Biochimica et Biophysica Acta 902 (1987) 109–127
Elsevier

BBA 73626

Dielectric properties of adsorption / ionization site of pentachlorophenol in lipid membranes

Pavel Smejtek, Arthur W. Barstad and Kwan Hsu

Environmental Sciences and Resources Doctoral Program and Department of Physics, Portland State University, Portland, OR 97207 (U.S.A.)

(Received 2 February 1987)

Key words: Pentachlorophenol toxicity; Phospholipid bilayer; Solvatochromic effect; Dielectric property; Pesticide–membrane interaction; Cholesterol

The results of three complementary studies focused on characterization of the local environment of the common pesticide pentachlorophenol (PCP) adsorbed to phosphatidylcholine (PC) and phosphatidylglycerol (PG) membranes are reported. The effect of cholesterol (Chol) was examined. These studies included: (1) Measurements of solvatochromic shifts of the ultraviolet absorption spectra of PCP in membranes and in polar non-hydrogen-bonding (a red shift) and hydrogen-bonding (a blue shift) solvents. Pi-pi* transition energies were analyzed in terms of the dielectric cavity models of (a) Onsager, (b) Block-Walker, which includes dielectric saturation, and (c) a soft dipole model of Suppan, which accounts for PCP's polarizability. The estimates of dielectric constant of the PCP adsorption site yielded 8.1–8.7 for the PC and 16.8–20.1 for PG membranes. Solvatochromic effects indicate hydrogen bonding between the membrane-bound ionized PCP molecule and water, which is enhanced by the presence of cholesterol. (2) Determinations of the pK_a of PCP adsorbed to PC, PG, PC/Chol, PG/Chol membranes and dissolved in dioxane-water solutions of a known dielectric constant. The pK_a value of PCP adsorbed to membranes was always greater than the standard pK_a value and it increased with the membrane's negative charge. The pK_a value sequence in 0.1 M KCl was 6.68 (PG), 6.32 (PG/Chol = 70:30 mole fractions), 5.97 (PC), and 5.75 (PC/Chol = 70:30). The intrinsic pK_a values of PCP in membranes were 5.2–5.4 (PG) and 5.5–6.0 (PC). Estimates of the dielectric constant of PCP's ionization site in membranes yielded 10–22 (PC) and 27–37 (PG). Cholesterol facilitated the release of the hydrogen ion from membrane-bound PCP. (3) Measurements of pH dependence of PCP-induced membrane electrical conductivity. pH values of conductivity maxima were always greater than the standard pK_a of PCP, and their sequence corresponded to that of the pK_a values of membrane-bound PCP. The anomalous properties of PCP as a Class 2 uncoupler are due to PCP's lipophilic character. In response to a low dielectric constant of the adsorption/ionization site, the physicochemical characteristics of PCP adsorbed to membranes are different from the standard values – a fact that needs to be taken into account in the development of models of PCP's toxicity.

List of symbols

W	= work of charging an ion	ϵ	= relative dielectric constant
		ϵ_0	= dielectric permittivity of vacuum
		b	= molecular or ionic radius
		p	= molecular dipole moment, ground state
		$p^\star$	= molecular dipole moment, excited state

Correspondence: P. Smejtek, Environmental Sciences and Resources Doctoral Program and Department of Physics, Portland State University, Portland, OR 97207, U.S.A.

V_d = electric potential difference across dipole layer
R = reaction field in the center of cavity created by the polarized medium
$f_O(\epsilon)$ = Onsager dielectric function
$f_{BW}(\epsilon)$ = Block-Walker dielectric function
$f_S(\epsilon)$ = soft dipole (Suppan) dielectric function
E_{tr} = pi–pi* transition energy of PCP
d = characteristic distance – a parameter in Block-Walker dielectric saturation model
α = electronic polarizability of solute
n = index of refraction
χ = scaling factor from Claussius-Mossotti equation
$\tilde{\pi}^*$ = medium polarity parameter in linear solvation energy model
$\tilde{\alpha}$ = hydrogen bond donating parameter
$\tilde{\beta}$ = hydrogen bond accepting parameter
$K_a(\,)$ = ionization constant
$pK_a(\,)$ = $-\log K_a(\,)$

Arguments in parentheses:
s = bulk solution
app = apparent
int = intrinsic

$[A^-]$ = volume density or concentration of ionized PCP in bulk phase
[HA] = volume density or concentration of neutral PCP in bulk phase

---

Subscripts:
s = bulk phase (solution)
w = water
if = membrane/water interface

$(A^-)_m$ = membrane surface density of ionized PCP
$(HA)_m$ = membrane surface density of neutral PCP
kT = thermal energy
V_{if} = electric potential difference between the membrane/water interface and bulk aqueous phase
q = elementary electric charge
v_i = valence of ion type i
C_i = volume density of ions type i
ΔG = work of transfer of proton from a standard state in water into a standard state in medium of dielectric constant ϵ

Introduction

This paper is concerned with the problem of the membrane toxicity of pentachlorophenol (PCP), specifically the local characteristics of PCP's adsorption site on phospholipid bilayers and the effect of the membrane environment on the physicochemical properties of membrane-bound PCP.

Pentachlorophenol, due to its antimicrobial activity, has been a widely used chemical compound in agriculture and industry. The primary use of PCP and its sodium salt has been in lumber mills for wood preservation. In the 1970's, PCP was the second most widely used pesticide in the United States and Canada [1,2], with a worldwide production of 280000 tons per year [3]. Due to its extensive use and the presence of other toxic impurities in the technical grade of PCP, concerns about PCP's presence of ecosystems have been raised. PCP is one of the contaminants of ground water [4,5], soils [6,7], and cattle – due to their contact with PCP-treated wood [8]. PCP is absorbed through the skin and digestive and respiratory systems and, due to its extensive past usage, PCP is present in the general population [9–11]. It was shown that PCP is also a product of the metabolic conversion of other pesticides, such as hexachlorobenzene and pentachloronitrobenzene [12,13], which provides the explanation as to why PCP was found in humans in countries where PCP use is low [14]. Van Ommen et al. [15] showed that PCP is the primary metabolic product of hexachlorobenzene due to the action of cytochrome *P*-450.

The biological response to PCP is rapid, which is typical for PCP-induced membrane toxicity [16]. It is now generally accepted that the major mode of PCP's toxicity is due to the uncoupling of the ATP synthesis from the electron transport [3,17]. In lipid bilayers, PCP induces electrical conduction [18–21] which is related to transmembrane proton translocation and the uncoupling effect [22]. It was shown that PCP's toxicity and induced membrane conductivity are related: the onset of

PCP toxicity, measured by the rate of carbon fixation in an alga, occurred at the measurable onset of PCP's adsorption to and loss of resistance of an egg phosphatidylcholine/cholesterol lipid bilayer membrane [23].

The ubiquitous presence of PCP in the environment as well as man stimulates the need to understand the molecular basis of PCP's toxic action. The present work addresses some questions about PCP–membrane interaction, which is seen as of a dual nature: On the one hand, PCP alters the membrane physical characteristics of membranes, loss of membrane function as hydrogen ion permeability barrier, and development of negative interfacial potential due to adsorption of ionized PCP. On the other hand, the membrane as a medium alters the physical properties of membrane-bound PCP, such as the PCP dissociation constant, which determines the distribution of the neutral and ionized forms of PCP in the membrane, and, subsequently, the membrane activity of PCP. This work deals with the second aspect of PCP–membrane interaction.

We are concerned with the dielectric and hydrogen bonding characteristics of the PCP adsorption site in membranes. The aspect of hydrogen bonding of membrane-bound PCP is relevant for the understanding of the mechanisms and kinetics of PCP-mediated proton translocation and the action of uncouplers in membranes [22,24,25] and the existence of localized proton transfer pathways in membranes [26]. The significance of the magnitude of the local dielectric constant for the energetics of membrane processes involving charged molecules can be illustrated by two examples: (1) The work of charging a sphere of radius b in a medium of dielectric constant ϵ, which can be related to the dissociation of membrane-bound PCP, is

$$W = \frac{q^2}{8\pi\epsilon\epsilon_0 b} \tag{1}$$

For a singly charged molecule of radius 0.2 nm, the work is either 360 meV or 52 meV in a medium with a dielectric constant of 10 or 70, respectively. This amount of work can be compared with the average thermal energy (25 meV at room temperature) and 170–350 meV of energy released on the transfer of typical lipophilic ions from water into the membrane core [27]. (2) Dipolar molecules, when adsorbed to membranes, become oriented [28–30] and change the electric potential difference between the membrane core and the aqueous solution, which is manifested by the change of the membrane's ionic permeability. This electric potential difference depends on the normal component of the dipole $p_\perp$, the surface density of dipoles N, and the dielectric constant ϵ,

$$V_d = \frac{Np_\perp}{\epsilon\epsilon_0} \tag{2}$$

For a $p_\perp$ of 2 debye and an interdipole separation of 1 nm, the electric potential difference across such a layer would be 75 mV or 22 mV if the dielectric constant were 10 or 70. In the former case, the ionic permeability would change by a factor of 20, whereas in the latter case only by a factor of 1.5. Furthermore, membrane is a very inhomogeneous dielectric, partly permeated by water [31] and, thus, knowledge of the dielectric constant at the membrane adsorption plane is needed for the development of predictive models of the adsorption of membrane-active molecules such as PCP. Flewelling and Hubbell [27] developed a simple and powerful total potential energy model for the phosphatidylcholine membrane which can reproduce the membrane's thermodynamic parameters for binding and translocation of tetraphenylphosphonium$^+$ and tetraphenylborate$^-$ ions. The model includes a dielectric transition region, Born, interfacial polarization, dipolar layer, and nonelectrostatic energy components. In the model the dielectric interface was located, on the basis of an 'educated guess,' in a membrane region with a dielectric constant of 10. These illustrations point out the need for experimentally determined local dielectric constants for the understanding and modeling of membrane processes, and, in the present case, for the characterization of the adsorption site of a toxic molecule in the lipid matrix of membranes.

In this paper we describe (1) the use of the solvatochromic shifts of the ultraviolet absorption spectra of PCP adsorbed to membranes for the characterization of the local environment of membrane-bound PCP, (2) the measurements of the

distribution of the neutral and ionized forms of membrane-bound PCP, from which we determine the apparent and intrinsic pK_a values of membrane-bound PCP, and show the effect of the membrane's surface charge and cholesterol on PCP dissociation, and (3) the comparison of the pK_a values of PCP obtained from the spectroscopic studies with the pH values of membrane conductivity maxima determined from electrical measurements on neutral and negatively charged planar membranes. The pH positions of the membrane conductivity maxima were shifted from the standard pK_a value of PCP, and the origin of those pH shifts was not understood. The results also provide support for the kinetic scheme of PCP-mediated proton transport in which the ionized PCP molecules are located within the interfacial region of lipid membranes and interact strongly with the aqueous phase.

The theoretical background for the interpretation of solvatochromic shifts of the ultraviolet-absorption bands of PCP

This study of the PCP-adsorption sites on membranes was based on the comparison of the pi-pi* transition energies of long-wavelength absorption bands of PCP adsorbed to membranes and PCP dissolved in model solvents. In order to develop a conceptual framework for the interpretation of experimental results, we outline a 'prototype Onsager model', describing the electrostatics of the problem of dipolar interaction between the solute molecule and its polar environment in simple terms. We consider this model to be a tool for the understanding of media effects that determine not only the solvatochromic shifts of PCP but other physicochemical properties as well.

Due to the absence of knowledge of charge distribution in the solute molecule and in the local environment, we will follow Onsager's concept of dipolar interactions [32] and will represent the solute molecule by a point dipole p located in a cavity of molecular radius b (Fig. 1). Inside the cavity the dielectric constant is assumed to be unity, whereas outside the cavity the dielectric constant is that of the medium. Thus, in the prototype model there is an abrupt, stepwise change of medium properties. For such a configuration, the distribution of electric potential in the space surrounding the dipole $V(r,\theta)$ is obtained by solving Laplace's equation, $\Delta V = 0$, for conventional boundary conditions $V(r,\theta)$ at $b-0 = V(r,\theta)$ at $b+0$ and dV/dr at $b-0 = dV/dr$ at $b+0$.

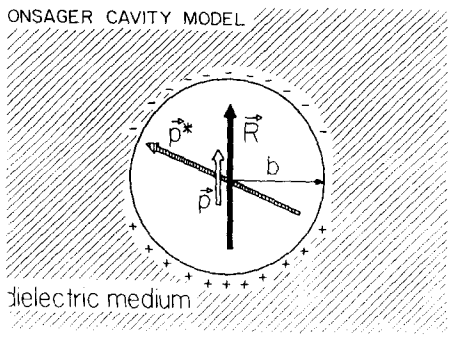

Fig. 1. Onsager cavity model of polar media depicting solute molecule represented by a point dipole p in the center of the cavity embedded in a medium of dielectric constant ϵ. b is the cavity radius and R the reaction field set up by the polarized medium.

In response to the dipole, the polarized medium sets up an electric field R, the so-called reaction field, given by

$$\vec{R} = \vec{p} f_O(\epsilon)/4\pi\epsilon_0 b^3 \tag{3}$$

where f_O, often called the Onsager dielectric function, is equal to

$$f_O(\epsilon) = 2(\epsilon - 1)/(2\epsilon + 1) \tag{4}$$

The term f_O can be regarded as a primitive measure of medium polarity. Other functions and a treatment of the electrostatic effects associated with membranes are presented in a review by Warshel and Russell [33].

The energy of the interaction of a solute molecule in a polar medium is $-\vec{p}\cdot\vec{R}$. If the electron distribution in the solute molecule in its ground and excited states is given by dipole moments $\vec{p}$ and $\vec{p}^*$, the energy of transition associated with the absorption of a photon is

$$E_{tr} = E_{tr\,ref} - (\vec{p}^* - \vec{p})\vec{R} = E_{tr\,ref} - (\vec{p}^* - \vec{p})\vec{p} f_O(\epsilon)/4\pi\epsilon_0 b^3 \tag{5}$$

where $E_{tr\,ref}$ corresponds to transition energy in some reference state.

We expect two types of solvatochromic shifts to occur on the transfer of a solute molecule from one to another, more polar medium: (a) a red shift if $\vec{p}(\vec{p}^{\star} - \vec{p}) > 0$, i.e., when the excited state of the solute is stabilized to a greater degree by the reaction field of the more polar medium compared to the ground state, and (b) a blue shift if $(\vec{p}^{\star} - \vec{p})\vec{p} < 0$. The latter case corresponds to greater stabilization of the ground state in the more polar medium. Both types of shifts have been observed in our studies, depending on the type of medium.

More elaborate Onsager-type models have been proposed [33], and here we will include two extensions of the prototype model:

(1) A model of Block and Walker [34] that includes local dielectric saturation in the vicinity of the solute molecule (BW model) since recent calculations of Kakitani and Mataga [35] suggested the existence of dielectric saturation in the first coordination shell. The BW model is attractive because the expression for the reaction field and the transition energy have the same form as that for the prototype Onsager model. Only the dielectric function is of different form, viz.,

$$f_{BW}(\epsilon) = 3\epsilon \ln \epsilon/(\epsilon \ln \epsilon - \epsilon + 1) - 6/\ln \epsilon - 2 \tag{6}$$

The qualitative difference between the BW and the prototype model is that the stepwise change of the dielectric constant at the edge of the Onsager cavity has been replaced by a transition region. The radial dependence of the dielectric constant is given by

$$\epsilon'(r) = \epsilon \cdot \exp(-d/r) \tag{7}$$

The characteristic distance d depends both on the medium dielectric constant and the cavity radius.

$$d = b \cdot \ln \epsilon \tag{8}$$

(2) A soft solute dipole which, in contrast to the prototype model, includes polarizability of the solute molecule. Suppan [36] has shown that a simple expression for the solvatochromic shifts can be obtained if the polarizability, α, is included in the reaction field according to

$$\vec{R} = (\vec{p} + \alpha\vec{R}) f_O(\epsilon)/4\pi\epsilon_0 b^3 \tag{9}$$

In the soft dipole model the polarizability of a solute molecule was related to the index of refraction of the solute n according to the Claussius-Mossotti equation.

$$\alpha/4\pi\epsilon_0 b^3 = (n^2 - 1)/(n^2 + 2) = \chi \tag{10}$$

The typical values of the scaling factor χ are $1/4$ and $1/3$ for the aliphatic and aromatic compounds, respectively. The reaction field according to the soft dipole model is

$$\vec{R} = (\vec{p}/4\pi\epsilon_0 b^3)(f_O(\epsilon)/(1 - \chi f_O(\epsilon))) \tag{11}$$

and the transition energy in this case also retains the Onsager-like form.

$$E_{trS} = E_{tr\,ref} - (\vec{p}^{\star} - \vec{p})\vec{p} f_S(\epsilon)/4\pi\epsilon_0 b^3 \tag{12}$$

where

$$f_S(\epsilon) = (f_O(\epsilon)/(1 - \chi f_O(\epsilon)))(1 + \chi f_O(\epsilon)/(1 - \chi f_O(\epsilon))) \tag{13}$$

We assume that the problem of dipolar interaction between the PCP molecule and the membrane can be regarded as a special case of solute–solvent interactions so that other approaches developed in physical organic chemistry can also be explored [37]. Among them is the concept of linear solvation energy relationships [38–43] which has the potential to be an excellent tool for the unraveling of the multiple solvent effects of molecules adsorbed to membranes. It was experimentally verified that some specific physicochemical parameters denoted as XYZ, such as solvatochromic shifts, can be expressed in terms of some reference values XYZ_0 and a set of empirical parameters representing nonspecific and specific solute–solvent interactions [44]. Three dominating interactions are of present concern: nonspecific, such as dipolar, interactions represented by a polarity parameter $\tilde{\pi}^{\star}$, and specific, such as hydrogen bond donating functions (acidities) represented by parameter $\tilde{\alpha}$, and hydrogen bond accepting functions (basicities) represented by parameter $\tilde{\beta}$,

$$XYZ = XYZ_0 = \tilde{s}\tilde{\pi}^{\star} + \tilde{a}\tilde{\alpha} + \tilde{b}\tilde{\beta} \tag{14}$$

where the coefficients $\tilde{s}$, $\tilde{a}$, and $\tilde{b}$ are regarded as susceptibilities. A comprehensive collection of up-

dated parameters can be found in Kamlet et al. [44].

In the following we report on the solvatochromic shifts of PCP in two series of model solvents: (a) polar non-hydrogen-bonding, and (b) polar hydrogen-bonding, and we show how the results can be used to calibrate the properties of the PCP adsorption site on membranes.

Materials and Methods

Methyl acetate, potassium phosphate dibasic trihydrate, boric acid (Mallinckrodt Chemicals, St. Louis, MO), potassium chloride, glacial acetic acid, chloroform, methanol (Baker Chemicals, Phillipsburg, NJ), potassium citrate monohydrate, anhydrous sodium acetate (EM Science, Cherry Hill, NJ), 1,4-dioxane, ethyl acetate, heptane, acetonitrile (Burdick and Jackson, Muskegon, ME) were of reagent grade or better. 1-Octanol, 1,2-dichloroethane, proprionitrile, hexadecane, pentachlorophenol (PCP) (Aldrich, Milwaukee, WI), phosphatidylcholine (PC), phosphatidylglycerol (PG) (Avanti Polar Lipids, Birmingham, AL) were at least 99% pure. Tetramethylammonium chloride (TMACl) (Eastman Kodak Co., Rochester, NY) was 98% pure with 2% water. Ethanol (Commercial Solvent Corp., Agnew, CA) was USP grade. Propylene carbonate (EM Science) purity was 98. All materials were used without further purification except: Water was purified using a Millipore Milli-Q reagent grade water apparatus. Cholesterol (Chol) (Em Science) was triply recrystallized from methanol. Recrystallized tetramethylammonium pentachlorophenolate (TMAPCP) was a gift of Dr. Alfred Levinson of the PSU Chemistry Department.

Ultraviolet absorption spectra of PCP were recorded over the wavelength range 200–450 nm on a Beckman Model Du-7HS UV/VIS spectrophotometer, interfaced to a DEC PCP-11/03 minicomputer used for data collection.

pK_a determination of PCP in dioxane-water solutions

For the determination of PCP ionization constants in mixtures of dioxane and water, a method similar to one reported earlier [45] was used. The solutions were prepared by mixing weighed amounts of dioxane and water at four concentrations from 20% to 80% by weight dioxane. Acetate buffer was used to control pH. Concentrations of acetate were 200-times that of PCP to minimize the effect of added PCP on the pH of the solutions. PCP was dissolved in solutions of acetic acid and sodium acetate to obtain reference spectra. 1 to 1 mole ratio mixtures of acetic acid to sodium acetate were used to obtain spectra with both HA and A^- present. Total buffer concentration was kept at 0.02 M. A sample blank was prepared for each solution containing buffer but no PCP. Aliquots of each PCP and blank solution were added to sample and reference UV cells and the spectrum was taken. To check the sensitivity of the method in determining a pK_a value for PCP at a pH far from the expected pK_a of PCP, a 5 to 1 buffer mixture was used in one of the dioxane-water mixtures. The pK_a of PCP determined for this buffer mixture was within 0.02 pK_a units of the value for the corresponding 1 to 1 buffer mixture. To calculate the pH of the solutions used, values for the ionization constants of acetic acid and water were interpolated from published data (Table 15-6-2A, [46]).

pK_a determination of PCP adsorbed on lipid membranes

For the determination of PCP's pK_a on unilamellar lipid vesicles, weighed amounts of PC, PG, and when needed, Chol, were dissolved in about 50 ml of chloroform. A Buchler Instruments flash evaporator was used to deposit a thin film of lipid in a round bottom flask (20–30 min). Residual solvent was removed by flushing the flask with dry nitrogen for several minutes. A solution containing buffer B-2 (0.02 M phosphate, 0.02 M citrate, and 0.005 M borate) and either 0.1 M KCl or 0.1 M TMACl was added and the mixture shaken under nitrogen until the lipid was suspended (5–10 min). This sample, containing 10 mg/ml lipid, was sonicated using a Branson Model S-75 sonifier at full power under dry nitrogen in an ice bath until the solution was as clear as possible (20–30 min). Aliquots of the vesicle suspension were then titrated to the desired pH and diluted to a final concentration of 5 mg/ml vesicles. pH values were chosen at 2–2.2, 8–9.5, and five intermediate pH values near the pK_a of

PCP on vesicles. Aliquots of concentrated buffer and salt solutions were added prior to titration to assure uniform buffer and salt concentrations in the final solutions. The pH values of the samples were recorded and aliquots added to sample and reference UV cells. A small amount ($< 0.1\%$) of PCP in methanol or pure methanol (reference) was slowly added to a cell while stirring vigorously for several minutes and the spectrum was taken. The PCP concentration was kept at 100–200 μM.

We have verified that the lipid content in the suspensions of lipid vesicles was sufficiently high so that the error due to the absorbance of PCP in the aqueous phase was negligible.

Spectral decomposition

Spectral decomposition was used to analyze all data for ionization constants. A DEC PDP-11/23 minicomputer with graphics capabilities was used to obtain the concentrations of the neutral (HA) and ionized (A^-) forms of PCP in solutions or adsorbed to the membranes. The high and low pH spectra of PCP were used to decompose the intermediate pH spectra. Absorbance values at 80 wavelengths (270 nm to 350 nm) were typically used in the analysis.

The pK_a of PCP was obtained by a method of chi-squared [47]. The decomposition was monitored visually as a check on the overall fitting. To improve the accuracy of the decomposition it was necessary to correct for the background absorbance due to light scattering by vesicles, compounded by the phenomenon of lipid vesicle swelling in the presence of PCP. This was accomplished by averaging the absorbance in a range of wavelengths where PCP does not absorb (400–450 nm), and subtracting this value from all absorbance data in the wavelength range of interest before analysis.

pH dependence of PCP-induced membrane conductivity

Steady-state electrical conductivity of planar egg phosphatidylcholine/cholesterol/decane and phosphatidylglycerol/cholesterol/decane membranes was measured by a conventional method [19]. Two levels of cholesterol, 20 and about 80 mole percent, were used. The conductance data were obtained by extrapolating the voltage dependent membrane conductance to applied voltage equal to zero. Aqueous solution contained 0.1 M potassium chloride and phosphate-citrate-borate (0.2 M/0.2 M/0.05 M) buffer.

Results and Discussion

The ultraviolet absorption spectra of pentachlorophenol in solvents and membranes

The long wavelength ultraviolet absorption bands of the neutral (HA) and the negatively charged PCP molecules (A^-) (structures shown in Fig. 2) in various solvents are depicted in Figs. 3a and 3b. These bands correspond to pi–pi* transitions, and the changes of the position of the absorption bands indicate the magnitude of the solvatochromic effects. As it follows from the results, the solvatochromic shifts for the ionized form of PCP are about one order of magnitude greater than those for the neutral form. Thus only the solvatochromic shifts of the ionized form provide information on the local environment. It is also important to note that the shapes of absorption bands of PCP adsorbed to membranes and those for the bulk solvents remained the same, with the exception of proprionitrile and propylene carbonate. The different shapes of the absorption bands in the latter two solvents are assumed to be due to different intermolecular interaction and these two solvents will be excluded from further analysis.

Two types of solvents have been chosen to gauge the solvatochromic effect of PCP adsorbed to membranes: (1) aprotic solvents, to estimate the effect of polarization interactions, and (2) hydrogen-bonding solvents, to estimate the effect of hydrogen bonding. In hydrogen-bonding solvents, in addition to nonspecific dipolar interactions, changes of electron distribution are to be expected

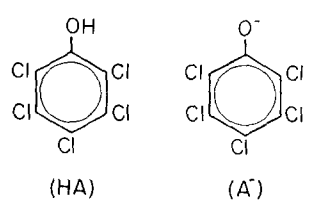

Fig. 2. Molecular structure of pentachlorophenol; HA denotes the neutral molecule and A^- the negatively charged ion.

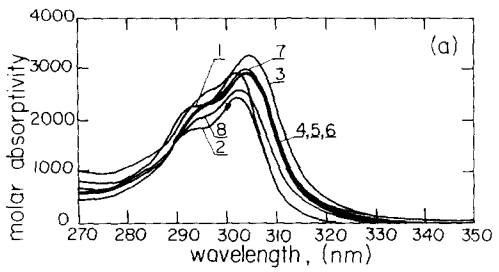

Fig. 3. (a) Long wavelength ultraviolet absorption band of the neutral molecule of pentachlorophenol in several typical solvents and adsorbed to membranes. 1, PCP in heptane; 2, PCP in hexadecane; 3, PCP adsorbed to PC vesicles at pH 2.10; 4, PCP adsorbed to PG vesicles at pH 2.10; 5, PCP in ethanol; 6, PCP in methanol; 7, PCP in 80.2% dioxane-water; 8, PCP in 20.8% dioxane-water.

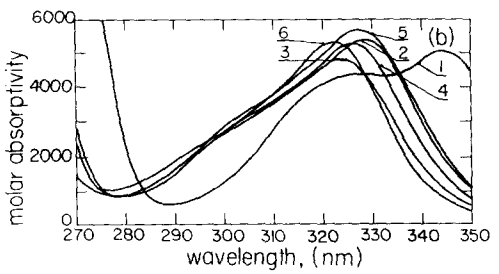

Fig. 3. (b) Long wavelength ultraviolet absorption band of the negatively charged pentachlorphenol molecule in several typical solvents and adsorbed to membranes. 1, TMAPCP in acetonitrile; 2, PCP adsorbed to PC vesicles at pH 7.92; 3, PCP adsorbed to PG vesicles at pH 8.38; 4, TMAPCP in ethanol; 5, TMAPCP in 80.2% dioxane-water; 6, TMAPCP in 20.8% dioxane-water.

in both the neutral and the ionized PCP molecules. The neutral PCP molecule acts as proton donor, and the negatively charged pentachlorophenolate as proton acceptor.

A complete set of the results of studies of solvatochromic shifts, the positions of the long-wavelength ultraviolet absorption bands of the neutral and the ionized forms of PCP in all solvents and membranes, is summarized in Tables Ia and Ib.

As we have noted earlier, if the excited state of the solute molecule is stabilized to a greater degree by the medium, a red shift will be observed with increasing polarity of the medium. This type of solvent effect has been observed for non-hydrogen-bonding media. Results for the 320–340 nm absorption band of ionized PCP are in Table Ib.

In contrast, in hydrogen-bonding solvents the effect of polarity is opposite. A blue shift and higher transition energies have been obtained for the transfer of pentachlorophenolate to more polar hydrogen-bonding media (Table Ib). Since the blue shift of PCP adsorbed to negatively charged membranes may be due to an electrochromic effect associated with the negatively charged polar head groups of PG, we have attempted to reduce the surface charge density of PG membranes by the adsorption of calcium. These studies were not successful because the suspensions of liposomes became opaque at elevated calcium concentrations (< 10 mM).

We have analyzed the transition energy of a pentachlorophenolate ion in solvents in terms of the four models mentioned earlier. Three were dielectric cavity models and one was based on linear solvation energy relationships. The cavity models included (1) Onsager, (2) Block-Walker, and (3) soft dipole. The models predict a linear relationship between the transition energy and the respective dielectric function: (1) $f_O(\epsilon)$, (2) $f_{BW}(\epsilon)$,

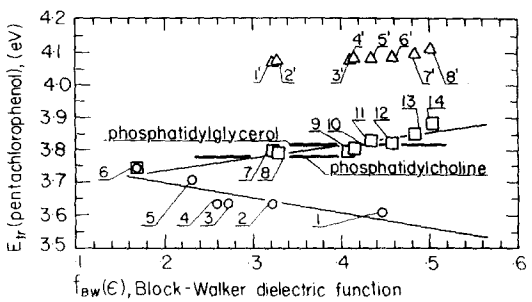

Fig. 4. Solvatochromic effect of long wavelength ultraviolet absorption spectra of PCP bound to membranes and in model solvents. The plot is of the pi-pi* transition energy versus the Block-Walker dielectric function. The lower sets of data give the transition energy of ionized PCP in non-hydrogen-bonding (negative slope) and hydrogen-bonding (positive slope) solvents. The horizontal lines give the transition energy observed for pentachlorophenolate adsorbed to neutral phosphatidylcholine membranes and negatively charged phosphatidylglycerol membranes. Note that they intersect the plot for hydrogen-bonding solvents. The upper set of data is that of neutral PCP in selected solvents. 1, TMAPCP in acetonitrile; 2, TMAPCP in 1,2-dichloroethane; 3, TMAPCP in methylacetate; 4, TMAPCP in ethylacetate; 5, TMAPCP in chloroform; 6, TMAPCP in dry phosphatidylcholine film; 7, TMAPCP in 1-octanol; 8, PCP in 80.2% dioxane-water; 9, TMAPCP in ethanol; 10, PCP in 60.4% dioxane-water; 11, TMAPCP in methanol; 12, PCP in 40.6% dioxane-water; 13, PCP in 20.8% dioxane-water; 14, PCP in 0.2 M TMACl, B-2, pH 8.0.

and $f_S(\epsilon)$, given by Eqns. 4, 6, and 13, respectively. From the data, E_{tr} versus the respective dielectric function, linear least-square fit parameters were obtained for each model and each group of solvents. Experimental E_{tr} for tetramethylammonium pentachlorophenolate in dry lipid film, representing the low polarity end point, was used in both data groups. Due to limitations in obtaining empirical parameters, the linear solvation energy relationship (Eqn. 14) was used only for non-hydrogen-bonding solvents ($\tilde{\alpha} = \tilde{\beta} = 0$). The performance of all models was about the same, and, since the Block-Walker model appeared to fit the data for hydrogen-bonding solvents better, we present a summary, given in Fig. 4, for the two groups of solvents in terms of the Block-Walker function $f_{BW}(\epsilon)$.

As it follows from Eqns. 5 and 12, the negative slope for the non-hydrogen-bonding solvents indicate (a) that the dipolar interactions dominate in non-hydrogen-bonding solvents, (b) that the dipole moment of the excited state of ionized pentachlorophenol is greater compared to that of the ground state, and (c) that the direction of the dipole moment in the excited state of pentachlorophenolate coincides with the direction of the reaction field, information useful for studies of media effects on electron density distribution in pentachlorphenol.

The positive slope of the least-square fit line (Fig. 4) indicates that in the case of hydrogen-bonding solvents it is the ground state of pentachlorophenolate which is stabilized. The stabilization is due to the formation of the hydrogen bond: the negatively charged pentachlorophenolate attracts a proton and the resulting electron redistribution in the ionized molecule, (A^-), approaches that of the neutral PCP molecule (HA). The stronger the hydrogen bond, the greater the sta-

TABLE Ia

ULTRAVIOLET ABSORPTION MAXIMA OF NEUTRAL PCP IN VARIOUS MEDIA

Medium	Dielectric constant	Wavelength (nm)	E_{tr} (eV)
Acetonitrile	37.5 (20)	302.5	4.099
Heptane	1.92 (20)	302.0	4.106
Hexadecane		302.5	4.099
Phosphatidylcholine dry film	3	305.2	4.062
Phosphatidylcholine vesicles	unknown	304.8	4.068
Phosphatidylcholine/ cholesterol (70/30) vesicles	unknown	304.8	4.068
Phosphatidylglycerol vesicles	unknown	304.5	4.072
Phosphatidylglycerol/ cholesterol (70/30) vesicles	unknown	304.8	4.068
Water	78.39 (25)	301.5	4.112
Methanol	32.70 (25)	303.8	4.081
Ethanol	24.55 (25)	304.2	4.076
1-Octanol	10.34 (20	304.5	4.072
80.2% Dioxane/water	10.88 (20)	304.0	4.078
60.4% Dioxane/water	26.26 (20)	303.8	4.081
40.6% Dioxane/water	43.65 (20)	303.5	4.085
20.8% Dioxane/water	61.65 (20)	302.8	4.095

TABLE Ib

ULTRAVIOLET ABSORPTION MAXIMA OF IONIZED PCP IN VARIOUS MEDIA

Medium	Dielectric constant	Wavelength (nm)	E_{tr} (ev)
Acetonitrile	37.5 (20)	343.8	3.606
Proprionitrile	27.2 (20)	330.5 [a]	3.751
1,2-Dichloroethane	10.36 (25)	341.2	3.634
Chloroform	4.81 (20)	335.0	3.701
Ethylacetate	6.02 (25)	341.5	3.631
Methylacetate	6.68 (25)	341.2	3.636
Propylenecarbonate	65.1 (25)	334.0 [a]	3.712
Phosphatidylcholine dry film	3	331.5	3.740
Phosphatidylcholine vesicles	unknown	328.8	3.771
Phosphatidylcholine/ cholesterol (70/30) vesicles	unknown	328.0	3.780
Phosphatidylglycerol vesicles	unknown	325.5	3.809
Phosphatidylglycerol cholesterol (70/30) vesicles	unknown	324.5	3.821
Water	78.39 (25)	319.5	3.881
Methanol	32.70 (25)	323.8	3.829
Ethanol	24.55 (25)	326.5	3.797
1-Octanol	10.34 (20)	326.5	3.797
80.2% Dioxane/water	10.88 (20)	327.2	3.789
60.4% Dioxane/water	26.26 (20)	326.0	3.803
40.6% Dioxane/water	43.65 (20)	324.5	3.821
20.8% Dioxane/water	61.65 (20)	322.2	3.848

[a] Anomalous peak shape.

bilization of the ground state and the greater the transition energy and the blue shift.

In Fig. 4 we compare the results obtained on pure PC and PG membranes with those for the calibrating solvents, for both the ionized and neutral forms of pentachlorophenol. The upper set of data represents the transition energy of the neutral molecule in hydrogen-bonding solvents. As it follows from the results, only a weak solvent effect has been detected, presumably because of the formation of the internal hydrogen bond with chlorine atoms [48]. For the pentachlorphenolate, there is a distinct energy gap between the upper and the lower sets of data that represents the effect of hydrogen bond formation. The horizontal lines in Fig. 4 give the transition energy of pentachlorophenolate on the phosphatidylcholine (lower line) and the phosphatidylglycerol (upper line) membranes. It follows that (1) the energy of transition of pentachlorophenolate adsorbed to both types of membranes is above the range of energies found for all non-hydrogen-bonding solvents. These results indicate without a doubt the presence of hydrogen-bonding interactions between the membrane-bound pentachlorophenol and the aqueous phase; (2) the hydrogen bonding interaction is stronger for pentachlorophenolate bound to the negatively charged (PG) membranes when compared to neutral (PC) membranes; and (3) presence of cholesterol in both PC and PG membranes enhanced the hydrogen bonding effect (blue shift, Table Ib).

The 'effective' dielectric constant of the adsorption site of ionized PCP in membranes was estimated from the intercept of the least-squares fit line and the horizontal line corresponding to the transition energy of pentachlorophenolate adsorbed to phosphatidylcholine and phosphatidylglycerol membranes. These estimates were obtained for the prototype Onsager, Block-Walker, and the soft dipole models; in the latter one, the scaling factors χ (Eqn. 10) of 1/4 and 1/3 were used. The range of dielectric constants obtained was 8.1–8.7 for the phosphatidylcholine and 16.8–20.1 for the phosphatidylglycerol membranes.

The above values of the dielectric constants can be compared with those of Kimura and Ikegami [49], who determined the local dielectric constant of synthetic dipalmitoyl- and distearoylphosphatidylcholine and bovine phosphatidylserine from shifts of the fluorescence spectra of dansylated phosphatidylethanolamine incorporated in the host membrane and calibrating solvents similar to ours. Their results were interpreted in terms of the Onsager dielectric function and they found that, above the phase transition of lipids, the dielectric constant of the interfacial region for both types of membrane was about 34, whereas, below the phase transition temperature, it decreased below 10, a value similar to ours found for the neutral phosphatidylcholine membranes. The experiments with fluorescent probes indicate substantial molecular rearrangements at the membrane interface associated with changes of the depth of insertion of dansylated PE. In the present work, the membrane active molecule itself provides information on its location in the membrane and it would be desirable to extend the present studies to find out how the properties of the PCP adsorption site change when membrane lipids undergo phase transition.

Dissociation of pentachlorophenol bound to membranes, dielectric and membrane charge effects

The process of dissociation of PCP

$$HA + H_2O \rightleftharpoons A^- + H_2O^+$$

is ionogenic, and for that reason sensitive to electrostatic effects. The work of the charging of an ion generated in the dissociation process increases with the decrease of the dielectric constant of the medium. Eqn. 1 provides a basis for the expectation that the release of a proton from membrane-bound PCP molecules will be affected by the local dielectric constant.

In this study, the pK_a values of PCP were experimentally determined according to

$$pK_a = pH - \log\left(\frac{[A^-]}{[HA]}\right) \tag{15}$$

where the ratio of concentrations of the ionized and the neutral forms was obtained by a numerical decomposition of the ultraviolet absorption spectrum of PCP adsorbed to liposomes into absorbances of the A^- and HA components.

A simple electrostatic theory of media effects [50.51] predicts an inverse relationship between

the pK_a and the dielectric constant of the medium

$$pK_a(2) = pK_a(1) + \text{const.}(1/\epsilon_2 - 1/\epsilon_1) \quad (16)$$

In order to test the applicability of simple electrostatic concepts to PCP dissociation, we first measured the pK_a values of PCP in a dioxane-water solution whose dielectric constant was set by the dioxane content. The results are shown in Fig. 5 where we plot the experimental pK_a values against the reciprocal of the dielectric constant of the solutions. The correction for the increased water activity coefficient at higher dioxane concentrations [52] did not appreciably increase the pK_a values in highly concentrated dioxane solutions, where some deviations from Eqn. 16 can be seen.

The results in Fig. 5 indicate that (a) dissociation of PCP is dominated by nonspecific electrostatic interactions, and the pK_a determined by the dielectric properties of the local environment, and (b) the magnitude of the dielectric effect is sufficiently large so that conclusions can be drawn about the properties of the PCP dissociation site in membranes from the pK_a shifts.

In Fig. 6 we present typical ultraviolet absorption spectra of PCP adsorbed to sonicated phosphatidylcholine and phosphatidylglycerol liposomes. The absorbance due to the neutral (HA) and the ionized (A^-) pentachlorphenol was obtained by numerical decomposition of the measured spectrum by means of the least-squares fit method. The total spectrum reconstructed from the components is also shown. The pK_a values obtained from these studies are summarized in Table II. There are several prominent features of the results: (1) In all cases the pK_a of membrane-bound PCP was found to be greater than the standard, aqueous pK_a; (2) pK_a values of PCP bound to membranes prepared from the negatively charged phosphatidylglycerol were greater than those for the electrically neutral phosphatidylcholine; and (3) the presence of cholesterol in a membrane facilitated PCP dissociation.

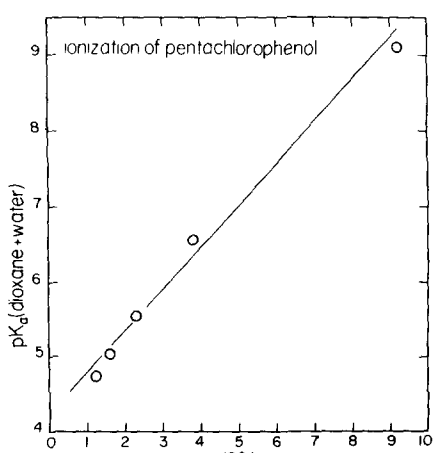

Fig. 5. Dissociation of PC in dioxane-water solution demonstrating the reciprocal relationship between the pK_a and the dielectric constant of the medium.

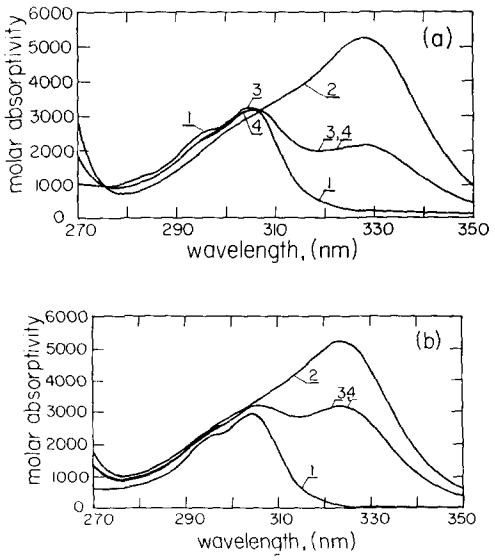

Fig. 6. Absorption spectra of PCP adsorbed to membranes at intermediate pH. (a) Phosphatidylcholine, 0.1 M KCl, 214 μM PCP. 1, vesicles at pH 2.10; 2, vesicles at pH 8.36; 3, vesicles at pH 5.70; 4, reconstructed spectrum of vesicles at pH 5.70. (b) Phosphatidylglycerol, 0.1 M KCl, 214 μM PCP. 1, vesicles at pH 2.06; 2, vesicles at pH 8.50; 3, vesicles at pH 6.88; 4, reconstructed spectrum of vesicles at pH 6.88.

For the purpose of further discussion, we define three types of dissociation constants. For the dissociation process in bulk solvents, such as dioxane-water solutions, the common definition applies

$$K_a(s) = [A^-]_s[H_3O^+]_s/[HA]_s \quad (17)$$

The apparent dissociation constant of membrane-bound PCP will relate the surface activities of A^- and HA with the activity of protons in the

TABLE II

DISSOCIATION OF MEMBRANE-BOUND PENTACHLOROPHENOL, EFFECT OF MEMBRANE CHARGE

Membrane	Aqueous solution (M)	pK_a(app)
Phosphatidylcholine	0.1 KCl	5.97 ± 0.07
Phosphatidylcholine + 30 mole % cholesterol	0.1 KCl	5.75 ± 0.03
Phosphatidylglycerol	0.1 TMACl	6.78 ± 0.01
Phosphatidylglycerol	0.1 KCl	6.68 ± 0.04
Phosphatidylglycerol + 30 mole % cholesterol	0.1 KCl	6.32 ± 0.07

bulk aqueous phase of the liposome suspension.

$$K_a(\text{app}) = (A^-)_m[H_3O^+]_w/(HA)_m \quad (18)$$

The round parentheses denote the surface related quantities and the square brackets the volume quantities. Thus the pK_a values shown in Table II are the apparent pK_a values since bulk pH was used in their determinations.

The intrinsic dissociation constant of a membrane-bound acid-base system takes into account the activity of hydrogen ions at the aqueous part of the interface, which may be different from that in the bulk.

$$K_a(\text{int}) = (A^-)_m[H_3O^+]_{if}/(HA)_m \quad (19)$$

We further assume that the distribution of protons between the membrane-water interface and the bulk aqueous phase is determined by a Boltzmann factor according to

$$[H_3O^+]_{if} = [H_3O^+]_w \cdot \exp(-qV_{if}/kT) \quad (20)$$

where V_{if} is the electric potential at the interface.

The intrinsic and apparent dissociation constant are related to each other, and their relationship includes the effect of a charged interface via the dependence of the interfacial activity of protons on the local electric potential

$$pK_a(\text{app}) = pK_a(\text{int}) - \log(e) \cdot qV_{if}/kT \quad (21)$$

Grahame's equation, which proved to be successful in the interpretation of the electrophoretic mobility data of liposomes and in studies of the adsorption of ions on membranes [53,54], is expected to be applicable in the present studies as well. It provides the relationship between the membrane charge density σ and the interfacial potential V_{if}

$$\sigma = (V_{if}/|V_{if}|)\left\{2kT\epsilon\epsilon_0 \sum_i [C_i]_w [\exp(-qV_{if}/kT) - 1]\right\}^{1/2} \quad (22)$$

The equation shows how the reduction of membrane surface charge density, either by the dilution of charged lipids, phosphatidylglycerol, by the addition of cholesterol, or by the adsorption of oppositely charged ions, affects the interfacial potential V_{if} that controls the activity of hydrogen ions at the interface.

Eqns. 21 and 22 predict an increase of the value of the apparent pK_a at a surface with the increase of the density of the negative charge. This theoretical expectation was confirmed by the experimental results. Apparent pK_a values and the change with the zeta potentials was determined from electrophoretic mobility studies under comparable conditions (Tables II and III). The largest pK_a was observed for phosphatidylglycerol membranes in 0.1 M solution of tetramethylammonium chloride followed by that for 0.1 M potassium chloride and cholesterol containing PG membranes. The pK_a values decrease as the zeta potential becomes less negative. A small but significant difference for the pK_a values of membrane-bound PCP was

TABLE III

ELECTROPHORETIC ZETA POTENTIAL AND INTRINSIC pK_a OF MEMBRANE-BOUND PENTACHLOROPHENOL

Membrane	Aqueous solution (M)	Zeta potential (mV)	pK_a(int)
Phosphatidylglycerol	0.1 TMACl	-91 [a]	5.2
Phosphatidylglycerol	0.1 KCl	-73 [a]	5.4
Phosphatidylglycerol	0.1 KCl	-0.8 [b,c]	5.9
Phosphatidylglycerol	0.1 KCl	-25 [c]	5.5

[a] Eisenberg, M. et al. [44].
[b] McLaughlin, A. et al. [65].
[c] Smejtek, P. et al. [57].

found for tetramethylammonium chloride and potassium chloride solutions. Tetramethylammonium ions are of special significance in membrane studies as their adsorption constant, even to negatively charged membranes, is practically zero whereas that for potassium is measurable [55]. In the presence of potassium, due to its adsorption, the membrane potential is less negative, which is manifested by a lower value of pK_a. The primary effect of cholesterol is the dilution of the negatively charged lipids, also resulting in a decrease of interfacial potential (Eqn. 22).

The lowest pK_a values of membrane-bound PCP were found for membranes prepared from the electrically neutral lipids. It is interesting that cholesterol was also active in neutral membranes. It decreased the pK_a value, i.e., it enhanced the ionization of PCP. This small effect may be related to a recent observation demonstrating that the presence of cholesterol in bilayers enhanced the transfer of protons [56].

As the next step, we use the observed values of pK_a values in combination with the available zeta potential data and estimate the intrinsic pK_a values of PCP bound to the two types of membranes by means of Eqn. 21. It has been established [55] that the zeta potential of 0.1 M salt (1 : 1) corresponds to the value of the electric potential at the plane of the hydrodynamic shear located 0.2 nm in front of the 'membrane surface'. In the absence of any information about the dissociation site of PCP in membranes, we assume that the interfacial potential determining the hydrogen ion activity at the membrane surface (Eqn. 20) is equal to the zeta potential obtained from the studies of electrophoretic mobility. It appears to be an acceptable assumption. The dissociation of membrane-bound PCP is a heterogeneous process whose energetics are determined by the final state corresponding to a solvated proton. The assumption $V_{if} = V_z$ is equivalent to stating that a proton released from the membrane is in a final solvation state at a distance of 1–2 layers of water in front of the membrane.

The intrinsic pK_a values obtained from Eqn. 21 are also given in Table III. The following considerations for zeta potentials were applied. Since, in phosphatidylglycerol membranes, each lipid molecule contributes one negative charge to the membrane surface, membrane charge density in the presence of PCP was assumed to be very similar to that for pure membranes and the published values of the zeta potential were used. For membranes prepared from phosphatidylcholine, we used a zeta potential of zero as an upper limit corresponding to the absence of PCP, and -25 mV as the lower limit. The latter corresponds to the saturation of the adsorption isotherm for PC membranes [57]. The ranges of intrinsic pK_a values of membrane-bound PCP obtained in this way are as follows: $pK_a(\text{int}) = 5.2–5.4$ for phosphatidylglycerol and $pK_a(\text{int}) \approx 5.5–6.0$ for the phosphatidylcholine membranes. The $pK_a(\text{int})$ values for the phosphatidylglycerol membranes turned out to be closer to the standard, aqueous pK_a – an observation suggesting that the dissociation plane of PCP in phosphatidylglycerol membranes is closer to the aqueous phase compared to that in phosphatidylcholine membranes. This conclusion is consistent with the observed blue shift of the ultraviolet absorption band of PCP adsorbed to the phosphatidylglycerol membranes discussed in the previous section.

The pK_a values of PCP obtained in dioxane-water solutions cannot be directly used for the determination of the dielectric constant of the PCP dissociation site in membranes. The fundamental difference between the dissociation processes of molecules dissolved in dioxane-water solutions and molecules adsorbed on membranes is the existence of the membrane-water boundary. In the case of the dioxane-water solution, the final state of the solvated proton is in the dioxane-water system, whereas in the case of liposomes, the final state of a proton released from the membrane is the aqueous phase.

It follows (consider Eqns. 17 and 19) that for the identical degrees of dissociation in the dioxane-water solution and membranes, i.e., $[A^-]_s/[HA]_s = (A^-)_m/(HA)_m$, the ratio of bulk and intrinsic dissociation constants is

$$K_a(s)/K_a'(\text{int}) = \exp(-\Delta G/RT) \tag{23}$$

where ΔG is the work of the transfer of a proton from a standard state in water into a standard state in a dioxane-water solution of dielectric constant ϵ. The primed quantity represents, then, a

dissociation constant in a medium with a dielectric constant ϵ as if the membrane/water interface were present [58]. In this way, we can obtain the primed intrinsic pK_a, given by

$$pK_a'(\text{int}) = pK_a(\text{s}) - \log(\text{e}) \cdot \Delta G/RT \qquad (24)$$

The term $pK_a'(\text{int})$ will be further used to calibrate the experimental intrinsic pK_a values obtained for membranes (Table III).

Fernandez and Fromherz [58] have also shown that pK_a shifts of identical magnitude for ionogenic and non-ionogenic dissociation processes can be obtained and accounted for by approximating the work of charging by that of transfer of hydrochloric acid between water and the dioxane-water solution. We have adopted this procedure, and the results are shown in Fig. 7.

The upper curve of Fig. 7 gives the raw experimental results, the dependence of the pK_a of PCP in a dioxane-water solution versus the dielectric constant of the medium. The lower curve gives the $pK_a'(\text{int})$ obtained from Eqn. 24. It represents the intrinsic dissociation process in a dielectric medium as if the dielectric medium-water interface were present. For the computation of $pK_a'(\text{int})$ we used the data for the free energy of the transfer of hydrochloric acid from water into a dioxane-water solution of Schwabe and Schwenke, compiled in a review by Bates [51].

The intrinsic pK_a values for membrane-bound PCP given in Table III can be compared with the

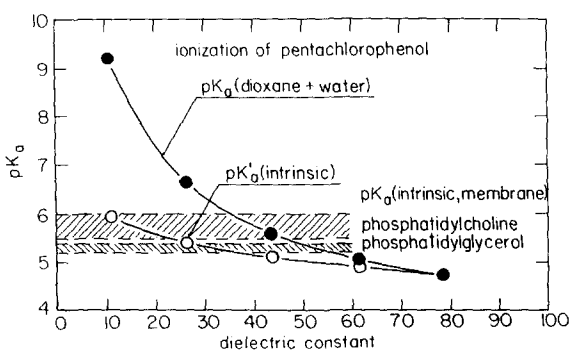

Fig. 7. Dependence of pK_a of pentachlorophenol on dielectric constant of the dioxane-water solution. The upper set of data gives the uncorrected, observed values. The lower set corresponds to $pK_a'(\text{int})$ computed according to Eqn. 24. It includes the correction for the transfer of protons across the interface between water and a medium of a given dielectric constant.

predicted pK_a values derived from the dioxane-water data and shown as the lower curve in Fig. 7. From this comparison, it follows that the dielectric constant of the dissociation site of PCP in the phosphatidylcholine membranes is within the range of 10–22 and in phosphatidylglycerol membranes in the range of 27–37. The curve $pK_a'(\text{int})$ versus ϵ is rather shallow, and more accurate values for the membranes are needed for a more precise determination of the dielectric constant of the dissociation site. Our results can be compared with the value of 32 obtained for fluorescent lipid pH indicators incorporated into micelles of sodium dodecylsulfate (SDS) and polyoxyethylene isooctyl phenyl ether (Triton X-100) [58].

In summary, we have shown in this section that a simple electrostatic model of the membrane/water interface satisfactorily explains the dissociation properties of PCP bound to phosphatidylcholine (electrically neutral) and phosphatidylglycerol (negatively charged) membranes. We have established two electrostatic effects which determine the dissociation of membrane-bound pentachlorophenol. The increased stability of the neutral form of PCP is due to (1) the lower dielectric constant of the PCP dissociation site in the membrane and (2) the effect of a negative membrane surface charge. One can associate pK_a shifts with these two processes, $\Delta pK_a(\epsilon)$ for the dielectric effect and $\Delta pK_a(V_{if}) = -\log(\text{e}) \cdot q \cdot V_z/kT$ for the enhancement of hydrogen ion density at the negatively charged membrane-water interface. These two quantities can be used to obtain the apparent pK_a

$$pK_a(\text{app}) = pK_a(\text{aqueous}) + \Delta pK_a(\epsilon) - \log(\text{e}) \cdot qV_z/kT \qquad (25)$$

a quantity of interest in biological and toxicity studies.

The effect of lipid composition of the membrane on the pH dependence of PCP-induced conductivity

The effect of the hydrogen ion concentration in the aqueous solution on the electrical conductivity of lipid bilayer membranes is shown in Fig. 8.

The conductivity of membranes prepared from negatively charged phosphatidylglycerol was lower than for those prepared from the neutral phos-

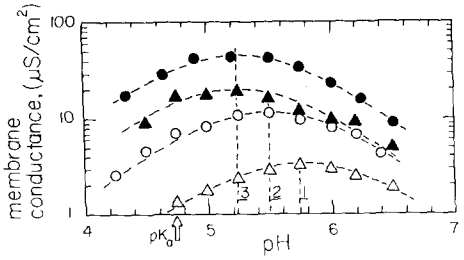

Fig. 8. Effect of pH on pentachlorophenol-induced electrical conductivity in lipid bilayer membranes. Open and closed symbols correspond to low and high cholesterol content in membrane forming solution. Circles, phosphatidylcholine (PC) containing membranes; triangles, phosphatidylglycerol (PG) containing membranes. The largest pH shift of conductivity maximum from the standard pK_a of PCP ($pH_{max\ 1}$) was observed from PG/Chol (80:20) membrane; $pH_{max\ 2}$ corresponds PC/Chol (80:20) membrane, and smallest shift ($pH_{max\ 3}$) to PC and PG membranes with the highest cholesterol content, about 80 mole percent.

phatidylcholine. Moreover, and what is more pertinent to the present work, is that (1) the conductivity maxima of all membranes studied occurred at a pH greater than the standard pK_a value of PCP (4.7–4.8) [19,59], and (2) the sequence of the pH values of the membrane conductivity maxima corresponded to that found for pK_a values of membrane-bound-PCP (Table II), viz., the pH values of the conductivity maxima of membranes having low cholesterol content were always higher than those having a high content of cholesterol. In fact, the conductivity maxima $pH_{max\ 1}$ and $pH_{max\ 2}$ (Fig. 8) for low cholesterol phosphatidylglycerol and phosphatidylcholine membranes were at 5.75 and 5.5, and the corresponding pK_a a values (Table II) were 6.32 and 5.75. The conductivity maxima of both types of membranes with a high level of cholesterol (about 80 mole %) were found at pH 5.25. In the latter case, the membrane environment of adsorbed PCP is dominated by cholesterol so that similar pH values of the conductivity maxima were to be expected, and the experimental results confirmed such an expectation.

The pH values of the membrane conductivity maxima in Fig. 8 cannot be directly compared with the pK_a values given in Table II because of the different ionic strengths used in these two types of studies. The conductivity of PCP-treated membranes had to be measured in highly buffered solutions in order to minimize the diffusion polarization artifacts. The consequence is that at high ionic strength the membrane charge is effectively screened (Eqn. 22), which results in a lower interfacial potential and a smaller shift of the apparent pK_a from the standard, aqueous value. The screening of a negatively charged surface decreases the excess of hydrogen ions at the interface and, subsequently, pK_a. For this reason, the pH value of the membrane conductivity maxima were lower than the data given in Table II.

The interfacial potentials of membranes in conductivity studies (Fig. 8) can be estimated according to Eqn. 25, using the dissociation data. It follows that

$$V_{if}(2) = V_{if}(1) + \frac{kT}{q} \cdot \ln(10) \cdot [pK_a(app,2) - pK_a(app,1)] \tag{26}$$

Using −73 mV as a reference for the pure phosphatidylglycerol membranes and an apparent pK_a = 6.68 (Table II), the position of $pH_{max\ 1}$ (Fig. 8) for the low-cholesterol membrane corresponds to −19 mV and that for the high-cholesterol PG membrane to −5 mV. The magnitude of the pH shifts and the above estimates of interfacial potential indicate that (a) the sensitivity of the pH shifts of conductivity related to membrane surface potential are sufficiently high to be observed in conventional membrane conductivity studies, and (b) PCP molecules adsorbed in membranes are effectively screened, as expected.

The combination of the results of membrane conductivity and PCP dissociation studies demonstrates that the transmembrane proton transfer characteristics are determined not by the distribution of the neutral and the ionized PCP in the aqueous phase, but by the dissociation characteristics of PCP adsorbed to membranes. This observation is of some significance for the interpretation of PCP-induced membrane conductivity and protonophoretic activity in membranes.

Pentachlorophenol-induced conductivity in lipid bilayers is very likely due to the presence of the molecular complex AHA^-, formed by the recombination of the HA and A^- species of PCP [19,22,60]. Results of earlier conductivity studies [19–21] are consistent with the kinetic scheme illustrated in Fig. 9. This molecular scheme de-

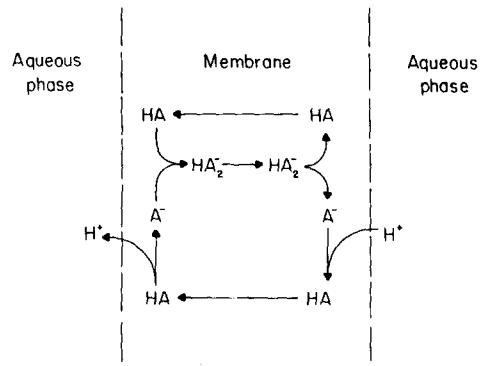

Fig. 9. Kinetic scheme of pentachlorophenol-induced electrical conductivity of lipid membranes. HA denotes the neutral, A^- the ionized PCP molecule, and AHA^- the membrane permeable complex. One kinetic cycle transfers one proton across the membrane.

picts the mechanism of PCP-induced conductivity in lipid bilayers: the interfacial hydrogen ion transfer process, the formation of and the diffusion of the membrane permeable complex across the membrane, and the return flow of the neutral molecules effecting the translation of protons in the direction of lower free energy. It also shows how the mechanisms of electrical conductivity and transmembrane proton transfer are linked. It then follows that the conductance is proportional to the product of the surface densities (A^-) and (HA), resulting in the pH-dependence of conductivity given by

$$G \propto \frac{y}{(1+y)^2} \quad \text{where} \quad y = 10^{(pH - pK_a)}$$

The conductivity has a maximum at $y = 1$, i.e., $pH_{max} = pK_a$.

Pentachlorophenol has been regarded as an irregular uncoupler because the pH of the conductivity maximum did not occur at the standard value of pK_a, as was the case for other Class 2 uncouplers 2 uncouplers [22], and the origin of the pH shift was not understood. In this study we have demonstrated that the pH values corresponding to the membrane conductivity maxima are determined by the dissociation of membrane-bound PCP and not by some kinetic effects related to the coupling of charge movement across the membrane and the accompanying chemical reactions.

Summary and Conclusions

The physical properties of the PCP adsorption/ionization site in membranes are one factor in determining the molecular basis of PCP's toxic activity – the loss of the membrane's function as a hydrogen ion permeability barrier. Studies of three related effects have been correlated: (1) solvatochromic shifts of the ultraviolet absorption spectra of PCP adsorbed to membranes; (2) membrane-lipid-dependent changes of PCP dissociation properties, and (3) membrane-lipid-dependent pH shifts of PCP-induced membrane conductivity.

Polar non-hydrogen-bonding and hydrogen-bonding solvents were used to define the dielectric and hydrogen-bonding properties of ionized PCP adsorbed to membranes. A gap in the long wavelength pi-pi* transition energy of ionized PCP between the non-hydrogen-bonding and hydrogen-bonding media was observed. It was found that the adsorption sites in both phosphatidylcholine and phosphatidylglycerol membranes belong to the group of hydrogen-bonding media. In addition, the absorption band of pentachlorophenolate in phosphatidylglycerol membranes was blue shifted compared to that for phosphatidylcholine, indicating stronger interaction with the aqueous phase.

The transition energies of pentachlorophenolate were analyzed in terms of the Onsager, Block-Walker, and soft dipole dielectric models, and the linear solvation energy relationship. The range of the dielectric constants of the adsorption site of PCP was estimated to be 8.1–8.7 for phosphatidylcholine and 16.8–20.1 for the negatively charged phosphatidylglycerol membranes.

The results of dissociation studies of PCP adsorbed to membranes indicated that (1) the membrane environment significantly contributes to the stabilization of neutral PCP, (2) the apparent pK_a of PCP adsorbed to negatively charged phosphatidylglycerol membranes was greater than that for phosphatidylcholine membranes, and (3) the presence of cholesterol in the membranes facilitates the dissociation of PCP.

It was shown that two components, (1) the dielectric, and (2) one related to membrane zeta potential, contribute to the shift of the apparent

pK_a of PCP from the standard aqueous value.

The dependence of the pK_a of PCP on the dielectric constant of the medium was demonstrated by using a water-dioxane solution of defined dielectric constant. These results, after correcting for the work of the transfer of protons across the membrane/water interface, were used to estimate the dielectric constant of PCP's dissociation site. A range of dielectric constants was obtained from the PCP dissociation studies: 10–22 for phosphatidylcholine membranes, and 27–37 for phosphatidylglycerol membranes.

It follows that the mid-range values of the dielectric constants estimated from the dissociation studies are about 80% higher than those obtained from the solvatochromic shifts. It is, however, important to realize that information about the local properties of the PCP adsorption/ionization site in membranes was obtained from two very different processes: changes of the electronic transition energy in the case of solvatochromic effects, and, in the case of the dissociation study, from the changes of the energetics of proton release.

A reviewer of this paper made an interesting proposal to rationalize the difference between the values of the dielectric constant obtained from the solvatochromic effects and the pK_a determination; that is, ϵ (solvatochromic) $< \epsilon$ (proton release). The proposal assumes the existence of a sharp dielectric constant gradient at the PCP adsorption plane. The solvatochromic effects are associated with the transition of pi electrons and therefore reflect the average dielectric constants of the ring region, whereas the pK_a values are determined primarily by the polar region in the vicinity of phenol oxygen

Studies of PCP-induced membrane conductivity revealed that the lipid composition of the membrane determined the pH dependence of PCP-induced membrane conductivity and that the pH shifts of membrane conductivity maxima correlated with the shifts of apparent pK_a values determined in the dissociation studies of PCP adsorbed to membranes. The results of conductivity studies provide support for the applicability of the kinetic scheme of membrane conductivity and net transmembrane transfer of protons proposed for Class 2 uncouplers.

The results of all three studies concerned with the effect of cholesterol in PCP-treated membranes:

(1) a blue shift of the ultraviolet absorption spectrum of pentachlorophenolate bound to both PC and PG membranes,

(2) an increase of the ionization constant of PCP, and

(3) occurrence of the membrane conductivity maximum at a lower pH value in membranes prepared from electrically neutral lipids,

indicated that cholesterol enhanced hydrogen bonding interaction between membrane-bound PCP and its local environment. One possible explanation is that PCP is located in membranes in the vicinity of cholesterol molecules and that cholesterol, in turn, introduces a 'defect' in the membrane's surface which facilitates the penetration of water manifested by the above-mentioned three effects.

The reason why PCP acts in membranes as an 'irregular' uncoupler is due to a rather low value of the dielectric constant at the PCP adsorption/ionization site. Results of both studies reported here point toward this conclusion.

It is the existence of the dielectric pK_a shift that causes the notable 'irregular' behavior of PCP in membranes. Auxiliary data are consistent with the above interpretation: PCP is known to be a highly lipophilic compound with a partition coefficient between octanol and water of neutral PCP on the order of $1.0 \cdot 10^5$ [61]. It is probable that PCP, in contrast to 'regular' Class 2 uncoulers, such as DTFB, TTFB [62,63], and 2,4-dinitrophenol [64], is adsorbed in a deeper, less polar, region of the lipid bilayer which causes larger dielectric pK_a shifts.

The combination of the results of our three studies illustrate the dual nature of interaction between PCP and a lipid membrane:

(1) PCP modifies the physical characteristics of lipid membranes by inducing electrical conductivity and proton permeability – the common origin of PCP's uncoupling activity.

(2) The low polarity membrane environment at the PCP adsorption/ionization site and the membrane surface charge alter the physicochemical characteristics of PCP with respect to standard values referenced to the aqueous medium.

The membrane-induced alteration of the physicochemical properties of membrane-active compounds, such as the pesticide PCP, should be included in the interpretation of membrane-related toxic effects.

Acknowledgement

We would like to thank Dr. A. Levinson of the PSU Department of Chemistry for his help in preparing tetramethylammonium pentachlorophenolate, Dr. M. Takeo of the PSU Department of Physics for discussions of dielectric cavity models, the reviewer for constructive comments, and Susan Iles for skillful assistance with the preparation of the manuscript. This research was supported by a Grant No. 5R01 ES 00937 from the National Institutes of Health. This is Environmental Sciences and Resources Program Publication No. 198.

References

1 Cirelli, D.P. (1978) in Pentachlorophenol, Chemistry, Pharmacology, and Environmental Toxicity (Rao, K.R., ed.), pp. 13–18, Plenum Press, New York
2 Hoos, R.A.W. (1978) in Pentachlorophenol, Chemistry, Pharmacology, and Environmental Toxicity (Rao, K.R., ed.), pp. 3–11, Plenum Press, New York
3 Ravanel, P., Taillandier, G., Tissut, M. and Benoit-Guyod, J.L. (1985) Ecotoxicol. Environ. Safety 9, 300–320
4 Buhler, D.R., Rassmuson, M.E. and Nahane, H.S. (1973) Environ. Sci. Tech. 7, 929–934.
5 McCarty, P.L., Reinhard, M. and Rittman, B.E. (1981) Environ. Sci. Tech. 15, 40–51
6 Tam, T.Y. and Trevors, J.T. (1981) Water Air Soil Pollut. 16, 409–414
7 Schellenberg, K., Leuenberger, C. and Schwarzenbach, R.P. (1984) Environ. Sci. Tech. 18, 652–657
8 Shull, L.R., Olson, B.A., Hughes, B.J., McKenzie, R.M. and Kinzell, J.H. (1986) Pestic. Biochem. Toxicol. 25, 31–39
9 Dougherty, R.C. (1978) in Pentachlorophenol, Chemistry, Pharmacology, and Environmental Toxicity (Rao, K.R., ed.), pp. 351–361, Plenum Press, New York
10 Kutz, F.W., Murphy, R.S. and Strassman, S.C. (1978) in Pentachlorophenol, Chemistry, Pharmacology, and Environmental Toxicity (Rao, K.R., ed.), pp. 363–369, Plenum Press, New York
11 Ohe, T. (1979) Bull. Environ. Contam. Toxicol. 22, 287–292
12 Engst, T., Macholz, R.M. and Kujawa, M. (1976) Bull. Environ. Contam. Toxicol. 16, 248–252
13 Renner, G. (1981) Xenobiotica 11, 435–446
14 Atuma, S.S. and Okor, D.I. (1985) Bull. Environ. Contam. Toxicol. 35, 406–410
15 Van Ommen, B., Van Bladeren, P.J., Temink, J.H.M. and Muller, F. (1985) Biochem. Biophys. Res. Commun. 126, 25–32
16 Senger, H., and Ruhl, D. (1980) Int. J. Biochem. 12, 1045–1048
17 Weinbach, E.C. and Garbus, J. (1965) J. Biol. Chem. 240, 1811–1819
18 Lebedev, A.V. and Boguslavski, L.I. (1973) Conf. Proc. Membrane Biophys., Palanga, Kaunas, pp. 406–411
19 Smejtek, P., Hsu, K. and Perman, W. (1976) Biophys. J. 16, 319–336
20 Pickar, A.D. and Amos, W.D. (1976) Biochim. Biophys. Acta 455, 36–55
21 Pickar, A.D. and Hobss, J. (1982) Biochim. Biophys. Acta 93, 221–236
22 McLaughlin, S.A. and Dilger, J.P. (1980) Physiol. Rev. 60, 825–863
23 Jayaweera, R., Petersen R. and Smejtek, P. (1982) Pestic. Biochem. Physiol. 18, 197–204
24 Kasianowicz, J., Benz, R. and McLaughlin, S. (1984) Biophys. J. 82, 179–190
25 Kasianowicz, J. Benz, R. and McLaughlin, S. (1986) Biophys. J. 49, 93a
26 Ferguson, S.J. (1985) Biochim. Biophys. Acta 811, 47–95
27 Flewelling, R.F. and Hubbell, W.L. (1986) Biophys. J. 49, 541–552
28 Andersen, O.S., Finkelstein, A., Katz, I. and Cass, A. (1976) J. Gen. Physiol. 67, 749–771
29 Smejtek, P. and Paulis-Illangasekare, M. (1978a) Biophys. J. 26, 441–466
30 Smejtek, P. and Paulis-Illangasekare, M. (1978b) Biophys. J. 26, 467–488
31 Griffith, H., Dehlinger, P.J., and Van, S.P. (1974) J. Membrane Biol. 15, 159–192
32 Onsager, L. (1936) J. Am. Chem. Soc. 58, 1486–1493
33 Warshel, A. and Russell, S.T. (1984) Q. Rev. Biophys. 17, 283–422
34 Block, H. and Walker, S.M. (1973) Chem. Phys. Lett. 19, 363–364
35 Kakitani, T. and Mataga, N. (1986) Chem. Phys. Lett. 124, 437–441
36 Suppan, P. (1983) Chem. Phys. Lett. 94, 272–275
37 Fowler, F.W., Katritzky, A.R. and Rutherford, R.J.D. (1971) J. Chem. Soc. (B), 460–469
38 Reichardt, C. (1979) Angew. Chem. Int. Edn. Engl. 18, 98–110
39 Taft, R.W. and Kamlet, M.J. (1976a) J. Am. Chem. Soc. 98, 377–383
40 Taft, R.W. and Kamlet, M.J. (1976b) J. Am. Chem. Soc. 98, 2886–2894
41 Kamlet, M.J., Abboud, J. and Taft, R.W. (1977) J. Am. Chem. Soc. 99, 6027–6038
42 Abboud, J., Kamlet, M.J. and Taft, R.W. (1977) J. Am. Chem. Soc. 99, 8325–8327
43 Abboud, J. and Taft, R.W. (1979) J. Am. Chem. Soc. 83, 412–419
44 Kamlet, M.J., Abboud, J., Abraham, M.H. and Taft, R.W. (1983) J. Org. Chem. 48, 2877–2887
45 Bell, R.P. and Robinson, R.R. (1960) Trans. Faraday Soc. 57, 965–970

46 Harned, H.S. and Owen, B.B. (1958) The physical Chemistry of Electrolytic Solutions, 3rd Edn., pp. 662, 756, Reinhold Publishing Corp., New York
47 Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, pp. 204-246, McGraw-Hill, New York
48 Pauling, L. (1936) J. Am. Chem. Soc. 58, 94–98
49 Kimura, Y. and Ikegami, A. (1985) J. Membrane Biol. 85, 225–231
50 King, E.J. (1965) in The International Encyclopedia of Physical Chemistry and Chemical Physics, Vol. 4 (Robinson, R.A., ed.), pp. 204, 256, 284, Macmillan, New York
51 Bates, R.G. (1968) in Hydrogen Bonded Solvent Systems (Covington, A.K. and Jones, P., eds.), pp. 49–86, Taylor and Francis, London
52 King, E.J. (1965) in The International Encyclopedia of Physical Chemistry and Chemical Physics, Vol. 4 (Robinson, R.A., ed.), p. 252, Macmillan, New York
53 McLaughlin, S. and Harary, H. (1976) Biochemistry 15, 1941–1948
54 McLaughlin, S. (1977) in Current Topics in Membranes and Transport (Bonner, F. and Kleinzeller, A., eds.), pp. 71–144, Academic Press, New York
55 Eisenberg, M., Gresalfi, T., Riccio, T. and McLaughlin, S. (1979) Biochemistry 18, 5213–5223
56 Davenport, L., Knutson, J.R. and Brandt, L. (1986) Biochemistry 25, 1186–1195
57 Smejtek, P., Wang, S., and Barstad, A. (1987) Biophys. J. 51, 485a
58 Fernandez, M.S. and Fromherz, P. (1977) J. Phys. Chem. 81, 1755–1781
59 Drahonovsky, J. and Vacek, Z. (1971) Coll. Czech. Chem. Commun. 36, 3431–3440
60 Smejtek, P., Barstad, W., Levinson, A. and Hsu, K. (1984) 8th Int. Biophys. Cong., Bristol, U.K., Book of Abstracts, pp. 242
61 Leo, A., Hansch, C. and Elkins, D. (1971) Chem. Rev. 71, 526–616
62 Cohen, F., Eisenberg, M. and McLaughlin, S. (1977) J. Membrane Biol. 37, 361–396
63 Dilger, J. and McLaughlin, S. (1979) J. Membrane Biol. 46, 359–384
64 McLaughlin, S. (1972) J. Membrane Biol. 9, 361–372
65 McLaughlin, A., Gratwohl, C. and McLaughlin, S. (1978) Biochim. Biophys. Acta 513, 338–357