface was well prepared the membrane lasted for more than 5 h. The progress of thinning was observed in reflected light through a microscope. In order to reduce the equilibration time and fluctuation of membrane area from membrane to membrane, we kept the thickness of the torus small by using minimal amounts of membrane forming solution to paint the membrane. We also kept the level of the fluid in the cell not more than 2–3 mm above the hole to minimize the hydrostatic pressure difference between the cell compartments caused by the displacement of some volume of the electrolytic solution by the paint brush.

Electrical Measurements

Membrane currents were measured with an electrometer (Princeton Applied Research [Princeton, N.J.] Model 135 or Keithley Picoammeter, Model 417 [Keithly Instruments, Inc., Cleveland, Ohio]). The current-voltage data were taken after the membrane became black and conductivity reached a constant level. This was judged from monitoring the current when a small voltage, typically 25 mV, was applied either continuously or intermittently across the membrane. Generally, fiber tip calomel electrodes (Corning Science Products, Corning, N.Y.) were used, either with or without agar bridges. We did not observe any significant difference in the current readings between the two cases. When the membrane resistance became comparable with the internal resistance of the calomel electrodes, they were replaced by Ag/AgCl electrodes (Annex Instruments, Santa Ana, Calif.). All measurements were carried out at temperatures between 22° and 23°C.

We had the option of stirring both cell compartments with magnetic stirrers, with speed up to 120 rpm, but did not find any appreciable dependence of conductivity on stirring. Since stirring increased the scattering of data points, we did not stir the solution except in the membrane potential and conductivity delay time experiments.

Each data point shown in the figures represents an average value obtained from at least four different membranes; the error bars denote one standard deviation. Generally we started to take data from the third or the fourth membrane for each experimental condition, because the first few membranes gave erratic current values. The specific conductivity was calculated from the area of the hole; therefore, the values in the figures represent the lower limits.

Determination of pK

The value of pK of PCP was determined from spectrophotometric measurements. Ultraviolet absorption spectra of buffered PCP solution (pH range 1.75–10.6) were taken using a Cary 14 spectrophotometer (Cary Instruments, Monrovia, Calif.). The spectrum of ionized PCP (PCP^-) was identical to that published by Lang (18) with two absorption peak maxima at 248 mμ 320 mμ, respectively. The absorption spectrum at pH 1.75 was similar to that of PCP in ethanol solution as reported by Lang (18), but with the absorption peak shifted from 304 mμ 302 mμ, presumably due to the solvent

effect. The extinction coefficient for the neutral species was found to be 2.28×10^3 liter $\cdot$ mol^{-1} $\cdot$ cm^{-1}. Since the ratio of the absorbance of neutral PCP molecules at 320 mμ to that of PCP$^-$ is insignificant (1/70), we plotted the absorbance of PCP$^-$ as a function of pH, and used the pH of the inflection point of the curve as the pK value. The pK value thus obtained was between 4.7 and 4.8 which agrees well with that of 4.8 reported by Kolesova et al. (19) and Blackman et al. (20) but disagrees with the value of 5.26 quoted in *Beilsteins Handbook* (21).

Determination of PCP Partition Coefficient

The partition coefficient of PCP between *n*-decane and aqueous solution was determined from two sets of experiments: (*a*) by mixing a decane solution of PCP with equal volume of buffer, and (*b*) by mixing a buffer solution of PCP with equal volume of decane. The mixing was done in separatory funnels using a wrist action shaker. After the separation of the two phases the PCP content in each was determined spectrophotometrically. The partition coefficient of PCP between decane and the aqueous phase for pH range from 6.5 to 7.5 was found to be $(1.9 \pm 0.3) \times 10^3 : 1$.

RESULTS AND DISCUSSION

pH Dependence of Membrane Conductivity

Fig. 1 illustrates the dependence of membrane conductivity on the pH of the aqueous phase for three phospholipids: phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), and phosphatidyl glycerol (PG). Two levels of cholesterol in the membrane forming solution were used: low—cholesterol mole fraction about 0.2 (Fig. 1*a*), and high—cholesterol mole fraction about 0.75 (Fig. 1*b*). The broken curves are plots of the function $S\{[H]/([H]_{max} + [H])^2\}$, where $[H]_{max}$ is the proton activity corresponding to that at the conductivity maximum. The scale factor S was adjusted to aid examining the data.

The data have several features. First, both the position of the maximum and the magnitude of conductivity depend on the type of lipids. Membrane formed from PG, which is negatively charged within the pH range, were less conductive as compared with those made from PC or PE. Second, with the exception of PE, the membrane conductivity maximum occur at pH > pK of PCP. Third, for all three phospholipids the conductivity increased with the cholesterol content in the membrane, and the maximum shifted toward lower pH.

The decrease of conductivity associated with PG membranes supports the hypothesis that negatively charged species formed from PCP are involved in membrane conduction. The increase of conductivity of PG membranes with high cholesterol content can be related to the decrease of the density of negatively charged PG head groups in the membrane. This electrostatic argument is, however, insufficient to explain similar increase of conductivity of PC and PE membranes with cholesterol since these lipids

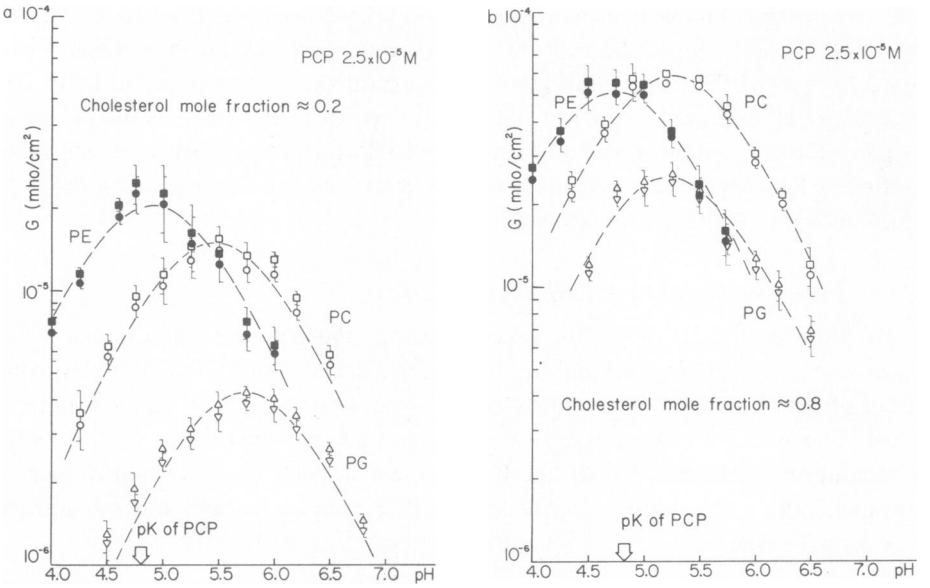

FIGURE 1*a* pH dependence of conductivity of phospholipid-cholesterol membranes having low cholesterol content. Lower values of each set represent conductivity at 25 mV and higher values, at 50 mV.

FIGURE 1*b* pH dependence of conductivity of phospholipid-cholesterol membranes having high cholesterol content. Lower values of each set represent conductivity at 25 mV and higher values, at 50 mV.

are neutral[1] within the pH range used in the experiment. Therefore, the increase of conductivity of these membranes is due to some specific action of cholesterol.

The above observation is consistent with that of Szabo who found that monoolein and glycerol dioleate membranes exhibit a decrease of the cation and an increase of the anion conductivity with the increase of the cholesterol content (23, 24). Further experimental studies of the cholesterol effect will be presented later in this paper.

Concentration Dependence of Membrane Conductivity

The experimental data on concentration dependence of PCP-induced conductivity in PC-cholesterol membranes are presented in Fig. 2. It is apparent that at low concentration ($<2\,\mu$M) and low pH (ph = 5) the dependence of conductivity on the concentration is quadratic, which is a characteristic of class II uncouplers. However, at higher concentration, the conductivity tends to saturate. Also when the aqueous phase becomes more basic the slope of the log C vs. log [PCP] curve decreases, and approaches unity. The concentration dependence studies at pH < pK were not done because of limited solubility of PCP at lower pH. Thus PCP appears to have "transitional" prop-

[1] PE becomes slightly positive charged at pH between 4 and 5 (22).

erties. Under some experimental conditions the membrane characteristics are similar to those produced by strictly class II uncouplers; for instance, TTFB and DTFB. At higher pH, however, it has characteristics which have also been noticed in the exceptional case of DNP. This latter compound, which has structural similarities to PCP has been extensively studied by McLaughlin (11) who despite the linear dependence of conductivity with its concentration classified it as a class II uncoupler.

We do not understand why at low concentrations the conductivity curves tend to merge and suspect some systematic error. If the effect were real one would expect a dependence of the pH values of conductivity maxima on PCP concentration. This is in keeping with Bruner's model (25) which predicts such shifts. Thus we investigated such a possibility in separate experiments and have not been able to detect any pH shift of the conductivity maximum down to PCP concentration of 2 μM. Below this value the experimental errors were too large to make a more definite conclusion.

Effect of Cholesterol

The enhancement of PCP-induced conductivity by cholesterol was studied in some detail, and the effect was observed for all three phospholipids. The increase of the rate of membrane ionic transport in the presence of cholesterol is an interesting phenomenon since, until recently, it has been generally accepted that cholesterol has an inhibitory effect on permeability of both biological and artificial membranes (26–28).

We report here on two sets of experiments: (*a*) Measurements of conductivity as a

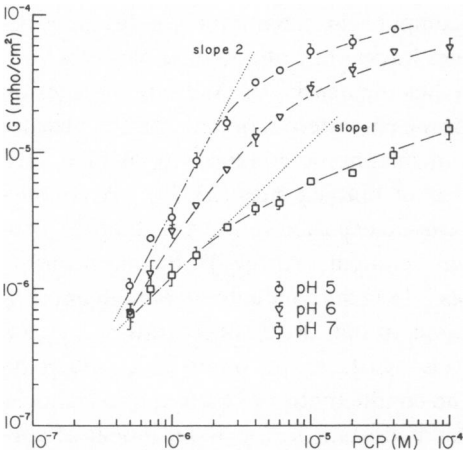

FIGURE 2

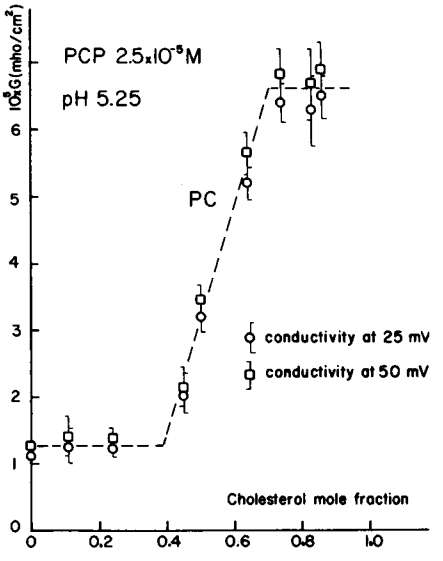

FIGURE 3

FIGURE 2 Conductivity of PC-cholesterol membranes (at 25 mV) as a function of PCP concentration at various pH. Cholesterol mole fraction is approximately 0.8.

FIGURE 3 The effect of cholesterol on PCP-induced conductivity of PC membranes.

function of cholesterol mole fraction, and (*b*) Measurements of the dependence of conductivity on the PCP concentration for membranes having low and high cholesterol content.

The result of the first experiment is shown in Fig. 3. In this set of measurements, the phosphatidyl choline content in the membrane solution was kept at 3 mg/ml of decane. In separate experiments we found that the effect was not due to the increase of the total lipid content in the membrane solution as the cholesterol mole fraction was increased. The data indicate that there are two membrane conduction levels, one corresponding to low and the other to high cholesterol content. The midpoint of the transition happens to occur at cholesterol mole fraction between 0.5 and 0.6; i.e., at cholesterol/PC molar ratio slightly greater than one. As Bunce and Hider (29) found, by direct determination of the composition of PC-cholesterol-*n*-decane membranes, the cholesterol to phosphatidyl choline molar ratio in the membrane is about the same as in the membrane forming solution. It suggests that the cholesterol scale in Fig. 3 represents the membrane composition.

The increase of membrane conductivity is approximately sixfold for PC and PG, and 2.5-fold for PE. These values are several times smaller than the almost 30-fold conductivity enhancement observed by Szabo (23) for monoolein membranes and tetraphenyl borate or CCCP anions.

The enhancement of anionic conductivity by cholesterol is presumably due to the change of orientation of the membrane dipole layer (23). From the comparison of the above conductivity data it follows that the orientating effect of cholesterol is greater for PC as compared with PE. The conductivity increase for PG should not be compared with that for PE and PC since PG is negatively charged and some of the conductivity increase can be attributed to the change of the surface charge density.

The aim of the second experiment was to compare the membrane conductivity dependence of PCP concentration for membranes having low and high cholesterol content. The intention was to find out as to whether the density of PCP binding sites on the membrane surface is affected by the membrane cholesterol content. In the absence of transport limitations, the saturation level of membrane conductivity at high PCP concentration should depend upon the number of binding sites (25, 30). A conductivity pattern similar to that shown in Fig. 4*a* can be expected in the case that the density of binding sites increases with the cholesterol content. At low PCP concentration, i.e., when the fraction of occupied binding sites is low, the conductivity of high and low cholesterol membranes would be about the same; at high PCP concentration the conductivity would saturate at two different levels. In fact, the observed conductivity pattern is qualitatively different (Fig. 4*b*). The conductivities of high and low cholesterol membranes are related to one another by a constant factor, independent of concentration. Thus the cholesterol effect is not a matter of limiting binding site density, rather the rate of transmembrane transport is enhanced by the presence of cholesterol. This is consistent with the hypothesis that there is an increased inward component of membrane dipole moments under these conditions (23).

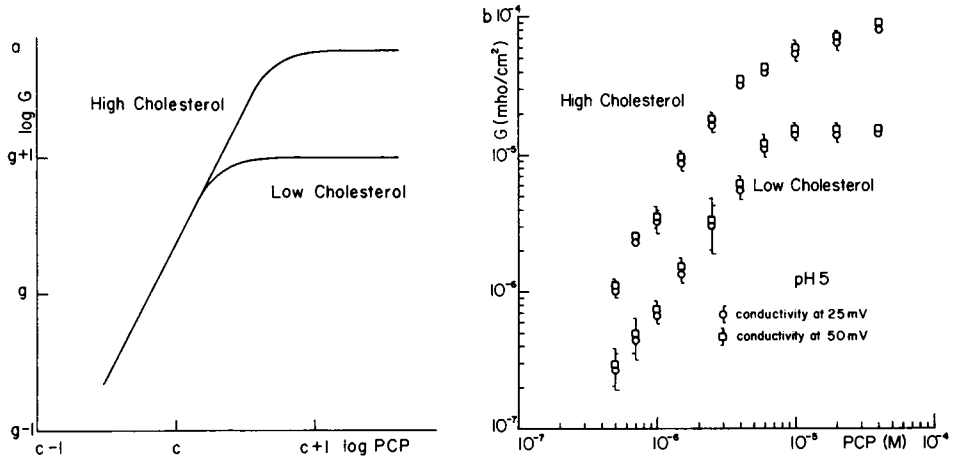

FIGURE 4*a* Expected relationship between the conductivity and PCP concentration for the density of binding sites dependent on the membrane cholesterol content.
FIGURE 4*b* Experimental results. The effect of cholesterol content on the concentration dependence of conductivity of PC membranes. High cholesterol: cholesterol mole fraction 0.8. Low cholesterol: cholesterol mole fraction 0.2.

Adsorption of PCP Anions

The charged complex formed from nonactin and potassium cation has been shown to be a suitable probe of membrane surface charge (31). Its use is based on the change of membrane conductivity due to the variation of density of nonactin-K^+ complexes at the membrane surface, which in turn is determined by the membrane surface potential. Thus, if PCP related anions are adsorbed at the membrane surface, an increase of nonactin-K^+ induced membrane conductivity is to be expected because the membrane surface potential would be more negative. We have observed such effect, and the experimental results are given in Fig. 5*a*. The lower set of data represents membrane conductivity as a function of PCP concentration when only PCP is present in the aqueous phase. The broken line in the middle marks the magnitude of membrane conductivity induced by the nonactin-K^+ complex in the absence of PCP in the system. The concentration of nonactin was 3.5×10^{-6}M. The upper set of data represents the conductivity when both nonactin and PCP are simultaneously present in the aqueous solution. The enhancement of membrane conductivity of positively charged complexes in the presence of PCP is significant. At pH 6 a PCP concentration of 20 μM increases the nonactin conductivity about a hundredfold; similar results are obtained at pH 7. This suggests that the effect may be associated with the adsorption of PCP anions although, under special conditions, the negatively charged dimers may also be involved.

Following McLaughlin's argument (11), we assume that the density of charged species at the membrane-water interface is solely determined by the surface electrostatic potential. Thus, in the absence of transport limitations, the nonactin-K^+ conductivity should be proportional to the Boltzmann factor $\exp(-q\psi/kT)$, where q is

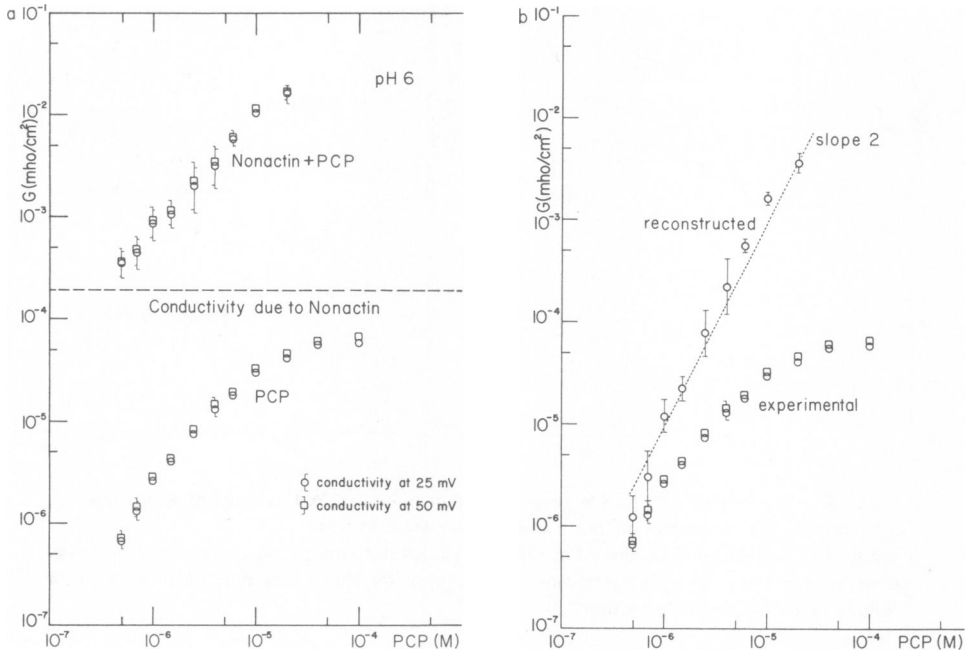

FIGURE 5*a* The effect of presence of PCP on membrane conductivity induced by nonactin-K^+ complex.

FIGURE 5*b* Concentration dependence of PCP-induced membrane conductivity reconstructed from the data shown in Fig. 5*a*.

the ionic charge and ψ is the surface potential. A decrease of membrane conductivity of negatively charged species associated with the increase of conductivity of positive ions is to be expected. Therefore, one can correct the PCP conductivity data in such a way as to eliminate the effect of surface potential. The result of such an analysis is given in Fig. 5*b*. The reconstructed conductivity data exhibit quadratic dependence of conductivity on PCP concentration, which is in agreement with our earlier finding that PCP belongs to class II uncouplers.

Conductivity Limit Due to Unstirred Layers

The experimental results presented in Figs. 1 and 2 are in agreement with the hypothesis that membrane conductivity is due to the transport of negatively charged dimers. A subsequent question arises as to where these postulated membrane permeable species are formed. To resolve this matter we shall consider first the case of formation of dimers in the aqueous phase and compare the expected conductivity with the experimental results.

We consider the dissociation of the neutral molecules (HA) and the formation of dimers (HA_2^-) according to the following reactions:

$$HA \rightleftharpoons H^+ + A^- \qquad (1)$$

$$HA_2^- \rightleftharpoons HA + A^- \tag{2}$$

The dimers present in the aqueous phase partition into the membrane and are then driven across by the electrochemical potential gradient. Further, for simplicity, we consider Eyring type transport across the membrane. Upon the application of a small voltage across the membrane, the initial current density ($t = 0$) is given by:

$$J(0) = e^2 k_i \beta [HA_2^-] V/kT, \tag{3}$$

where e is the electronic charge, k_i is the rate constant of transfer of dimers across the membrane, β is the partition coefficient of dimers between the membrane surface and the aqueous phase, $[HA_2^-]$ is the equilibrium concentration of dimers in the aqueous solution, and V is the applied voltage. At steady state, after the concentration gradients in the unstirred layers have fully developed, the current density is:

$$-J = eD([HA_2^-] - [HA_2^-]')/\delta = eD([HA_2^-]'' - [HA_2^-])/\delta. \tag{4}$$

The primed and double primed quantities are the aqueous concentrations of dimers at the left and right membrane surfaces, D is the diffusion coefficient of dimers in water, and δ is the thickness of the unstirred layer. Using the condition of current continuity we obtain the steady-state current density in the system

$$J = J(0)/(1 + 2k_i\beta\delta/D). \tag{5}$$

Assuming that $2k_i\beta\delta/D \gg 1$, which implies that the membrane current is fully limited by the aqueous unstirred layers, the steady-state conductivity limit is given by:

$$G_{\text{lim}} = e^2 D[HA_2^-]/(2kT\delta). \tag{6}$$

The concentration of dimers in the aqueous phase can be obtained from reactions 1 and 2 in terms of the total uncoupler concentration C_T, where $C_T = [HA] + [A^-] + [HA_2^-]$. The result is

$$[HA_2^-]/\left(1 - \frac{[HA_2^-]}{C_T}\right)^2 = C_T^2\{K_1/[H]/K_2(1 + K_1/[H])^2\}, \tag{7}$$

where K_1 and K_2 are the two dissociation constants for reactions 1 and 2. Eq. 7 should be satisfied at least up to PCP concentration of 1.5×10^{-6} M at pH 5, since the concentration dependence of conductivity is quadratic.

At pH = pK_1 the dimer density has a maximum value equal to

$$[HA_2^-]_{\text{max}} = C_T^2/(4K_2).$$

The corresponding maximum conductivity is

$$G_{\text{lim}} = e^2 DC_T^2/8kT\delta K_2. \tag{8}$$

Up to this point we have been assuming that dimer formation is taking place in the bulk of aqueous phase, outside the unstirred layers. When this restriction is lifted, the

conductivity limit (Eq. 8) should be increased by a factor of two. This is a consequence of the equilibrium between dimers and their components HA and A^-. The factor of two is related to the existence of two parallel fluxes, one of HA and one of A^- in the unstirred layers (9). The conductivity limit corrected for the dimer formation within the unstirred layers, is therefore, given by:

$$G_{\text{lim}} = e^2 D C_T^2 / 4kT\delta K_2. \tag{9}$$

We will now use this equation to evaluate the magnitude of conductivity and compare it with the experimental data. The lower limit of K_2 can be obtained from the requirement of the existence of the quadratic dependence of conductivity; as follows from Eq. 7 the condition $[HA_2^-] \ll C_T$ is equivalent to $K_2 \gg C_T$. For $K_2 > 10^{-6}$ M, $D = 5 \times 10^{-6}$ cm^2/s, which is half of the diffusion constant of sodium ions in water (32), $\delta = 0.2$ mm (33), $C_T = 1.5 \times 10^{-6}$ M, the expected conductivity limit $G_{\text{lim}} < 5.4 \times 10^{-7}$ mho/cm^2. This value is lower by a factor of 20 as compared with the experimental data (Fig. 2, curve pH 5) at the same PCP concentration. This indicates that the above mechanism of PCP-induced membrane conductivity, namely, formation of dimers in the aqueous phase with subsequent partition into the membrane and release on the other side, does not occur.

Membrane Potentials and Current-Voltage Characteristics

Membrane potentials of PC-cholesterol membranes generated under the two following sets of condition were measured: (*a*) the solutions separated by the membrane have different concentrations of PCP but the same pH and (*b*) the solutions have the same PCP concentrations but different pH. In the former case the potentials developed were small ($\simeq$10 mV or less) and erratic. In the second case the membrane potentials were reproducible, and their magnitude and polarity corresponded to that calculated from the pH gradients. The apparent proton permeability implied by such agreement and the absence of significant membrane potentials under condition *a* have also been noted for other uncouplers (6). This must be a consequence of high membrane permeability of neutral molecules and ionization reactions in the aqueous phase (15).

In separate experiments we added PCP solution into both cell compartments after membrane thinning had been completed, and observed the delay and rise time of conductivity for pH between 5 and 8. We have found that both the delay and rise time become longer at higher pH. This suggests that the membrane has to adsorb a sufficient amount of neutral PCP molecules to become conductive. The existence of such effect is consistent with the observation of relatively high partition coefficient (1.9×10^3: 1) of neutral PCP molecules between the hydrocarbon phase and the aqueous phase. This supports the conclusion drawn from the membrane potential measurements, namely, that the PCP molecules are highly permeable in the membrane.

It is a generally accepted notion that if the ion transport is limited by the membrane and not by diffusion through unstirred layers or by a slow interface process, the

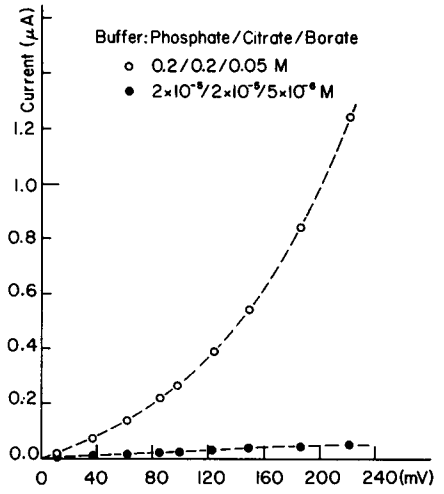

FIGURE 6

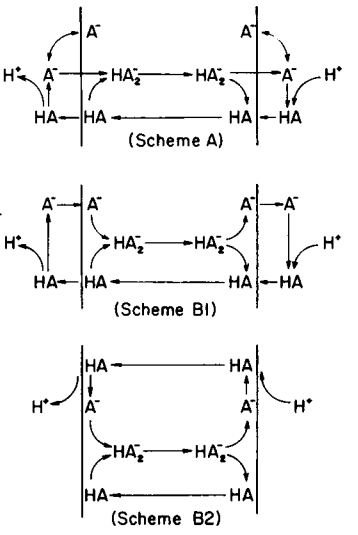

FIGURE 7

FIGURE 6 The effect of buffer strength on the current-voltage characteristics of PC-cholesterol membranes. PCP 1.0×10^{-4} M, KCl 0.1 M, and pH 5.25.

FIGURE 7 Schemes of charge transport across the membrane and of dimer formation processes at the membrane-water interface. Scheme A: dimers are assumed to be formed by recombination of PCP anions from the aqueous phase with neutral PCP molecules located at the membrane interface. Schemes B1 and B2: dimers are assumed to be formed within the membrane surface.

current-voltage characteristics of the membrane are superlinear (34). This type of *I-V* characteristics has been also observed for PCP for all three phospholipids, under the condition that the buffer concentration was of the order of 10^{-1} M. A typical *I-V* characteristic curve obtained under "normal" conditions is given in Fig. 7 (upper curve). The curvature was found to be essentially independent of pH; the current reaches steady state within 1 ms, and the conductivity is practically independent of the rate of stirring. In contrast, the properties of the membrane system at very low buffer concentrations are quite different. The steady-state conductivity is considerably lower, and the current-voltage characteristics saturates. After the application of a voltage step the transient current reveals $t^{-1/2}$ dependence, which is indicative of the development of the diffusion polarization regime (35). This result suggests that in the vicinity of the membrane the charge transport is maintained by protons.

The Scheme of Charge Transport

The experiments on concentration and pH dependence of membrane conductivity have indicated that both forms of PCP, neutral and anionic, are involved in the membrane conduction. In addition, it has been found that the experimental value of conductivity is greater than that expected from the model based on dimer formation in the aqueous phase. Thus none of the dimer components is being transported from one of the cell

compartments to the other. In order to maintain the observed level of membrane conductivity one has to assume that there must exist a return flow of the dimer components. The results of membrane potential measurements (supported by partition coefficient determination and the rise time experiments) show that the membrane permeability of neutral PCP molecules must be high (15). We have also found, from the conductivity measurements at high pH, that the flux of PCP anions across the membrane is insignificant.

Based on the foregoing conclusions, the transport model we use has to have the following characteristics. As there must be a return flow of dimer components which are unlikely to be anions, we assume that only a return flow of neutral PCP molecules is present in the membrane. In addition, the scheme of charge transport across the membrane should not contradict the observation that proton transport takes place in the vicinity of the membrane. There are several schemes of transport which appear at first sight to be consistent with the above criteria. These are depicted in Fig. 7. They have been considered recently by Neumcke and Bamberg (9), Borisova et al. (12), and by Cohen et al. (36) in their analysis of membrane conduction due to TTFB. All schemes have in common the existence of charged dimers as the current carrying species inside the membrane and proton fluxes outside. They differ, however, in the mode of dimer formation and in the type of charge transfer across the interface. According to scheme A, the dimer formation takes place by a heterogeneous process. In contrast, in scheme B1 and B2 dimers are formed by recombination of the neutral and the dissociated PCP molecules both located within the membrane surface. The difference between B1 and B2 is only in the type of charges crossing the interface, they are either PCP anions (scheme B1) or protons (scheme B2).

We will now briefly compare the membrane conductivity characteristics predicted by the above two schemes A and B of dimer formation. Since under typical experimental conditions we have not observed any significant limitation of conductivity due to some interface process, we will consider the interface at equilibrium. The distribution of neutral PCP molecules (HA) and PCP anions (A^-) between membrane and water is assumed to be given by:

$$[HA]_{im} = (\beta_{HA}) \frac{N_F}{N_T} [HA]_{ia}, \tag{10}$$

$$[A^-]_{im} = (\beta_{A^-}) \frac{N_F}{N_T} [A^-]_{ia}, \tag{11}$$

where N_F is the density of the free binding sites and N_T is the density of the total binding sites at the membrane surface; β_{HA} and β_{A^-} are the partition coefficients; $[HA]_{ia}$ and $[A^-]_{ia}$ are the concentrations of HA and A^- species at the membrane interface in the aqueous phase, and $[HA]_{im}$ and $[A^-]_{im}$ are the corresponding densities at the membrane surface.

The formation of dimers takes place either by a heterogeneous process (scheme A) according to:

$$[HA_2^-]_{im} = K_2^A[A^-]_{ia}[HA]_{im}, \tag{12A}$$

or within the membrane surface layer (schemes B1 and B2)

$$[HA_2^-]_{im} = K_2^B[A^-]_{im}[HA]_{im}. \tag{12B}$$

Due to finite density of binding sites at the membrane surface, N_T, we require that:

$$N_T = N_F + [A^-]_{im} + [HA]_{im} + [HA_2^-]_{im}. \tag{13}$$

The negatively charged species A_{im}^- and HA_{2im}^- produce an electric field at the interface which affects the ion distribution. As a result, the aqueous concentration $[A^-]_{ia}$ at the interface will be lower compared with that in the bulk according to the expression:

$$[A^-]_{ia} = [A^-]_o \exp(e\psi/kT), \tag{14}$$

where ψ is the surface potential ($\psi < 0$), and $[A^-]_o$ is the concentration in the bulk aqueous phase. The electrostatic repulsion influences the density of dimers at the membrane surface directly, by changing the local density $[A^-]_{ia}$, as well as indirectly, through a change in the types of species which are occupying the binding sites. To maintain the models simple, we will not include the effect of repulsive interaction between the negative species adsorbed at the membrane surface on the adsorption and dimer formation processes; such an extension would make β_A^- and K_2 also dependent on ψ. We further assume that distribution of neutral molecules is not affected by the surface potential, i.e.,

$$[HA]_{ia} = [HA]_o. \tag{15}$$

The bulk concentrations, which appear in Eqs. 14 and 15, are obtained from the dissociation equilibrium equations

$$[A_o^-] = (C_T K_1/[H]_o)/(1 + K_1/[H]_o), \tag{16}$$

$$[HA]_o = C_T/(1 + K_1/[H]_o). \tag{17}$$

$[H]_o$ is the proton bulk activity, and K_1 is the dissociation constant defined by reaction 1.

The surface potential ψ must satisfy the following condition:

$$-e\{[A^-]_{im} + [HA_2^-]_{im}\} + \sigma_m = (2kT\epsilon/e\lambda)\sinh(e\psi/2kT), \tag{18}$$

which follows from the solution of Poisson's equation for a simple electrolyte. σ_m is the membrane surface charge associated with phospholipid head groups, ϵ is the dielectric constant of the aqueous phase, and λ is the Debye length.

We have numerically solved the above system of equations for a large variation of parameters. By making the assumption that the membrane conductivity is proportional to the dimer density one can compare the predictions for these two models with the experimental data. The major qualitative difference is that the heterogeneous

process A does not predict the saturation of conductivity at high PCP concentration unless the binding sites are filled with dimers. The numerical calculations show that for any particular set of the parameters, PCP concentration, partition coefficients, and density of the binding sites N_T, the saturation of conductivity occurs only at a specific value of the dissociation constant K_2. In contrast, the scheme B can reproduce the conductivity saturation regardless how small the dimer dissociation constant K_2 is. In addition, the schemes B1 and B2 predict different conductivity saturation levels at different pH, a feature indicated by the experimental data, without any severe restrictions on the parameters. Thus, in view of the above properties, it appears that in the case of PCP the formation of charged, membrane permeable species takes place within the membrane surface layer rather than by the heterogeneous process. Unfortunately, the available experimental data do not allow any differentiation between schemes B1 and B2. Perhaps more insight into this problem will be gained from the studies of pH shifts at the membrane-water interfaces at various pH and buffer strength.

The deviation of the position of the conductivity maximum from the pK value of PCP and its dependence on the type of phospholipids and cholesterol content can be related to different proton activity and different energetics of dissociation (neutral molecules and anions are stabilized differently at the membrane surface). Since the neutral PCP molecules partition very strongly into the nonpolar medium, it is to be expected that their density within the region of polar head groups will be higher than in the bulk of aqueous solution. In addition, due to the negative surface charge (exposed phosphate groups and adsorbed negative ions) the local proton activity is probably higher. Under these circumstances the first dissociation constant K_1 at the membrane water interface is expected to be shifted toward higher pH. The observation of largest pH shifts of the conductivity maximum for negatively charged PG membranes (Fig. 1) and smaller shifts for cholesterol-rich membranes is in agreement with this hypothesis.

Conclusion

As follows from the scheme of membrane transport selected on the basis of the present experimental results, one hydrogen atom is being transported across the membrane as a part of the neutral PCP molecule in one direction while simultaneously a unit of one electronic charge is being transported in the opposite direction. The net result is equivalent to a transfer of one proton. Thus, if the biological activity of PCP is related to the movement of protons across the membrane, it should reflect the characteristic features of membrane conduction. The present work provides such information on the apparent proton transfer across the membrane induced by PCP, and its dependence, among other factors, on PCP concentration, pH, lipid composition, and cholesterol content.

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