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**MECHANISMS OF PENTACHLOROPHENOL
INDUCED CHARGE TRANSPORT IN
LIPID MEMBRANES**

by

WILLIAM CHARLES BROWN

**A dissertation submitted in partial fulfillment of the
requirements for the degree of**

**DOCTOR OF PHILOSOPHY
in
ENVIRONMENTAL SCIENCES AND RESOURCES: PHYSICS**

Portland State University

1996

DISSERTATION APPROVAL

The abstract and dissertation of William Charles Brown for the Doctor of Philosophy in Environmental Sciences and Resources: Physics was presented May 22, 1996 and accepted by the dissertation committee and the doctoral program.

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ABSTRACT

An abstract of the dissertation of William Charles Brown for the Doctor of Philosophy in Environmental Sciences and Resources: Physics presented May 22, 1996

Title: Mechanisms of Pentachlorophenol Induced Charge Transport in Lipid Membranes

Pentachlorophenol (PCP) is one of the prominent environmental pollutant that has penetrated into food chain and is present in humans. Health concerns have been raised since daily intake of PCP by the US population is estimated to be 16-19 μg .

PCP facilitates dissipation of electrochemical potential gradients of hydrogen ions across energy transducing membranes, which are the energy sources for the conversion of adenosine diphosphate into adenosine triphosphate. Closely linked to these dissipative effects is the development of electrical conductivity in lipid membranes, induced by the presence of PCP.

Three modes of PCP - induced membrane electrical conductivity were theoretically analyzed and experimentally verifiable formulations of each models were developed. Experimental studies using the charge - pulse method involved characterization of the time dependent transmembrane voltage over a wide pH range, from 1.8 to 9.5, for 30 μM concentrations of PCP. Lipid membranes were prepared from dioleoyl phosphatidylcholine.

It was shown that three PCP molecular species were determining the

transmembrane transfer of hydrogen ions: electrically neutral PCP molecules (HA), negatively charged pentachlorophenolate ions (A^-) and negatively charged heterodimers (AHA^-).

It was found that at $pH > 9$ the membrane electrical conductivity was determined by the transmembrane movement of A^- ions, whenever $pH < 7$ the conductivity was determined by the AHA^- species. Two new membrane surface reactions were proposed as supplementary mechanisms for the generation of AHA^- in addition to the formation of AHA^- by the recombination of HA and A^- , $HA + A^- \rightarrow AHA^-$. These new reactions are, (i) $2HA \rightarrow H^+ + AHA^-$, and (ii) $H_2O + 2A^- \rightarrow OH^- + AHA^-$. Reaction (i) provides formation of membrane permeable heterodimers AHA^- at $pH < 5.5$ and reaction (ii) at $pH > 5.5$.

The maximum surface density of AHA^- heterodimers was 0.09 pmol/cm^2 . The rate constant of formation of AHA^- by recombination, $HA + A^- \rightarrow AHA^-$ was estimated to be $k_f = 2.6 \times 10^9 \text{ cm}^2 \text{ mol}^{-1} \text{ s}^{-1}$ and the dissociation rate constant for $AHA^- \rightarrow HA + A^-$, $k_d = 72 \text{ s}^{-1}$.

Further, it was possible to determine the rate constants of transmembrane translocation for A^- and AHA^- ions to be $k_a = 6.6 \times 10^{-5} \text{ s}^{-1}$ and $k_{aha} = 1200 \text{ s}^{-1}$, respectively.

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This project has taken many years and there are three people who were absolutely critical to a successful outcome.

Dr. Arnold Pickar set me on my journey and taught me all the fundamentals whether it was how to make membranes or the nuances of all the classic papers that are the foundation of this research. Together we constructed much of the equipment that was and is the basis of this project. He always had time and a quiet patience when I needed it.

Dr. Pavel Smejtek started me thinking about PCP. His many years of research on PCP and his insight were the keystones making my studies possible. There was always a suggestion and a new thought. There were many time this project seemed hopelessly and endlessly stalled, then a little encouragement and a new view point would move it forward.

The theoretical and mathematical difficulties surrounding this project never seemed to end or, at times, have an answer. Dr. Makoto Takeo had a sharp eye and a penetrating mind. We talked and, sometimes, argued strongly. But that is when science is at its best. His rigor and his thoughts provided more than a few sharp edges to cut the Gordian knots.

In the end, it matters most of all that those you love and cherish are still there when all the years of long nights away from home have consumed themselves. To my wife Carole, to my children, you can turn the light off, I'm back.

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MECHANISMS OF PENTACHLOROPHENOL-INDUCED CHARGE TRANSPORT IN LIPID MEMBRANES

I. INTRODUCTION

A. ENVIRONMENTAL SIGNIFICANCE OF PCP

PCP is an effective biocide (Rao, 1978). The environmental significance of PCP became evident during the 1970's due to its wide spread use since the 1930's as a major pesticide, disinfectant, and wood preservative (Butte et al, 1985). PCP is a common byproduct of bleaching processes used in the pulp and paper industries (Paasivirta et al, 1992) as well as an agricultural pesticide or intermediate in herbicide production (Crosby, 1981). Most of the annual production of PCP, 80%, was used in the wood industry as an anti-sap stain agent, in wood preservation and for remedial treatment against fungi attack, or in the treatment of masonry (Crosby, 1981).

An important consideration with respect to the toxic effects of PCP is its accumulation in ecosystems. Studies of the distribution of toxic chemicals between biota and soil or water were based on their distribution between water and octanol as the adsorbing medium because of octanol's similarity to the average properties of

biological tissues (Connell, 1988; Hawker and Connell, 1989). A high linear correlation, for instance, is found between octanol adsorption of over 50 organic compounds as a function of their aqueous concentration and similar experiments involving the lipid tissue of fish (Chiou, 1985). Some of those studies have included PCP (Veith et al, 1979; Mackay, 1982).

For natural environments such as lake sediments and aquifer materials the sorption of PCP is very dependent on the organic content of the sediment or aquifer material. This is presumably linked to PCP's high octanol/water partition coefficient. PCP has the highest octanol/water partition coefficient of all the chlorinated phenols which is related to its lower pK_a , about 4.75 (Schellenberg et al, 1984). At physiologically and environmentally relevant pH values, it is the ionic form of PCP that dominates in aqueous media.

Studies of human exposure to PCP in the U.S.A. suggest that virtually all of that exposure is through the food chain (99.9%). As a result, through fruits, vegetables and grains, PCP is ingested by humans at a rate of about 16 $\mu\text{g}/\text{day}$ resulting in a residual of 17 $\mu\text{g}/\text{kg}$ of adipose (fat) tissue (Hattermer - Frey and Travis, 1989) with a half life of about 20 days (Uhl et al, 1986). Studies of human exposure in other countries have confirmed similar conclusions (Geyer et al, 1987; Mussalo - Rauhamaa et al, 1989; Wild et al, 1992; Wild and Jones, 1992).

The physiological effects of PCP have been shown to range from acute --

anaesthesia, coma -- to chronic -- rashes, sinusitis and eye disease (IRAC, 1979). PCP manufacture has been restricted in the United States, United Kingdom, and many other countries as the result of public pressure to reduce exposure to organic chemicals. However, due to its continued use and on-going presence in the environment it continues to be present in humans. The PCP burden in humans today is $\approx 550 \mu\text{g/person}$. Thus PCP continues to maintain a significant presence.

B. MEMBRANE TOXICITY OF PCP AS AN UNCOUPLER OF OXIDATIVE PHOSPHORYLATION

1. Energy Transducing Organelles

a. Mitochondrion

PCP has been shown to be similar to other compounds whose biological effect is associated with the uncoupling of phosphorylation from electron transport with inhibition of respiration in mitochondrion (p. 5 below).

As Fig. 1 shows mitochondria are characterized by a mitochondrial matrix surrounded by an inner membrane and an outer membrane with a region between the two membranes called the intermembrane space. The intermembrane space connects

Figure 1

The mitochondrial membrane showing the complex reactions leading to the phosphorylation of ADP beginning with the pumping of 2 protons from the inner membrane to the intermembrane space between the inner and outer membranes. Eventually a pH difference of -1.4 units and an electrical potential of 140 mV, implying a total electrochemical potential of 220 mV, is established which allows for the formation of ATP from ADP by an ATP synthesizing complex, ATPase, as protons passively flow back into the mitochondrial complex.

to the cytoplasm of the host cell through pores in the outer membrane which allows passage to molecules with molecular weights up to 5,000.

Unlike the porous outer membrane, the inner membrane is impermeable to most ions and molecules and has a high ratio of proteins to phospholipids. Therefore, while molecules and ions are relatively free to move between the host cell cytoplasm and the inner membrane space, passage through the inner membrane is very restricted.

The restrictive permeability of the inner membrane chemically isolates the mitochondrial matrix and allows the matrix to carry on its various biochemical functions. Of particular significance to this proposal are molecular complexes embedded in the inner membrane which allow protons to be pumped across the inner membrane from the mitochondrial matrix as part of a larger process which results in the phosphorylation of ADP (adenosine diphosphate) to form ATP (adenosine triphosphate).

As illustrated in Fig. 1, the pumping of protons from the mitochondrial matrix through the inner membrane into the intermembrane space begins in the mitochondrial matrix as NADH (nicotinamide adenine dinucleotide) and one proton interact with the first of a series molecular complexes embedded in the inner membrane, $\text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+ + 2\text{H}^+ + 2\text{e}^-$. The latter two protons are pumped across the inner membrane into the intermembrane space. The two electrons are passed through a

series of molecular complexes embedded in the inner membrane and eventually transferred back into the mitochondrial matrix and molecular oxygen: $\frac{1}{2}\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}$. During the sequential transfer of the two electrons by molecular complexes embedded in the inner membrane four more protons are pumped across the inner membrane into the intermembrane space.

The effect of pumping protons across the inner membrane is to decrease the pH and increase the electrical potential of the intermembrane space relative to the mitochondrial matrix. Also embedded in the inner membrane is an ATP-synthesizing complex, ATPase, which provides a channel for protons to passively flow back into the mitochondrial matrix. When a proton gradient is established by the pumping action of respiration, protons migrate back into the matrix through the ATPase channel. As a result of this migration process through ATPase channel, ADP is phosphorylated into ATP.

To quantify the conversion of metabolic energy into ATP the electrochemical potential of protons was used by Mitchell (Mitchell, 1961; Mitchell and Moyle, 1969). It takes into account both the pH difference and electric potential difference across the inner membrane:

$$\Delta\mu = \Delta\psi - \frac{RT}{F} \Delta pH \quad (1)$$

where $\Delta\mu$ is the electrochemical potential difference across the inner membrane, $\Delta\psi$ and ΔpH is the electric potential and pH difference, respectively, between the intermembrane space and the mitochondrial matrix, R is the gas constant, T is the absolute temperature, and F is the faraday. The above definition suggests the pumping of protons into the intermembrane space from the mitochondrial matrix causes the electric potential of the intermembrane space to be relatively higher and its pH relatively lower compared to the mitochondrial matrix.

Typically, during respiration the difference in pH is -1.4 units and the electric potential difference is 0.14 V. Eq. 1, then, implies an electrochemical potential of about 0.22 volts or 5.2 kcal/mole of protons.

b. Chloroplasts

Photosynthesis in plants is involved in a similar chemiosmotic scheme involving a proton gradient and ATP formation (Mitchell, 1966). Within a plant cell photosynthesis takes place in chloroplasts. Chloroplasts are more complicated than mitochondria and have three membranes instead of two separating them from the host

cell. The inner most membrane, called the thylakoid membrane, functions in a similar manner as the inner membrane in mitochondrion. Inside the thylakoid membrane is the thylakoid space and on the other side of the membrane the stroma. The thylakoid membrane, like the inner membrane of mitochondrion, is relatively impermeable and can likewise sustain a pH and electric potential difference.

In the case of plants, however, the initial energy is supplied by the absorption of light. Embedded in the thylakoid membrane are chlorophyll complexes. The absorption of light results in exciting a chlorophyll electron followed by a series of step-wise electron transfer reactions resulting in the pumping of protons across the thylakoid membrane and ultimately resulting in the formation of ATP.

The above process actually involves two forms of chlorophyll and two separate light absorption events. Proton pumping across the thylakoid membrane results when electrons are transferred from one light absorbing chlorophyll complex to the other light absorbing chlorophyll complex.

An important difference, however, between the above photosynthetic coupling of phosphorylation and the pumping of protons across the inner membrane to the analogous process in mitochondrion is that the polarity of the electrochemical potential is reversed. In the photosynthetic version of the chemiosmotic hypothesis protons are pumped into the thylakoid space.

2. Effect of Lipophilic Uncouplers in Mitochondria

When weak lipophilic acids are introduced into the inner membrane space of mitochondrion, the concentration gradient causes the acid to migrate across the inner membrane. On the matrix side of the inner membrane the pH is higher and the neutral acid readily gives up its proton while the resulting negatively charged ion is driven back across the membrane to pick up another proton. This transport of protons across the inner membrane into the matrix causes the collapse of the metabolically generated electrochemical potential of protons (Mitchell and Moyle, 1967; VanDam, 1967) even if respiration continues. As a result, the synthesis of ATP is uncoupled from the respiration process.

The toxicity of uncouplers lies in their ability to circumvent the formation of ATP by providing an alternative protonic back flow. PCP, in particular, has been shown to have biological effects specifically as a weak lipophilic acid uncoupler (Weinbach and Garbus, 1965; Senger and Ruhl, 1980; Ravanel et al, 1985). At micromolar concentrations PCP can stop ATP synthesis and the production of chlorophyll (Senger and Ruhl, 1980).

C. STUDIES OF PCP IN LIPID BILAYER MEMBRANES

1. Studies of Adsorption of PCP to Lipid Membranes and Thermotropic Transitions

A study of PCP has been conducted utilizing sonicated vesicles of the lecithin membranes dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) by measuring their electrophoretic mobility. In addition, the effect of PCP on the gel to fluid transition temperature of the membranes' hydrocarbon chains using photometric methods was conducted (Smejtek et al, 1989). The main parameter monitored in this study was the membranes' ζ -potential, essentially the difference in electrostatic potential between the aqueous side of the membranes' surface and the bulk aqueous phase suspending the lipid vesicles. For both types of membranes the magnitude of the ζ -potential increased step-wise upon approaching the melting point of the hydrocarbon chains of the lipids as more PCP was adsorbed in the fluid phase. Also the gel to fluid transition temperature decreased with PCP concentration. It was concluded from the studies of adsorption of PCP that PCP is adsorbed at the interface between the polar head and hydrocarbon chain regions of the membrane.

A further study indicated the adsorption plane for PCP lie 0.4 - 0.6 nm below

the water/dielectric interface and that the dielectric constant of the adsorption plane region was 8 - 10 (Smejtek and Wang, 1990).

Spectrophotometric studies of PCP adsorbed on PC membranes (Smejtek et al, 1987a) indicated that the pK_a for PCP adsorbed to phosphatidylcholine (PC) - membranes is shifted from its aqueous value of 4.75 (Drahonovsky and Vacek, 1971) to at least 5.5. Also the partition coefficient for neutral and ionized PCP was found to be 1.5×10^{-4} m and 3.0×10^{-5} m, respectively. The results of studies of adsorption of ionized PCP are very compatible with the environmental studies involving the absorption of PCP into fish tissue (Mackay, 1982) where the partition coefficient of ionized PCP was found to be 10^{-5} m. The similarity of results for PCP adsorption in lipid membranes and in fish tissue supports the environmental relevance of biophysical studies using artificial lipid membranes.

2. Studies of PCP - Induced Electrical Conductivity

PCP induces electrical conduction in lipid bilayer membranes (Smejtek et al, 1976; Pickar and Amos, 1976; Pickar and Hobbs, 1982) which is related to transmembrane proton translocation in a manner similar to that described in the previous section for weak lipophilic acid uncouplers (McLaughlin and Dilger, 1980).

Also involved in such conductance effects is the hydrogen bonding of membrane

bound PCP creating a dimer AHA^- (Smejtek et al, 1976; McLaughlin and Dilger, 1980; Smejtek et al, 1987b). AHA^- is formed from HA and A^- where HA represents the neutral PCP molecule and A^- the pentachlorophenate. All three forms are illustrated in Fig. 2.

Figure 2

The pentachlorophenate (A^-), molecular (HA) and dimer (AHA^-) forms of PCP

D. MECHANISMS OF CHARGE TRANSPORT INDUCED IN LIPID
MEMBRANES BY UNCOUPLERS

1) The Lipophilic Ion Model

The simplest way to describe the translocation of A^- across the membrane interior is to first neglect both the formation of AHA^- , and the interaction between HA and A^- . The kinetic scheme characterizing this lipophilic ion model is shown in Fig. 3 below.

Figure 3

General lipophilic ion model for PCP

When an electric potential is imposed across the membrane the resulting electric field induces ions to flow across the membrane interior. A net flux of ions will continue to flow across the membrane interior in response to the electric field until the concentration difference between ions adsorbed on one side of the membrane compared to the other side causes an equal diffusive back flux of ions to flow in the opposite direction.

As ions accumulate on one side of the membrane, a net flux of ions will also flow from their adsorption plane on the membrane into the aqueous solution. On the other side of the membrane, a deficiency of adsorbed ions will cause additional ions to flow from the aqueous solution and resulting in their adsorption onto the membrane. If the driving electric field is still significant, aqueous A^- ions continue to flow across the membrane interior.

The system of differential equations which describe the lipophilic model have been solved (Benz et al, 1976). A complete mathematical analysis of the lipophilic model is given in Appendix A.

The lipophilic model is a useful approximation for PCP at $pH \gg pK_a$. In this region the surface density of HA is very low compared to the surface density of A^- . Under these conditions the reaction between HA and A^- can be neglected and the initial surface density of A^- on both sides of the membrane can be considered to be set by the aqueous concentration of PCP.

2. The Class - 1 Uncoupler Model

Fig. 4 illustrates the transport scheme for standard class - 1 uncouplers.

Figure 4

General class - 1 model for PCP

The imposition of an electric potential across the membrane induces ions to transport across the membrane interior in the same manner as described for the lipophilic model. In the standard class - 1 uncoupler model, however, the movement of A^- ions between the aqueous solution and their adsorption on the lipid is neglected.

Unlike the lipophilic model the class - 1 uncoupler model includes the presence of HA . Due to the transmembrane transport of A^- in response to an external electric

field, the reaction $A^- + H^+ \rightleftharpoons HA$ creates an excess of HA on one side of the membrane and a deficiency of HA on the other side. As a result of this concentration difference, HA diffusively migrates across the membrane interior in a direction opposite that of the accumulating ions. This back flux of HA dissipates the concentration difference of A^- ions caused by the external field and allows more A^- ions to be transported across the membrane under the influence of the remaining electric field.

The differential equations characterizing standard class - 1 uncouplers have been described and solved (McLaughlin and Dilger, 1980). In addition to the movement of A^- and HA across the membrane interior, the standard class - 1 uncoupler model includes the reaction of H^+ with A^- on the surface of the membrane,

where k_R ($\text{cm}^2 \text{ mole}^{-1} \text{ s}^{-1}$) and k_{RR} (s^{-1}) are the indicated rate constants.

Appendix B expands the standard mathematical analysis suggested by McLaughlin and Dilger to include the hydroxyl ion, OH^- , and the reaction

where k_D ($\text{cm}^2 \text{ mole}^{-1} \text{ s}^{-1}$) and k_{DD} (s^{-1}) are the indicated rate constants.

It has been shown (Kasianowicz et al, 1987) that the removal of H^+ from HA can also be significantly induced by OH^- and that the rate of H^+ removal is very fast and dependent only on the aqueous pH and the local pK_a of HA and A^- .

However, a complete mathematical theory of class - 1 uncouplers including both the effects of H^+ and OH^- as characterized by Eqs. 2 and 3 has not previously been published. Appendix B details such a complete theory involving the above process.

3. The Class - 2 Uncoupler Model

The standard class - 2 uncoupler model also includes the formation of the dimer, AHA^- , formed from HA and A^- . The resulting transport scheme, illustrated in Fig. 5, is more complicated.

Figure 5

General class - 2 model for PCP

When an electric potential is imposed across the membrane, the class - 2 uncoupler responds in a manner analogous to a class - 1 uncoupler. The essential difference is that in the standard class - 2 uncoupler kinetic scheme the dimer AHA⁻ is the only membrane permeable species.

In order to solve the differential equations that characterize class - 2 uncouplers it is assumed by most investigators (Smejtek et al, 1976; McLaughlin and Dilger, 1980; Kasianowicz et al, 1984; Kasianowicz et al, 1987; Smejtek et al, 1987b) that the removal of H⁺ from HA is very fast compared to the transport of A⁻, AHA⁻ or HA across the interior of the membrane. As a result, the ratio N_a/N_{ha} on either side of the membrane is considered a constant dependent only on the aqueous pH and the pK_a .

the membrane is considered a constant dependent only on the aqueous pH and the pK_a of the uncoupler adsorbed in the membrane. Therefore, the surface reaction between HA and A^- with H^+ or OH^- are not characterized by rate constants and are excluded from the mathematical analysis. Only the reaction between A^- and HA is considered in the formation of the dimer, that is,

where k_f ($\text{cm}^2 \text{ mole}^{-1} \text{ s}^{-1}$) and k_d (s^{-1}) are the indicated rate constants.

Appendix C expands the published mathematical theory of class - 2 uncouplers to include not only the above reaction between A^- and HA, Eq. 4, and the surface reactions between H^+ and OH^- with HA, Eqs. 2 and 3, but H^+ and OH^- with AHA^- as given below,

E. PRELIMINARY CONSIDERATIONS CONCERNING THE CHARGE PULSE METHODOLOGY

The theory of the charge pulse experimental method which is used exclusively in this investigation has been described for lipophilic ions (Benz et al, 1976) and used to investigate a number of lipophilic ions (Benz et al, 1976; Benz and Lauser, 1977; Pickar and Benz, 1978). Appendix A also describes the theory of charge pulse measurements.

The theory of the charge pulse experimental method for class - 1 uncouplers has also been described McLaughlin and Dilger (McLaughlin and Dilger, 1980). Using their analysis many class - 1 uncouplers have been investigated besides PCP (Benz and McLaughlin, 1983; Kasianowicz et al, 1984; Kasianowicz et al, 1987).

However, the theoretical development of the charge pulse experimental method for class - 1 uncouplers has not included interactions between HA and A⁻ with H⁺ and OH⁻, that is, the reactions suggested by Eqs. 2 and 3. Appendix B, therefore, also expands the theory of the charge pulse experimental method for class - 1 uncouplers to include both the reactions suggested by Eqs. 2 and 3.

There is no published theory for the charge pulse experimental method applicable to class - 2 uncouplers. Therefore, Appendix C, additionally, develops the mathematical theory for the charge pulse experimental method and includes all the

reactions of HA, A⁻ and AHA⁻ with H⁺ and OH⁻, plus the dimer forming reaction between HA and A⁻. In other words, the charge pulse theory developed in Appendix C for class - 2 uncouplers includes all the reactions characterized by Eqs. 2 - 6.

F. THE PRINCIPAL RESULTS OF CLASS - 2 AND CHARGE PULSE THEORY AS DETAILED IN APPENDIX C

Since PCP has been shown to be a class - 2 uncoupler and was also confirmed as such by this investigation it will be useful to describe the principal results of the mathematical analysis of Appendix C.

The differential equations of class - 2 theory involve the transport of HA, A⁻ and AHA⁻ across the interior of the membrane each characterized by three interior rate constants, k_{ha} , k_a and k_{aha} , respectively. Furthermore, before the charge is placed on the membrane the above three adsorbed forms of PCP are characterized by surface densities on each side of the membrane, in moles per cm², namely, N_{ha}^0 , N_a^0 and N_{aha}^0 , respectively. Likewise, the surface densities of H⁺ and OH⁻ within the reaction layer on each side of the membrane are characterized by N_h^0 and N_{oh}^0 , respectively.

In addition all the surface reactions suggested in Eqs. 2 - 6 are included, along with their respective rate constants.

During the time course of charge pulse experiment, after the initial charge has been placed on the membrane, the surface densities on either side of the membrane will change, as A^- and AHA^- move across the interior from one side of the membrane to the other in response to the electric field now established across the membrane by the charge pulse. As surface reactions, Eqs. 2 - 6, on either side of the membrane become significant a difference in pH between one side of the membrane and the other could also develop.

If Y_{ha} , Y_a , Y_{aha} , Y_h and Y_{oh} represent the difference in surface densities on one side of the membrane compared to the other, then, class - 2 theory, as developed in Appendix C, implies that in matrix notation,

$$\begin{pmatrix} \frac{dY_{ha}}{dt} \\ \frac{dY_a}{dt} \\ \frac{dY_{aha}}{dt} \\ \frac{dY_h}{dt} \\ \frac{dY_{oh}}{dt} \end{pmatrix} = A \begin{pmatrix} Y_{ha} \\ Y_a \\ Y_{aha} \\ Y_h \\ Y_{oh} \end{pmatrix} - u^0 \begin{pmatrix} 0 \\ 2k_a N_a^0 \\ 2k_{aha} N_{aha}^0 \\ 0 \\ 0 \end{pmatrix} \quad (7)$$

where t is time, $u^0 = V^0 F / RT$, V^0 = the voltage at $t = 0$ across the membrane due to the initial charge placed on the membrane, F is Faraday's constant, R is the gas

constant and T is the temperature in Kelvin.

The term A in Eq. 7 is a 5x5 matrix with the following columns, 1 through 5, respectively,

$$\begin{aligned}
 & -2k_{ha} - k_{RR} - k_D N_{oh}^0 - k_f N_a^0 - k_{zha} N_{ha}^0 \\
 & k_R N_h^0 + k_{DD} - k_f N_{ha}^0 \\
 & k_d N_{ha}^0 + 2k_{haha} N_h^0 \\
 & k_R N_a^0 + 2k_{haha} N_{aha}^0 \\
 & -k_D N_{ha}^0
 \end{aligned} \tag{8}$$

$$\begin{aligned}
 & k_{RR} + k_D N_{oh}^0 - k_f N_a^0 \\
 & -2k_a - k_f N_{ha}^0 - k_R N_h^0 - k_{DD} - k_{raha} N_a^0 - bk_a N_a^0 \\
 & k_d + 2k_{poh} N_{oh}^0 - bk_a N_a^0 \\
 & -k_R N_a^0 + bk_a N_a^0 \\
 & k_D N_{ha}^0 + 2k_{poh} N_{aha}^0 - bk_a N_a^0
 \end{aligned} \tag{9}$$

$$\begin{aligned}
 & k_f N_a^0 + k_{zha} N_{ha}^0 \\
 & k_f N_{ha}^0 + k_{raha} N_a^0 - bk_{aha} N_{aha}^0 \\
 & -2k_{aha} - k_d - k_{haha} N_h^0 - k_{poh} N_{oh}^0 - bk_{aha} N_{aha}^0 \\
 & -k_{haha} N_{aha}^0 + k_{aha} N_{aha}^0 \\
 & -k_{poh} N_{aha}^0 - bk_{aha} N_{aha}^0
 \end{aligned} \tag{10}$$

$$\begin{aligned}
& k_{RR}N_a^0 + k_{2ha}N_{ha}^0 \\
& - k_R N_h \\
& - k_{haha}N_h^0 \\
& - k_R N_a^0 - k_{haha}N_{aha}^0 \\
& 0
\end{aligned} \tag{11}$$

$$\begin{aligned}
& - k_D N_{oh}^0 \\
& k_{DD} + k_{raha}N_a^0 \\
& - k_{poh}N_{oh}^0 \\
& 0 \\
& - k_D N_{ha}^0 - k_{poh}N_{aha}^0
\end{aligned} \tag{12}$$

where $b = F^2/CRT$ and C = the membrane's total electrical capacitance.

Charge pulse theory as developed in Appendix C characterizes the experimental time course of the decaying voltage as the sum of decaying exponentials and associated amplitudes, namely,

$$\begin{aligned}
u(t) &= u^0 \sum_{j=1}^5 \beta_j e^{-\frac{t}{\tau_j}} \\
\text{where } \sum_{j=1}^5 \beta_j &= 1
\end{aligned} \tag{13}$$

Once numerical values are assumed for the variables in matrix A , the eigenvalues of A , λ_j , are related to the τ_j 's of Eq. 13.

$$\lambda_j = \tau_j^{-1} \quad (14)$$

The associated amplitudes, the β_j 's of Eq. 13, are derived from the respective eigenvectors of matrix A associated with each λ_j . The detailed relationship is described at the end of Appendix C.

II. MATERIALS AND METHODS

A. SOLUTIONS

The solutions were buffered aqueous solutions of 30 μ M PCP brought to 0.1 M ionic strength with KCl.

Stock solutions of 3 mM PCP (99%, Aldrich Chemical Company, Milwaukee, WI) were prepared in 0.02 M KOH. Stock solutions of buffered 1.0 M KCl were prepared with B⁻¹ buffer, 0.2M/0.2M/0.05M potassium phosphate/potassium citrate/boric acid, respectively.

Experimental solutions were then made by mixing the buffered stock KCl solutions and stock PCP solutions, diluting them with de-ionized water (Millipore Q2 system, Millipore Corp., Bedford, MA) in ratios of 10:1 and 100:1, respectively. Concentrated HCl and KOH were used to bring the final solutions to the desired pH.

B. LIPIDS

Lipid solutions of L- α -dioleoyl lecithin (1,2-dioleoyl-sn-glycerol-3-phosphocholine) 20 mg lipid/ml chloroform, were obtained from Avanti (Avanti Polar-Lipids, Alabaster, AL). The chloroform was evaporated and the lipid was redissolved in n-decane (Aldrich Gold 99+ %, Aldrich Chemical Company, Milwaukee, WI) in equal volume to the chloroform evaporated.

C. THE CHARGE PULSE EXPERIMENTAL METHOD

In the charge pulse experimental method a membrane immersed in an aqueous solution has an electrical charge placed on it and then is immediately isolated electrically from its surroundings. The voltage across the membrane, resulting from the initial electrical charge, is monitored through a very high impedance circuit and the time course of its decay constitutes the experimental data upon which charge pulse measurement theory is based. Generally, two electrodes are immersed in the aqueous solution on either side of the membrane to both apply the initial electrical charge and to monitor the subsequent decaying voltage.

The experimental setup for this series of experiments is illustrated below in Fig. 6.

Figure 6

Experimental setup: Square pulse, 20 V p-p, closed FET allowing 5 V across membrane for 50 ns charging the membrane to ≈ 10 mV; the high impedance amplifier input resistance was $10\text{ G}\Omega$ with amplification of 20.

C. THE EXPERIMENTAL APPARATUS

The membrane was formed across a hole, about 1 mm in diameter, in a teflon cup separating the two saline solutions. By means of a syringe the lipid was drawn into a 0.1 x 100 mm capillary tube. One end of the tube was then placed near the hole and the syringe used again to blow lipid into the hole where the membrane formed. Cylindrical silver - silver chloride electrodes (6 mm diameter, 2 mm length,

Annex Instruments, Santa Ana, CA) were used to both place the initial charge on the membrane and monitor the resulting voltage decay. The charging procedure began when a square pulse of 50 ns and an amplitude of 20 V was generated by a 5010 Tektronix Programmable Function Generator. The pulse was directed to the gate of a FET switch (Signetics 2N4392) causing it to close and allow 5 V DC on the FET's drain to appear across the cell - membrane system for the duration of the square pulse (50 ns), after which the FET again opened to once more isolate both the function generator and the 5 V DC from the membrane and the rest of the circuit through an open resistance of $5 \times 10^{10} \Omega$. This generally resulted in a potential across the membrane of about 10 mV before the membrane was electrically isolated. The electrodes then follow the time course of the subsequent voltage decay across the membrane which is recorded by a Nicolet 4094 Digital Oscilloscope.

Before the 50 ns current pulse was introduced the membrane was monitored using a 100 mV peak to peak, 1 kHz AC voltage generated by a Hewlett Packard 4284A Precision LCR Meter. The measurement routine began after the membrane had stabilized and reached a capacitance of approximately 400 nF/cm². At this capacitance the dissolving decane had presumably been completely drained from between the membrane surfaces.

The membrane was isolated from the Oscilloscope by a high impedance operational amplifier with an input impedance of $10^{12} \Omega$ (Burr-Brown OPA102BM)

which also amplified the signal by a factor of 20.

Theoretically, the time resolution of the charge pulse method is limited only by the external circuitry measuring the decaying voltage across the membrane. The Nicolet 4094 Digital Oscilloscope had a minimum limit of $0.5 \mu\text{s}$ per point and thus the lower time limit was a few μs .

The upper time resolution of the charge pulse experiment is limited by the input impedance of the high impedance amplifier isolating the membrane after it has been charged. In these experiments the input resistance of the isolating impedance was $10^{12} \Omega$ which when combined with a membrane capacitance of about 10 nF implied a RC time of 10^4 s , well beyond any of the observed results.

D. MEASUREMENT ROUTINE

After the membrane was formed it was allowed to stabilize, generally, around 400 Nf/cm^2 which, given the area of the membrane, was $6 - 8 \text{ Nf}$ as monitored by the LCR Meter.

The entire measurement routine was under the control of a DEC PDP 11/03 computer which removed the LCR Meter from the circuit after it was determined by the experimenter that the membrane had stabilized charge pulse measurements could begin using the experimental configuration shown in Fig. 6. At this point, therefore,

the membrane was electrically isolated through the high impedance operational amplifier.

A 50 ns pulse from the function generator momentarily closed the FET switch so that the membrane could be charged. Immediately after the pulse passed the switch opened isolating the membrane, once more, through the operational amplifier. The monitoring of the time course of the membrane's voltage decay was begun before the 50 ns pulse was allowed to activate the FET switch so that a zero voltage base line could be established.

For each membrane two records were made. One record was made with the Nicolet set for a time resolution of 1 ms per point (total sweep time 16 seconds) or lower if a sufficiently long time constant was not observed. This record was taken to measure the slow processes. A second record was then made at the shortest time resolution the Nicolet oscilloscope was capable of making, 0.5 μ s per point (total sweep time 8 ms). This record was taken to measure the fast processes.

Analysis of the digitized data was carried out by a DEC PDP 11/23 computer using a program based on a sequential deconvolution of the transient signal. The program used a semi-log graph of transmembrane voltage vs. time to display the data. This allowed the computer to generate a least squares fit to the voltage trace in order to determine the relevant time constant and its associated amplitude. Once the longest

time constant and its amplitude was determined, it was subtracted from voltage trace and shorter time constants and amplitudes determined in a similar fashion. This method of successively peeling away time constants and their amplitudes was useful and accurate to the extent that the time constants were exponential and sufficiently separated. To facilitate this analysis the program was capable of expanding any part of the voltage record and, if necessary, even resolving individual data points.

E. PRELIMINARY TEST RUNS WITH RC CIRCUITS

Preliminary test runs were made using the above routine to insure the apparatus had sufficient resolution. Membranes were simulated utilizing three parallel RC circuits each consisting of resistor and capacitor in parallel. All three parallel circuits were linked in series with each other, so they would generate three time constants. An additional single resistor was put in series to simulate the aqueous solution.

The overall capacitance of the circuit was adjusted to 10 nF and its overall resistance adjusted to 100 k Ω which was approximately the bulk capacitance and resistance of the membranes to be considered under the assumed experimental conditions.

It was found that the above apparatus could accurately discriminate time constants of a few μs even if their amplitudes were only a few percent of the whole. Also, time constants differing by a factor of 2 could be accurately determined even if their amplitudes also only differed by the same factor.

III. RESULTS

A. GENERAL COMMENTS

Charge pulse measurements were taken over a wide range of pH values, from pH 1.8 to 9.5 to be compared to the theoretical analyses developed in Appendices A, B and C.

In each case at least three time constants were observed. At pH 5.0 and 5.5 four time constants were observed. Except for the shorter observed time constants, τ_3 and τ_4 , the least squares fit to the data for each membrane showed little error. The goodness of the fit was better than 1% for the longest time constant, τ_1 , and better than 3% for the next longest time constant, τ_2 . For the shorter time constants the fitting error with respect to each membrane was larger, typically 10% - 20%.

B. THE EXPERIMENTAL DATA

Table 1 below summarizes the results for the three time constants observed at all pH values. Table 2 summarizes the results for the fourth time constant observed at pH 5.0, 5.5 and 6.0. The data in both tables is an average of 3 - 5 membranes.

Charge Pulse Measurements of PCP-Modified Lipid Membranes τ_1 , τ_2 , τ_3						
pH	τ_1 (s)	β_1	τ_2 (ms)	β_2	τ_3 (μ s)	β_3
1.8	1.94 ± 0.81	0.890 ± 0.042	31.5 ± 16.3	0.0996 ± 0.0423	366 ± 52.9	0.0107 ± 0.0020
2.9	1.74 ± 0.36	0.866 ± 0.019	27.8 ± 13.6	0.120 ± 0.021	432 ± 110	0.0139 ± 0.0020
3.7	0.398 ± 0.179	0.862 ± 0.078	65.8 ± 20.8	0.126 ± 0.070	437 ± 116	0.0123 ± 0.0083
4.5	0.113 ± 0.007	0.869 ± 0.039	35.4 ± 2.9	0.131 ± 0.039	None	Observed
5.0	0.0693 ± 0.0090	0.716 ± 0.075	16.1 ± 5.4	0.245 ± 0.081	535 ± 91	0.0260 ± 0.0151
5.5	0.0478 ± 0.0063	0.721 ± 0.065	19.1 ± 1.3	0.197 ± 0.064	526 ± 32	0.0164 ± 0.008

Charge Pulse Measurements of PCP-Modified Lipid Membranes τ_1 , τ_2 , τ_3						
6.0	0.0442 ± 0.0040	0.982 ± 0.001	24.1 ± 4.1	Combine with β_1	None	Observed
6.5	0.119 ± 0.021	0.904 ± 0.056	31.4 ± 4.7	0.0951 ± 0.056	302 ± 25	0.018 ± 0.002
7.0	0.332 ± 0.065	0.923 ± 0.015	0.0945 ± 0.005	0.0343 ± 0.0007	4.58 ± 1.4	0.0426 ± 0.0145
7.5	0.324 ± 0.056	0.890 ± 0.010	0.150 ± 0.042	0.0357 ± 0.0043	3.11 ± 0.48	0.0744 ± 0.0078
7.75	1.20 ± 0.31	0.927 ± 0.002	0.218 ± 0.042	0.0346 ± 0.0045	4.92 ± 0.56	0.0388 ± 0.0067
8.0	2.83 ± 0.64	0.942 ± 0.013	0.357 ± 0.034	0.0393 ± 0.0092	19.6 ± 2.6	0.0189 ± 0.0053
8.5	2.75 ± 2.12	0.953 ± 0.005	0.679 ± 0.119	0.0336 ± 0.0043	52.0 ± 11.0	0.0138 ± 0.0040

Charge Pulse Measurements of PCP-Modified Lipid Membranes τ_1 , τ_2 , τ_3						
9.0	5.29 ± 2.30	0.955 ± 0.007	0.916 ± 0.153	0.0318 ± 0.0041	81.6 ± 10.8	0.0127 ± 0.0040
9.5	7.18 ± 2.2	0.945 ± 0.015	13.8 ± 3.4	0.0327 ± 0.0125	691 ± 266	0.0217 ± 0.0082

Table 1

Charge Pulse Measurements of PCP- Modified Lipid Membranes τ_4		
pH	τ_4 (μs)	β_4
5.0	38.5 ± 25.6	0.0166 ± 0.008
5.5	12.3 ± 3.8	0.0461 ± 0.0239
6.0	32.1 ± 14.3	0.0271 ± 0.005

Table 2

Experimental conditions both tables: 30 μ M PCP, 0.1 M KCl, phosphate/citrate/borate buffer
 (0.02/0.02/0.005 M), 25° C.

C. THEORETICAL ANALYSIS

The results summarized in Table 1 were evaluated with respect to the charge pulse methodology in terms of the three models described in Appendices A, B, and C: the lipophilic ion model, the class - 1 uncoupler model, the class - 2 uncoupler model.

For each of the three models, theoretical curves for the time constants and associated amplitudes as a function of pH were constructed by assuming values of each of the relevant rate constants and surface densities in matrix A of Eq. 7. This allowed determination of the eigenvalues and eigenvectors associated with matrix A upon which theoretical curves could be constructed as detailed in Appendices A, B and C. These curves were then compared to the experimental data in Table 1.

As shown in the Appendices not all the rate constants and surface densities of matrix A were considered independent. Boundary conditions allowed k_{RR} , k_{DD} and N_{aha}^0 to be dependent variables. Boundary conditions also implied that N_0 = total amount of PCP adsorbed onto the membrane = $2N_a^0 + 4N_{aha}^0 + 2N_{ha}^0$ is a constant, resulting from the assumption that no PCP passes into the aqueous solution or is adsorbed from the aqueous solution during the time course of the experiment. The value of N_0 was taken from the partition coefficient for PCP measured by Smejtek et

al (Smejtek et al, 1976) and assumed to be 100 pmol/cm².

In a separate investigation Smejtek et al (Smejtek et al, 1987a) also determined the pK_a of PCP on the adsorbed surface of the membrane to be 5.5, the value also adopted here. The pK_a allowed the determination of the ratio N_a^0/N_{ha}^0 as a function of pH. The ratio N_a^0/N_{ha}^0 along with a value for N_0 and N_{aha}^0 as a function of N_a^0 and N_{ha}^0 , allowed N_a^0 to be calculated as a function of pH and the various rate constants. Appendix C contains all the relevant details of these calculations.

In addition, Smejtek et al (Smejtek et al, 1987a) determined the depth at which the adsorption of PCP took place was between 0.4 and 0.6 nm. It was assumed that the effective thickness of the reaction layer was equal to the adsorption depth of PCP in the membrane which was set at 0.5 nm. This made it possible to determine the surface densities for both the H^+ and OH^- ions on the membrane surface as a function of pH.

As a result, boundary conditions made it possible to evaluate all the surface densities and two of the rate constants (k_{RR} and k_{DD}) in matrix A before the curve fitting routine was begun.

Theoretically, then, the remaining eleven rate constants could be determined from the 5 experimentally observable time constants and their associated 5 amplitudes that Appendix C implies should be present if also included is the boundary condition

on the amplitudes that $\sum_j \beta_j = 1$, $j = 1 - 5$, Appendix C, Eq. C32.

Experimentally the eleven rate constants above were varied until the best least squares fit was obtained for all the observed time constants and amplitudes. As Tables 1 and 2 illustrate not all the theoretically presumed time constants and amplitudes were observed, a problem which will be discussed in the Discussion section.

Each of the three observed time constants shall now be examined in turn.

C. τ_1 AND ITS ASSOCIATED AMPLITUDE

1. τ_1

As Fig. 7 demonstrates, neither of the τ_1 's suggested by the lipophilic ion or class - 1 models yielded a reasonable fit to the observed values of τ_1 .

Figure 7

Theoretical curves for the class - 1 and lipophilic ion models as a function of pH compared to the experimental values of τ_1

The theoretical lipophilic long time constant associated with the movement of A^- across the interior of the membrane due to the initial external voltage placed across the membrane can be expressed, Eq. A44, Appendix A,

$$\tau_{lipOA}^{-1} = 2k_a + bk_a N_a^0 \quad (15)$$

where $b = F^2/(CRT)$. F is Faraday's Constant, R is the gas constant, T is the temperature in Kelvin and C is the bulk electrical capacitance per unit area of the membrane which normalizes the given rate constant with respect to the membrane's bulk electrical capacitance and surface area.

The lipophilic movement of A^- across the interior because of the externally applied voltage is limited by the back diffusion of A^- in the reverse direction due to the increasing concentration difference.

Due to the back diffusion of A^- or a low adsorbed initial concentration of A^- such lipophilic movement may be insufficient to equilibrate the external voltage. In this case, a net flow of A^- across the membrane interior can be supported if A^- is removed from the high concentration side and supplied to the low concentration side. In the lipophilic case A^- is supplied from and diffuses into the aqueous solution.

In the case of class - 1 uncouplers, the removal of excess A^- is accomplished by the formation of HA from A^- . HA back diffuses across the membrane and the breakup of HA to supply A^- to the low A^- concentration side.

For the class - 1 model τ_1 , then, becomes the serial or sequential sum of four times, hereafter called flux times: the time required for A^- to move across the interior, for HA to form from A^- , for HA to diffuse back across the membrane

interior and for A^- to form from HA. Eqs. 3 - 4 characterize the formation/dissociation of HA.

If the characteristic flux time for the formation/ dissociation of HA is considered very short compared to the movement of A^- and HA across the membrane interior, τ_1 becomes

$$\tau_{class1} = \frac{1}{bk_a N_a^0} + \frac{1}{bk_{ha} N_{ha}^0} \quad (16)$$

where bN^0 normalizes the given rate constant with respect to the membrane's bulk capacitance and surface area.

At pH values greater than the point in Fig. 7 where the two models diverge ($\approx$ pH 4), given the experimental conditions, both Eqs. 15 and 16 are dominated by $bk_a N_a^0$. Eventually, however, as N_a^0 decreases and N_{ha}^0 increases with decreasing pH, the first term $2k_a$ begins to dominate Eq. 15 while the second term, $bk_{ha} N_{ha}^0$, begins to dominate Eq. 16. Thus their eventual divergence.

The class - 2 model also includes the dimer AHA^- . Thus, the flux times involve, sequentially, the heterodimer's formation, its movement across the membrane's interior, the dimer's dissociation into A^- and HA and diffusion of HA back across the interior. All the Eqs. 3 - 8 are, therefore, involved.

The class - 2 theoretical curve shown in Fig. 8 below clearly fits the experimental better than those of Fig. 7.

The results showed that throughout the pH range an increase in k_f decreased τ_1 while an increase in k_d had the opposite affect on τ_1 . Both k_a or k_{raha} inversely affected τ_1 above the pK_a , with the effects of k_{raha} restricted to pH values greater than 8. Below the pK_a the inverse effect of k_{2ha} was very strong.

In analyzing all the rate constants both here and below for significance, each was varied by a factor of 2 and then 1/2 to judge the effect on the parameter in question.

Figure 8

Theoretical τ_1 curve for the class - 2 model as a function of pH compared to the observed values of τ_1

2. β_1

In Fig. 9 below the class - 2 theoretical curve for the amplitude, β_1 , is compared to the experimental values.

Figure 9

Theoretical β_1 curve for the class - 2 model as a function of pH compared to the observed values of β_1

Particularly significant is the sharp decrease in amplitude around the pK_a of PCP where the concentration of AHA^- is at a maximum. The decrease in amplitude is dependent almost exclusively on k_f and k_d . The theoretical amplitude at 5.5 is lowered from 0.73 to 0.40 when k_f is doubled and k_d is halved. When k_f is halved and k_d is doubled the theoretical amplitude at 5.5 is increased to 0.92. These changes correspond to changes in the dimer surface density suggesting that changes in N_{aha}^0 have a dramatic and inverse affect on the amplitude near the pK_a .

The theoretical decrease in β_1 , described above when k_f was doubled and k_d halved, was taken up by a time constant in the range of 10^{-4} s which depended almost exclusively on k_{aha} .

At pH 6.0 the difference between τ_1 and τ_2 and their respective amplitudes (presumably ≈ 0.8 and 0.2 , respectively) made it difficult to experimentally separate the amplitudes with sufficient accuracy. Therefore, as noted, the amplitudes were combined and the data point at pH 6.0 excluded from Fig. 9.

D. GENERAL COMMENTS ON τ_2 AND τ_3 AND THEIR ASSOCIATED AMPLITUDES

Difficulties arose with respect to τ_2 and τ_3 above the pK_a of PCP. None of the

theoretical time constants derived from the matrix matched quantitatively or qualitatively their respective associated data in Table 1. Since the theoretical amplitudes of τ_2 and τ_3 were very small away from the pK_a of PCP compared to their experimental counterparts, the theoretical amplitudes were of marginal use in identifying relevant time constants.

Although the theoretical time constants below the pK_a of PCP could be made to agree with their respective experimental time constants, in the case of τ_2 ($pH < pK_a$) the associated amplitude was too small by many orders of magnitude.

E. τ_2 AND ITS ASSOCIATED AMPLITUDE

1. τ_2

The data for τ_2 seems widely separated above and below pH 7.0, as Fig. 10 clearly shows. The theoretical curve shown in Fig. 10 was dependent on k_D . The value of k_D was adjusted to give the best fit to τ_2 below pH 7.

No theoretical adjustment could explain the dependence of τ_2 on $pH \geq 7.0$ or the sudden increase in amplitude below pH 7.0.

Figure 10

Theoretical τ_2 curve for the class - 2 model as a function of pH compared to the observed values of τ_2 .

The theoretical curve was based on $\tau_2^{-1} = k_D N_{\text{ba}}^0$.

2. β_2

The amplitude for the theoretical curve in Fig. 10 was of the order of 10^{-10} below the pK_a . Therefore, no graph of β_2 is shown.

F. τ_3 AND ITS ASSOCIATED AMPLITUDE

1. τ_3

As indicated in Table 1 in two cases, pH 6.0 and 4.5, no time constants were observed in the presumed range of a few hundred μ s. Given the small amplitudes of all the τ_3 measurements, such missing data points might be expected.

Like τ_2 there is a similar discontinuity in τ_3 above and below pH 7.0. The discontinuity is similar in that at and above pH 7.0 the pH dependence of τ_3 is very strong, while below pH 7.0 τ_3 is comparatively constant.

The theoretical curve in Fig. 11 below depended essentially on k_{aha} .

Figure 11

Theoretical τ_3 curve for the class - 2 model as a function of pH compared to the observed values of τ_3 .

The theoretical curve was based on $\tau_3^{-1} = 2k_{\text{aha}} + bk_{\text{aha}}N_{\text{aha}}^0$.

2. β_3

The amplitudes for the theoretical curve in Fig. 11 ranged from 6×10^{-4} at pH 9.5, 0.27 at pH 5.5 and 5×10^{-3} at 1.8. Although they matched the observed amplitudes in a few instances, given their wide range little significance could be given to them. Therefore, no plot is shown.

G. τ_4 AND ITS ASSOCIATED AMPLITUDE

Although a fourth time constant was observed around the pK_a no theoretical counterpart was found. The significance of τ_4 and β_4 was not clear.

H. EVALUATION OF THE RATE CONSTANTS

Not all the rate constants were sensitive to the curve fitting routines described above. In particular, k_{haha} and k_{poh} could only be found in their upper limit where they both degraded the theoretical fit for τ_1 . In a similar way the theoretical curves were insensitive to k_{ha} , except in this case only a lower limit could be found.

The curve fitting routine for τ_1 was particularly sensitive to k_a , k_{raha} , k_{2ha} , k_f and k_d . To a lesser extent, but still significant, a reasonable evaluation of the parameters k_R and k_{aha} could be made, the latter also affecting the theoretical curve for τ_3 dramatically. The rate constant k_D was entirely determined from its effect on the theoretical curve for τ_2 below the pK_a .

The rate constants, as evaluated by the lipophilic, class - 1 and class - 2 models are given below in Table 3.

Rate Constants As Determined By The Three Proposed Models						
Lipophilic ion model						
ka						
1.93×10^{-4}						
Class - 1 model						
ka	kha	kD	kDD	kR	kRR	
1.93×10^{-5}	2.76×10^4	$\approx 2 \times 10^{11}$	≈ 200	$\approx 4 \times 10^{12}$	$\approx 6 \times 10^4$	
Class - 2 model						
ka	kha	kD	kDD	kR	kRR	
6.65×10^{-5}	> 200	2.43×10^{11}	$\approx 2 \times 10^{-8}$	$\approx 6 \times 10^{12}$	$\approx 4 \times 10^{-5}$	
kaha	khaha	k2ha	kpoh	kraha	kf	kd
1.24×10^3	< 10^{10}	8.80×10^6	< 10^{14}	8.00×10^5	2.58×10^9	71.9

TABLE 3

Estimated values of the rate constants providing the best least squares fit to the observed data as illustrated in Figs. 8 - 11

III. DISCUSSION

A. GENERAL MODEL FOR PCP TRANSPORT

The results for τ_1 are particularly significant because the mechanisms involving τ_1 experimentally carry over 70% of the membrane's conductivity due to PCP and exceeds 85% of the conductivity only one pH unit in either direction away from the pK_a . Therefore, understanding those mechanisms is of primary concern.

From the theoretical curves of the three proposed models, Figs. 7 and 8, clearly the class - 2 model is the most viable description.

A further insight can be gathered by considering the flux times implied by the class - 2 model, namely, the flux times involved in the formation of the dimer, its transport across the membrane interior, its dissociation and, finally, the transport of HA back across the membrane.

The formation of the dimer involves three rate constants, k_f , k_{2ha} and k_{raha} , Eqs. 4 - 6. All three processes act in parallel and therefore their characteristic times add reciprocally,

$$\tau_{formation}^{-1} = bk_f N_a^0 N_{ha}^0 + bk_{2ha} N_{ha}^0 N_{ha}^0 + bk_{raha} N_a^0 N_a^0 \quad (17)$$

In a similar way the dissociation of the dimer involves k_d , k_{haha} and k_{poh} and should likewise be added reciprocally. However, only k_d is given below since only upper limits could be established for the other two rate constants, suggesting they have little effect on τ_1 .

$$\tau_{dissociation}^{-1} = bk_d N_{aha}^0 \quad (18)$$

The flux time across the interior of the membrane becomes simply

$$\tau_{interior}^{-1} = bk_{aha} N_{aha}^0 \quad (19)$$

The flux time for the back flow of HA, involving the k_{ha} process in a fashion similar to the k_{aha} process, is neglected since the results showed that changing k_{ha} did not affect τ_1 significantly.

Therefore, combining Eqs. 17 - 19, a rough estimate of the characteristic time for class - 2 movement of the dimer becomes,

$$\tau_{class2-AHA} = \tau_{formation} + \tau_{interior} + \tau_{dissociation} \quad (20)$$

Operating in parallel with the class - 2 movement of AHA^- is the lipophilic movement of A^- , Eq. 15, which may be important at high pH. Thus, the test function for τ_1 becomes

$$\tau_{1\text{-test}}^{-1} = \tau_{\text{class2-AHA}}^{-1} + \tau_{\text{lipoA}}^{-1} \quad (21)$$

As Fig. 12 demonstrates $\tau_{1\text{-test}}$ is reasonable fit to the data.

Figure 12

A comparison of $\tau_{1\text{-test}}$, Eq. 21, and the theoretical class - 2 τ_1 curve to the observed values of τ_1

At pH 9 $\tau_{\text{class2-AHA}}$ and τ_{lipoA} are comparable, 15 s and 12 s, respectively. Above pH 9 τ_{lipoA} dominates and below pH 9 $\tau_{\text{class2-AHA}}$ dominates.

This implies that above pH 9, PCP acts very much like a lipophilic ion with A₁ dominating the conduction. Below pH 9, the class - 2 movement of AHA₁ starts to dominate the conduction as the concentration of dimer begins to increase.

It was noted above that the k_{raha} process also influences τ_1 at high pH. Table 4 below illustrates k_{raha} 's effect with the third column of Table 4 being the value of k_{raha} implied by the theoretical curve of Fig. 8.

Influence of k_{raha} on τ_1			
pH	$2k_{\text{raha}}$	$k_{\text{raha}}/2$	k_{raha}
6.0	63.9 ms	64.0 ms	64.0 ms
7.0	0.310 s	0.315 s	0.314 s
8.0	2.05 s	2.29 s	2.20 s
9.0	5.43 s	7.45 s	6.63 s
10.0	6.51 s	9.66 s	8.32 s

11.0	6.44 s	9.99 s	8.54 s
12.0	6.66 s	9.99 s	8.56 s
13.0	6.66 s	9.99 s	8.56 s

Table 4

Effect on τ_1 of changing k_{rha} by a factor of 2 and 1/2 between pH 6 - 13

At high pH the surface concentration of AHA^- is very low due to the lack of HA. The involvement of the k_{rha} process at high pH would suggest, given Eq. 6, that sufficient AHA^- is supplied by the collision of A^- ions to significantly affect the conduction of PCP, even at pH 13, in a region where the conduction would otherwise be exclusively lipophilic conduction of A^- .

In a similar fashion, at low pH, where the concentration of AHA^- would be considered very low due to the lack of A^- , the k_{2ha} process enhances the dimer conduction. In this case, as Eq. 5 implies, collisions between HA molecules supply the necessary AHA^- ions. Table 5 illustrates this effect.

Influence of k_{2ha} on τ_1			
pH	$2k_{2ha}$	$k_{2ha}/2$	k_{2ha}
6.0	63.9 ms	64.0 ms	64.0 ms
5.0	62.6 ms	63.4 ms	63.1 ms
4.0	25.3 ms	28.9 ms	27.5 ms
3.0	0.816 s	1.40 s	1.12 s
2.0	1.41 s	4.65 s	2.62 s
1.0	1.38 s	5.39 s	2.73 s

Table 5

Effect on τ_1 of changing k_{2ha} by a factor of 2 and 1/2 between pH 1 - 6

Given the effects of the k_{raha} and k_{2ha} processes it is interesting to note what fraction of the membrane's conductivity is carried by the class - 2 transport of AHA^- compared to the combined class - 2 transport of AHA^- and the lipophilic transport of A^- as a function of pH. This can be done by evaluating the following formulation

$$\text{Fraction of Flux} = \frac{\tau_{\text{class2-AHA}}^{-1}}{\tau_{\text{class2-AHA}}^{-1} + \tau_{\text{lipoA}}^{-1}} \quad (22)$$

Table 6 below gives the fraction of flux due to class - 2 conduction of AHA⁻, in terms of a percentage, over a range of pH from pH 13 to 1.

Conductivity Due To AHA ⁻ (%)													
pH	13	12	11	10	9	8	7	6	5	4	3	2	1
%	27	29	29	31	45	82	98	100	100	100	100	100	100

Table 6

Percent of ion flux across the interior of the membrane due to AHA⁻ compared to the total ion flux due to both AHA⁻ and A⁻

Thus, even at the extremes of pH away from the pK_a of PCP dimer conduction is significant and essentially due to the reactions, Eqs. 5 and 6, characterized by the k_{rthA} and k_{2thA} processes.

The effects of k_r and k_d were significant throughout the full range of pH not

only on τ_1 but also on β_1 as Table 7 illustrates.

Influence of k_f and k_d on τ_1 and β_1						
pH	$2k_f \quad k_d/2$		$k_f/2 \quad 2k_d$		$k_f \quad k_d$	
	τ	β	τ	β	τ	β
13.0	8.53 s	1	8.63 s	1	8.56 s	1
12.0	8.52 s	1	8.63 s	1	8.56 s	1
11.0	8.48 s	1	8.61 s	1	8.54 s	1
10.0	8.06 s	1	8.50 s	1	8.32 s	1
9.0	5.37 s	1	7.55 s	1	6.63 s	1
8.0	1.27 s	0.98	3.57 s	1	2.20 s	1
7.0	0.178 s	0.85	0.604 s	0.99	0.314 s	0.99
6.0	52.5 ms	0.47	0.110 s	0.94	64.0 ms	0.79

5.0	51.7 ms	0.47	0.108 s	0.94	63.1 ms	0.78
4.0	0.160 s	0.83	0.502 s	0.99	0.275 s	0.95
3.0	0.743 s	0.84	1.62 s	0.99	1.12 s	0.97
2.0	2.52 s	0.97	2.74 s	0.99	2.62 s	0.98
1.0	2.70 s	0.99	2.80 s	1	2.73 s	1

Table 7

Effect of doubling and halving k_f and k_d on τ_1 between pH 1 - 13

The effects of k_f and k_d on τ_1 become more understandable when considering their effect on the surface density of the dimer. From Appendix C,

$$N_{aha}^0 = \frac{k_f N_a^0 N_{ha}^0 + k_{raha} N_a^0 N_a^0 + k_{2ha} N_{ha}^0 N_{ha}^0}{k_d} \quad (23)$$

where k_{poh} and k_{haha} have been neglected.

Their effect, then, results from the influence they have on the surface density of the dimer.

The decrease in β_1 may seem a contradiction given the maximum value of $k_f N_a^0 N_{ha}^0 / k_d$ near the pK_a . A theoretical analysis of this problem shows the decreasing amplitude of β_1 is taken up, theoretically at least, by β_3 discussed below. It follows from the model that τ_3 is exclusively identified with the lipophilic transport of AHA^- across the membrane interior before the class - 2 chain of kinetically coupled reactions has had time to establish itself. Thus, it is precisely the increased surface density of AHA^- around its pK_a that allows the lipophilic mode of transport to depress β_1 .

The decrease in β_1 has a logical theoretical and physical basis. However, even a casual inspection of the experimental data in Table 1 shows the decreasing amplitude of β_1 is taken up by β_2 , not β_3 . This is a question which remains unanswered.

It is useful to calculate the surface density of AHA^- as a function of pH, which has been done in Fig 13.

Figure 13

The surface density (mol/cm 2) of the dimer AHA $^{\cdot}$ calculated from Eq. C26, Appendix C, which is essentially Eq. 23 above

The leveling off of N_{aha}^0 at low pH is due to dimer creation produced by the by the k_{2ha} process, Eq. 5. This is an unexpected and, hitherto, unobserved result mainly because previous investigators have not measured the conductivity of PCP at such low pH values.

The inability to measure any effects due to the k_{haha} and k_{poh} processes, the

lack of measurable effects due to the k_D process on τ_1 or β_1 and the very marginal effect of the k_R process is not without implication. All these rate constants involve the interaction of H^+ and OH^- directly with HA , A^- or AHA^- . Presumably these direct reactions would be very fast as suggested by work of McLaughlin's laboratory (McLaughlin and Dilger, 1980; Kasianowicz et al, 1987), certainly less than a few μs which was the lower limit of this investigation.

B. BOUNDARY CONDITIONS AND THE UNIQUENESS OF THE DETERMINED RATE CONSTANTS

In the previous section the determination of all the surface densities and two of the rate constants were derived from the data of other investigators or from the boundary conditions implied by the class - 2 model. The remaining eleven time constants were presumably to be determined from one additional boundary condition on the amplitudes and from ten observed time constants and their associated amplitudes. As discussed above and shown explicitly in Table 3 only upper or lower limits could be given to three of the rate constants, k_{ha} , k_{haha} and k_{poh} , and one rate constant, k_R , was known only marginally. Thus, of the eleven remaining rate constants, four were essentially eliminated from the curve fitting

routine since they had no effect on the results. Physically they likely characterized processes that were too fast and/or of such low amplitude that the experimental apparatus was unable to detect their effect. One of the remaining seven rate constants was determined from τ_2 , k_D , and another from τ_3 , k_{aha} (discussed below).

As a result, five rate constants were left, k_a , k_f , k_d , k_{raha} and k_{2ha} to be analyzed in terms of τ_1 and β_1 . Mathematically, this would imply that the five rate constants could be found explicitly only in terms of two ratios and, therefore, considerable ambiguity would remain.

Experimentally, however, the situation was more favorable. At very low pH only the k_{2ha} process was effective in determining τ_1 as Tables 4, 5 and 7 illustrate. The same Tables show that at high pH k_{raha} was significant and Table 6 shows the significance of k_a at high pH. The influence of the k_a process, transport of A^- across the membrane interior, was effective at lower pH values than was the k_{raha} process and thus the two could be separated. The main effect of the k_f and k_d processes was around the pK_a where they effected both τ_1 and β_1 and could thus be separated. Experimentally, then, data from τ_1 and β_1 was able to distinguish the five remaining rate constants. Physically, this separation of the rate constants follows from the fact that k_{2ha} , k_{raha} , k_a , and the pair $k_f - k_d$ are derived from very different processes effective over different pH ranges.

B. DIFFICULTIES WITH THE SHORTER TIME CONSTANTS

The essential difficulties with τ_2 and τ_3 are contained in Figs. 10 and 11. None of the models discussed here, including the class - 2 model, fit the data even qualitatively. The theoretical curves shown in Figs. 10 and 11, derived in the same manner as the other theoretical curves, from the eigenvalues of matrix A, Eq. 7, also corresponded within a few percent in the range shown to the following formulations,

$$\tau_2^{-1} = k_D N_{ha}^0 \quad (24)$$

$$\tau_3^{-1} = 2k_{aha} + bk_{aha}N_{aha}^0 \quad (25)$$

The value of k_D was adjusted to fit the low pH data for τ_2 . Since k_D had little effect on any other theoretical parameter the adjustment was simple. However, given the difficulties surrounding τ_2 and its interpretation, the estimated numerical value of k_D and its implications are suggestive rather than persuasive.

With τ_3 and Eq. 25, however, the adjustable limits of k_{aha} were not as

arbitrary since even adjustments of 10% marginally began to affect τ_1 .

Eq. 25 is, in fact, the theoretical formulation for the lipophilic movement of AHA^- across the membrane interior as would be derived in Appendix A. This observation is the rationale for the above comment that, theoretically, the decrease in β_1 around the pK_a is due to the contribution of the lipophilic ion mode of transport of AHA^- .

The major qualitative problem, shown in Figs. 10 and 11, is the pH dependence of the observed shorter time constants at $\text{pH} > \text{pK}_a$. It appears that there are two processes involved, one at higher pH which is strongly pH dependent and the other at low pH which is pH independent.

No resolution to this problem was found. The possibility of protonic leakage was considered despite investigations (Gutman et al, 1995; Deamer and Volkov, 1995) showing that protonic leakage does not depend on pH. The idea that aqueous channels spontaneously open up across the membrane allowing a net flux of protons to be transported through the channels due to concentrations differences on either side of the membrane or due to an imposed electric field, such as a charge pulse experiment would induce, is mathematically modeled in Appendix D. Computed results based on the theory outlined in Appendix D, in fact, degraded the theoretical fit for τ_1 and showed no pH dependence.

The suggestion that the hydrolysis of water, $\text{H}^+ + \text{OH}^- \leftrightarrow \text{H}_2\text{O}$, might be limiting in the context of membrane surfaces was also modeled in Appendix D with no useful experimental results.

C. COMPARISONS WITH THE RESULTS OF OTHER INVESTIGATORS

1. Previous Investigations

As indicated previously (section I C) the class - 2 nature of PCP has been invoked by investigators. Some of those results have been used in this study (Smejtek et al, 1976; Pickar and Hobbs, 1982; Smejtek et al, 1987a; Smejtek et al, 1989, Smejtek and Wang, 1990). The results presented here provide greater insight into the nature of PCP - induced membrane electrical conductivity.

Barstad et al (Barstad et al, 1993) attempted to measure the formation constant of AHA^- dimers in various lipid membranes but their results were somewhat inconclusive because the dimer surface density proved too small to measure directly. The lower detection limit of their spectrophotometric method was $\approx 10^{-11}$ mol/cm². The results here, as illustrated in Fig. 13, imply that the maximum dimer surface density reached 9×10^{-14} mol/cm².

However, Smejtek et al (Smejtek et al, 1976) did directly measure the steady state conductivity induced by PCP (25 μ M) between pH 4 and 7 in phospholipid-cholesterol membranes. The conductivity per unit area was assumed to be

$$G = \frac{F^2 k_{aha} N_{aha}^0}{RT} \quad (26)$$

Their results for phosphatidylcholine (egg lecithin) membranes are reproduced below along with the theoretical conductivity curve using Eq. 26 and the kinetic constants contained in the present work. The agreement between predicted and experimental electrical conductivity is quite remarkable considering the different kinds of lipids and methods used in these two types of studies

Figure 14

Theoretical conductivity (S/cm²) based on Eq. 26 and the results of this investigation for 30 μ M PCP compared with the experimental results of Smejtek et al (Smejtek et al, 1976) for 25 μ M PCP

Pickar and Hobbs (Pickar and Hobbs, 1982) used alternating current methods at pH 5.4 to investigate the effects of PCP on the electrical conductivity of egg-lecithin membranes and concluded that processes that involved the creation of A^- independent of the dimer AHA^- , K_{RR} , Eq. 2, dominated over the destruction of A^- as the result of dimer formation, $k_f N_{HA}^0$, Eq. 4. Conversely, they concluded, that processes that involved the destruction of A^- ions independent of the dimer

AHA^- , $k_R N_h^0$, Eq. 2, were dominated by creation of A^- due to dimer dissociation, k_d , Eq. 4. Their investigation did not include the OH^- ion and thus was based on Eqs. 2 and 4. They suggested that, in terms of the parameters defined in this report, $k_{RR} \gg k_f N_{ha}^0$ and $k_d \gg k_R N_h^0$ which implies the A^- species was long lived compared to the dimer AHA^- . At pH 5.4 the first inequality is not supported by this report.

However, if their suggestion is expanded to include the OH^- ion, the implication is that $k_{RR} + k_D N_{ha}^0 \gg k_f N_{ha}^0$ and $k_d \gg k_R N_h^0 + k_{DD}$, a result that includes Eq. 3. The first inequality is now true at any pH and the second is true for any $pH > 3$. Therefore, it would seem, based on this report, that their assumptions masked the effects of the OH^- ion but also that their essential conclusion is supported here.

2. New Advances

The class - 1 and class - 2 models presented in Appendices B and C, respectively, expand previous models by including the effects of both H^+ and OH^- .

The inclusion of both H^+ and OH^- was made possible by a mathematical approach to class - 1 and class - 2 modeling using matrix theory and taking

advantage of the resulting eigenvalues and eigenvectors to determine the time constants and amplitudes of interest. Also new in both cases is the development of charge pulse measurement theory utilizing the matrix approach.

Theoretically, then, all the effects suggested by Eqs. 2 - 3 and Eqs. 2 - 6 can be analyzed using the expanded models of Appendices B and C, respectively. And also, as a consequence, the surface densities of HA, A⁻ and AHA⁻ can potentially be determined explicitly as a function of pH and compared to the results of A⁻ and AHA⁻ membrane sorption studies.

Experimentally, the situation is more difficult since even the theory suggests that many of the theoretical time constants and amplitudes would likely be too small to observe.

However, several new aspects of PCP - induced membrane conduction did emerge from this investigation that have not been previously reported.

The reactions involving the k_{raha} and k_{zha} processes, Eqs. 5 and 6, have not been proposed in the literature. The determination of these rate constants and their effect, demonstrated by Tables 3, 4 and 5, have now been shown to be significant even at pH values far from the pK_a of PCP where the surface concentration of AHA⁻ formed by the conventional recombination of HA and A⁻ would seem to exclude any conduction at all.

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APPENDIX A

LIPOPHILIC IONS

Part I

Derivation of Fully Linearized Kinetic Equations for Lipophilic Ions

Following Benz, et al, (Benz, et al, 1976) the basic differential equations describing the transmembrane transport of lipophilic ions are:

$$\frac{dN_a'}{dt} = k_{aqm}'c - k_{maq}'N_a' - k_a'N_a' + k_a''N_a'' \quad A1$$

$$\frac{dN_a''}{dt} = k_{aqm}''c - k_{maq}''N_a'' - k_a''N_a'' + k_a'N_a' \quad A2$$

where t is time, k_a' , k_a'' and N_a' , N_a'' are the transmembrane rate constants (s^{-1}) and adsorbed anion surface densities (moles per unit area), respectively, for A^- . k_{aqm}' and k_{aqm}'' are the rate constants (m/s) associated with movement of A^- from the aqueous solution to its adsorption on the membrane, k_{maq}' and k_{maq}'' are the rate constants (s^{-1}) associated with the movement of A^- from its adsorption on the membrane into the

aqueous solution and c is the molar aqueous concentration of A^- .

To transform Eqs. B1 and B2 into a more useful linear form, it is first assumed that the single and double primed rate constants for A^- are related in the following way:

$$k'_a = k_a e^{\frac{\alpha}{2}u} = k_a + \frac{\alpha}{2}k_a u \quad A3$$

$$k''_a = k_a e^{-\frac{\alpha}{2}u} = k_a - \frac{\alpha}{2}k_a u \quad A4$$

$$k'_{aqm} = k_{aqm} e^{\frac{1-\alpha}{4}u} = k_{aqm} + \frac{1-\alpha}{4}k_{aqm} u \quad A5$$

$$k''_{aqm} = k_{aqm} e^{-\frac{1-\alpha}{4}u} = k_{aqm} - \frac{1-\alpha}{4}k_{aqm} u \quad A6$$

$$k'_{maq} = k_{maq} e^{-\frac{1-\alpha}{4}u} = k_{maq} - \frac{1-\alpha}{4}k_{maq} u \quad A7$$

$$k''_{maq} = k_{maq} e^{\frac{1-\alpha}{4}u} = k_{maq} + \frac{1-\alpha}{4}k_{maq} u \quad A8$$

where u is the reduced external electric potential imposed across the membrane, $u = VF/RT$, where V is the actual imposed external voltage across the membrane, F is Faraday's constant, T is the temperature in Kelvin, and R is the gas constant. The term α is the fraction of the reduced voltage, u , which appears across the interior of

the membrane and $(1 - \alpha)/2$ is the fraction of u which appears across each aqueous - membrane interface.

The above linearization assumes the reduced externally imposed electric potential, u , is $\ll 1$ so that the exponential can be expanded, $e^x \rightarrow 1 + x$. The next step is to formulate a new parameter:

$$Y_a = N'_a - N''_a \quad A9$$

Because of the small voltage restriction it is assumed that during the time course of the experiment none of the surface densities depart significantly from their equilibrium states, and that cross terms between Y_a and u are insignificant and can be set to zero.

Also let N_a^0 be the equilibrium surface densities on each side of the membrane before the external electric potential is applied, that is, half the sum of the single and double primed surface densities at $t = 0$. Then at equilibrium

$$k_{aqm}c = k_{maq}N_a^0 \quad A10$$

Allow

$$r(t) = N_a' + N_a'' \quad A11$$

then adding Eqs. A1 - A2 and allowing $t \rightarrow \infty$

$$\frac{d(r)}{dt} \Big|_{t \rightarrow \infty} = 2k_{aqm}c - k_{maq}r(t \rightarrow \infty) = 0 \quad A12$$

Comparing Eqs. A10 and A12 it follows that

$$r(t \rightarrow \infty) = r(0) = 2N_a^o \quad A13$$

In general adding Eqs. A1 - A2 yields

$$\frac{dr}{dt} = 2k_{aqm}c - k_{maq}r \quad A14$$

with the general solution

$$r(t) = r(t \rightarrow \infty) + (r(0) - r(t \rightarrow \infty))e^{-\lambda t} \quad A15$$

Comparing Eqs. A13 and A15

$$r(t) = r(\infty) + (r(0) - r(\infty))e^{-\lambda t} \quad A15$$

Comparing Eqs. A13 and A15

$$r(t) = r(0) = r(\infty) = N_a' + N_a'' = 2N_a^o \quad A16$$

Using Eqs. A3 - A9, Eq. A10, and Eq. A16, Eqs. A1 - A2 are reduced to the following:

$$\frac{dY_a}{dt} = -(2k_a + k_{maq})Y_a + ((1 - \alpha)k_{maq} - 2\alpha k_d)N_a^o u \quad A17$$

which is now fully linearized.

For a membrane of electric capacitance C, the time course of the reduced potential in the charge pulse experimental method is

$$\frac{du}{dt} = \frac{F^2}{CRT} \left(\frac{1 - \alpha}{2} (k_{maq}' N_a' + k_{aqm}'' c - k_{maq}'' N_a'' - k_{aqm}' c) + \alpha (k_a'' N_a'' - k_a' N_a') \right) \quad A18$$

using the above linearizations

$$\frac{du}{dt} = b\left(\frac{1-\alpha}{2}k_{maq} - \alpha k_d\right)Y_a - b\left(\frac{(1-\alpha)^2}{2}k_{maq} + \alpha^2 k_d\right)N_a^o u \quad A19$$

where $b = F^2/CRT$.

In general, Eqs. A17 and A19 comprise a complete set of linearized differential equations with constant coefficients characterizing the lipophilic mode of ion transport.

In the case where $\alpha \approx 1$ Eqs. A17 and A19 reduce to

$$\frac{dY_a}{dt} = -(2k_a + k_{maq})Y_a - 2k_a N_a^o u \quad A20$$

$$\frac{du}{dt} = -bk_a Y_a - bk_a N_a^o u \quad A21$$

As $u(t \rightarrow \infty) du/dt \rightarrow 0$. From Eq. A19

$$\begin{aligned} \frac{du}{dt} \Big|_{t \rightarrow \infty} &= b\left(\frac{1-\alpha}{2}k_{maq} - \alpha k_d\right)(N_a' - N_a'') \Big|_{t \rightarrow \infty} \\ &- b\left(\frac{(1-\alpha)^2}{2}k_{maq} + \alpha^2 k_d\right)N_a^o u(\infty) = 0 \end{aligned} \quad A22$$

or

$$u(\infty) = \frac{\left(\frac{1-\alpha}{2}k_{maq} - \alpha k_d\right)(N_a' - N_a'') \Big|_{t \rightarrow \infty}}{\left(\frac{(1-\alpha)^2}{2}k_{maq} + \alpha^2 k_d\right)N_a^o} + u^0 \quad A23$$

Thus, to the extent that enough A^- transports across the membrane to equilibrate u^0 , $u(\infty) \rightarrow 0$. In the case of lipophilic ions where, essentially a reservoir of A^- that can potentially cross the membrane for subsequent transmembrane transport, it can be assumed that $u(\infty) \rightarrow 0$. In Part II, Eq. A24, $u(\infty)$ assumed to be zero.

Part II

Determination of the Rate Constants by the Charge Pulse Method

In the charge pulse methodology an electric charge is placed on the membrane which is then electrically isolated from the charging circuit through a high impedance switch while the voltage decay across the membrane is measured. Therefore, Eq. A19 is a theoretical expression for the time course of the voltage decay in the charge pulse method.

The property of the charge pulse method is that for any kinetically linear system, where $u(\infty) \rightarrow 0$, the time dependence of the membrane potential difference is the sum of exponentially decaying components. In the case of lipophilic ions (Eqs. A17 and A19) two time constants are implied:

$$u = u^0(\beta_1 e^{-\frac{t}{\tau_1}} + \beta_2 e^{-\frac{t}{\tau_2}}) \quad A24$$

where u^0 is the imposed reduced voltage across the membrane at $t = 0$ and the β 's and τ 's are the amplitudes and associated time constants, respectively, of the observed voltage decay. Obviously,

$$\beta_1 + \beta_2 = 1 \quad \text{A25}$$

In matrix notation Eqs. A17 and A19 become

$$\begin{pmatrix} \frac{dY_a}{dt} \\ \frac{du}{dt} \end{pmatrix} = \begin{pmatrix} -2k_a - k_{maq} & (1 - \alpha)k_{maq}N_a^0 - 2\alpha k_a N_a^0 \\ b\frac{1 - \alpha}{2}k_{maq} - b\alpha k_a & -b\frac{(1 - \alpha)^2}{2}k_{maq}N_a^0 - b\alpha^2 k_a N_a^0 \end{pmatrix} \begin{pmatrix} Y_a \\ u \end{pmatrix} \quad \text{A26}$$

The eigenvalue solution to Eq. A26 generates eigenvalues which are identical to the above reciprocal time constants in Eq. A24. Defining λ_i to be the i th eigenvalue implied by Eq. A26, and therefore the i th reciprocal time constant, τ_i^{-1} , given in Eq. A24, the eigenvalues of Eq. A26 are determined from the following determinant equation:

$$\det \begin{vmatrix} \lambda - 2k_a - k_{maq} & (1 - \alpha)k_{maq}N_a^0 - 2\alpha k_a N_a^0 \\ b\frac{1 - \alpha}{2}k_{maq} - b\alpha k_a & \lambda - b\frac{(1 - \alpha)^2}{2}k_{maq}N_a^0 - b\alpha^2 k_a N_a^0 \end{vmatrix} = 0 \quad \text{A27}$$

Expansion of the above determinant in powers of λ yields

$$\lambda^2 - Q1\lambda + Q2 = 0 \quad A28$$

where

$$Q1 = 2k_a + k_{maq} + Q4 \quad A29$$

$$Q2 = bk_{maq}k_a N_a^o \quad A30$$

The expression Q4 above is derived from Eq. A19.

$$Q4 = -\frac{1}{u^o} \frac{du}{dt} \Big|_{t=0} = b \left(\frac{(1 - \alpha)^2}{2} k_{maq} + \alpha^2 k_d \right) N_a^o \quad A31$$

From the time derivative of Eq. A24 and from Eq. A25 and A31,

$$\beta_1 = \frac{Q4 - \lambda_2}{\lambda_1 - \lambda_2} \quad A32$$

The reason for the form of Eq. A28 and for deriving the expression Q4 is to obtain expressions for the rate constants and the surface density (Eqs. A17 and A19) in terms of observed time constants and associated amplitudes defined by Eq. A24. This is done first by defining two new parameters:

$$P1 = \lambda_1 + \lambda_2 \quad A33$$

$$P2 = \lambda_1 \lambda_2 \quad A34$$

where λ_i is the i th eigenvalue of Eq. A27.

From P1 and P2 the following identity, valid for any $\lambda = \lambda_i$, can be immediately formed

$$\lambda^2 - P1\lambda + P2 \equiv 0 \quad A35$$

which is analogous to Eq. A28 for the same eigenvalues. From the time derivative of Eq. A24, a new parameter is defined:

$$P4 = -\frac{1}{u^o} \frac{du}{dt} \Big|_{t=0} = \beta_1 \lambda_1 + \beta_2 \lambda_2 \quad A36$$

Obviously, $Q_i = P_i$ where $i = 1, 2$, and 4 . Thus, though the algebra is tedious the rate constants and surface density in expressions Q_1 , Q_2 , and Q_4 can be solved in terms of P_1 , P_2 , and P_4 :

$$k_a = \frac{1}{4P_4} ((P_1 - P_4)P_4 + (1 - 2\alpha)P_2 + \sqrt{(P_1 - P_4)^2P_4^2 - 2(2\alpha^2 - 2\alpha + 1)(P_1 - P_4)P_4P_2 + (1 - 2\alpha)^2P_2^2}) \quad A37$$

$$k_{maq} = P_1 - P_4 - 2k_a \quad A38$$

$$N_a^o = \frac{P_2}{bk_a k_{maq}} \quad A39$$

$$k_{aqm} = \frac{k_{maq}N_a^o}{c} \quad A40$$

$$Q_4 = P_4 \quad A41$$

A special case is when $\alpha \approx 1$. This case is useful if the reduced voltage u is considered to be primarily across the interior of the membrane with little or no electric potential difference being present across the aqueous - membrane interface. For PCP, in particular, this is the conventional assumption. The expressions for k_a , and k_{maq} are greatly simplified.

$$k_a = \frac{PIP4 - P4^2 - P2}{2P4} \quad A42$$

$$k_{maq} = \frac{P2}{P4} \quad A43$$

Generally, for the case of PCP, it is not only assumed that $\alpha = 1$ but that $k_{maq} = 0$ since it is assumed that PCP does not diffuse into or adsorb from the aqueous solution during the time course of the experiment. Following the methods described above only one time constant is found:

$$\tau^{-1} = 2k_a + bk_a N_a^0 \quad A44$$

It should be noted that the classic lipophilic theory derived here follows the development first given by Benz et al (Benz et al, 1976) has been questioned on theoretical grounds (M. Takeo, private communication).

APPENDIX B

CLASS - 1 UNCOUPLERS

Part I

Derivation of Fully Linearized Kinetic Equations for Class - 1 Uncouplers

The basic differential equations describing transmembrane transport of class - 1 uncouplers for a weak acid (HA) with its conjugate anion (A⁻) include surface reactions involving A⁻ and HA, respectively. Also included are the following surface reactions with respect to H⁺ and OH⁻, their respective rate constants being noted to the right.

where the units for k_{RR} and k_{DD} are s⁻¹ and k_R and k_D are cm² s⁻¹ moles⁻¹.

The complete set of differential equations describing class - 1 uncouplers becomes, then,

$$\frac{dN'_{ha}}{dt} = -k_{RR}N'_{ha} + k_R N'_a N'_h + k_{DD}N'_a - k_D N'_{ha} N'_{oh} + k_{ha} N''_{ha} - k_{ha} N'_h \quad B3$$

$$\frac{dN''_{ha}}{dt} = -k_{RR}N''_{ha} + k_R N''_a N''_h + k_{DD}N''_a - k_D N''_{ha} N''_{oh} - k_{ha} N''_{ha} + k_{ha} N'_h \quad B4$$

$$\frac{dN'_a}{dt} = k_{RR}N'_{ha} - k_R N'_a N'_h - k_{DD}N'_a + k_D N'_{ha} N'_{oh} + k''_a N''_a - k'_a N'_a \quad B5$$

$$\frac{dN''_a}{dt} = k_{RR}N''_{ha} - k_R N''_a N''_h - k_{DD}N''_a + k_D N''_{ha} N''_{oh} - k''_a N''_a + k'_a N'_a \quad B6$$

$$\frac{dN'_h}{dt} = k_{RR}N'_{ha} - k_R N'_a N'_h \quad B7$$

$$\frac{dN''_h}{dt} = k_{RR}N''_{ha} + k_R N''_a N''_h \quad B8$$

$$\frac{dN'_{oh}}{dt} = k_{DD}N'_a - k_D N'_{ha} N'_{oh} \quad B9$$

$$\frac{dN''_{oh}}{dt} = k_{DD}N''_a - k_D N''_{ha} N''_{oh} \quad B10$$

where k_{ha} is the rate constant (s^{-1}) associated with the movement of HA across the membrane interior, N'_{ha} , N''_{ha} with the surface densities (in moles per unit area) of HA. N'_h , N''_h , and N'_{oh} , N''_{oh} are associated with the surface densities (in moles per unit area) of H^+ and OH^- , respectively.

The transformation Eqs. B3 to B10 into a more useful linear form proceeds in

the same manner as in Appendix A with the formation of four new parameters.

$$Y_{ha} = N'_{ha} - N''_{ha} \quad B11$$

$$Y_a = N'_a - N''_a \quad B12$$

$$Y_h = N'_h - N''_h \quad B13$$

$$Y_{oh} = N'_{oh} - N''_{oh} \quad B14$$

Let N_{ha}^0 , N_a^0 , N_h^0 and N_{oh}^0 be the equilibrium surface densities HA, A⁻, H⁺ and OH⁻, respectively, on each side of the membrane before the external electric potential is applied, where, if they do not cross the membrane interface,

$$N_{ha}^o = \frac{N_{ha}^{o'} + N_{ha}^{o''}}{2} \quad B15$$

$$N_a^o = \frac{N_a^{o'} + N_a^{o''}}{2} \quad B16$$

$$N_h^o = \frac{N_h^{o'} + N_h^{o''}}{2} \quad B17$$

$$N_{oh}^o = \frac{N_{oh}^{o'} + N_{oh}^{o''}}{2} \quad B18$$

Using Eqs. B11 - B18, Eqs. B3 - B10 are reduced to the following:

$$\frac{dY_{hs}}{dt} = -(2k_{hs} + k_{RR} + k_D N_{oh}^0)Y_{hs} + (k_R N_h^0 + k_{DD})Y_a + k_R N_a^0 Y_h^0 - k_D N_{hs}^0 Y_{oh} \quad B19$$

$$\frac{dY_a}{dt} = (k_{RR} + k_D N_{oh}^0)Y_{hs} - (2k_a + k_{DD} + k_R N_h^0)Y_a - k_R N_a^0 Y_h + k_D N_{hs}^0 Y_{oh} - 2k_a N_a^0 u \quad B$$

$$\frac{dY_h}{dt} = k_{RR}Y_{hs} - k_R N_h^0 Y_a - k_R N_a^0 Y_h \quad B21$$

$$\frac{dY_{oh}}{dt} = -k_D N_{oh}^0 Y_{hs} + k_{DD}Y_a - k_D N_{hs}^0 Y_{oh} \quad B22$$

For a membrane of electric capacitance C, the decaying time course of the reduced potential in the charge pulse experimental method is

$$\frac{du}{dt} = b(k_a'' N_a'' - k_a' N_a') = \frac{b}{2} \left(\frac{dY_a}{dt} + \frac{dY_{oh}}{dt} - \frac{dY_h}{dt} \right) \quad B23$$

using the above linearizations

$$\frac{du}{dt} = -bk_a Y_a - bk_a N_a^0 u \quad B24$$

where $b = F^2/CRT$.

Eq. B23 can be used to solve for u ,

$$u = \frac{b}{2}Y_a + \frac{b}{2}Y_{oh} - \frac{b}{2}Y_h + u^\circ \quad B25$$

This result will be useful later to eliminate u from Eq. B20 in terms of Y_a and the initial reduced voltage, u° .

Under equilibrium conditions, before the external voltage is applied across the membrane, Eqs. B7 - B10 immediately suggest two boundary conditions. Before the external voltage is applied across the membrane, $N' = N''$, regardless of the specific surface density in question. Adding Eqs. B7 and B8, the following result is easily obtained,

$$k_{RR} = \frac{N_a^0 N_h^0}{N_{ha}^0} k_R \quad B26$$

Likewise, adding Eqs. B9 and B10,

$$k_{DD} = \frac{N_{ha}^0 N_{oh}^0}{N_a^0} k_D \quad B27$$

A third boundary condition can be found by adding Eqs. B3 - B6 resulting in

$$\frac{d(N'_{ha} + N''_{ha} + N'_a + N''_a)}{dt} = 0 \quad B28$$

If N_0 = the total adsorbed concentration of HA and A⁻ at $t = 0$, then,

$$N_0 = 2N_a^0 + 2N_{ha}^0 \quad B29$$

By examining Eq. B1 at $pH = pK_a$ of PCP it is seen that

$$\frac{N_a^0}{N_{ha}^0} = 10^{pH - pK_a} \quad B30$$

Combining Eqs. B29 and B30,

$$N_a^0 = \frac{N_0}{2 (1 + 10^{(pK_a - pH)})} \quad B31$$

As $t \rightarrow \infty$, from Eq. B25,

$$u(\infty) = \frac{b}{2} [N'_a - N''_a]_{t \rightarrow \infty} + \frac{b}{2} [N'_{oh} - N''_{oh}]_{t \rightarrow \infty} - \frac{b}{2} [N'_h - N''_h]_{t \rightarrow \infty} + u^0 \quad B32$$

Thus, to the extent that the initial voltage across the membrane, u^0 , is equilibrated by concentration gradients of A^- , OH^- and/or H^+ ions across the membrane, $u(\infty) \rightarrow 0$. Particularly for small voltages, $u^0 < 1$, there would be, at least, a large reservoir of H^+ ions. In part II below, therefore, $u(\infty)$ is taken to be zero.

Part II

Determination Of The Rate Constants by the Charge Pulse Method

Proceeding as in Appendix A, with the assumption that $u(\infty) = 0$, the four linearly independent equations, Eqs. B19 - B22 in the charge pulse methodology imply 4 time constants:

$$u = u^0(\beta_1 e^{-\frac{t}{\tau_1}} + \beta_2 e^{-\frac{t}{\tau_2}} + \beta_3 e^{-\frac{t}{\tau_3}} + \beta_4 e^{-\frac{t}{\tau_4}}) \quad B33$$

$$\beta_1 + \beta_2 + \beta_3 + \beta_4 = 1 \quad B34$$

The matrix equation suggested from Eqs. B19 - B22, when Eq. B26 is used to eliminate u , takes the form

$$\begin{pmatrix} \frac{dY_{ha}}{dt} \\ \frac{dY_a}{dt} \\ \frac{dY_h}{dt} \\ \frac{dY_{oh}}{dt} \end{pmatrix} = A \begin{pmatrix} Y_{ha} \\ Y_a \\ Y_h \\ Y_{oh} \end{pmatrix} - u^0 \begin{pmatrix} 0 \\ 2k_a N_a^0 \\ 0 \\ 0 \end{pmatrix} \quad B35$$

where A is a 4x4 matrix whose columns are shown below

$$\begin{array}{cc}
-2k_{ha} - k_{RR} - k_D N_{oh}^0 & k_R N_h^0 + k_{DD} \\
k_{RR} + k_D N_{oh}^0 & -2k_a - bk_a N_a^0 - k_{DD} - k_R N_h^0 \\
k_{RR} & -k_R N_h^0 \\
-k_D N_{oh}^0 & k_{DD}
\end{array}
\quad
\begin{array}{cc}
(1) & (2)
\end{array}$$

$$\begin{array}{cc}
k_R N_a^0 & k_D N_{ha}^0 \\
bk_a N_a^0 - k_R N_a^0 & -bk_a N_a^0 - k_D N_{ha}^0 \\
-k_R N_a^0 & 0 \\
0 & k_D N_{ha}^0
\end{array}
\quad
\begin{array}{cc}
(3) & (4)
\end{array}$$

The eigenvalue solutions, λ_i ($i = 1-4$), to the determinant of matrix A are the reciprocal time constants suggested in Eq. B33, that is, $\lambda_i = \tau_i^{-1}$.

Finding the associated amplitudes is a more involved process which utilizes the eigenvectors of matrix A.

The first step is to make a change of variables such that the constant term in Eq. B35 is eliminated. This is done by solving Eq. B35 for Y_{ha} , Y_a , Y_h and Y_{oh} in the case where the LHS of the equation is set to zero. This can be done by Gaussian elimination using determinants,

$$C_i = \frac{\det | A' |}{\det | A |} \quad B36$$

where matrix A' is matrix A with the ith column replaced by the constant term in Eq. B35, namely,

$$- \begin{pmatrix} 0 \\ 2k_a N_a^0 u^0 \\ 0 \\ 0 \end{pmatrix}$$

and i is, respectively, ha, a, h and oh . The proper change of variable is, then,

$$Y'_{ha} = Y_{ha} - C_{ha} \quad B37$$

$$Y'_a = Y_a - C_a \quad B38$$

$$Y'_h = Y_h - C_h \quad B39$$

$$Y'_{oh} = Y_{oh} - C_{oh} \quad B40$$

where the primed variables are the new variables.

With respect to the new primed variables the constant term in Eq. 35 is

eliminated and, thus, the differential equations have a simple solution

$$Y'_i = \sum_j \alpha_{i,j} e^{-\lambda_j t} \quad B41$$

$$\frac{dY'_i}{dt} = -\sum_j \alpha_{i,j} \lambda_j e^{-\lambda_j t} = \frac{dY_i}{dt} \quad B42$$

where $i, j = ha, a, h$ and oh , respectively, and the RHS of Eqs. B41 and B42 are summed over j . Thus, each Y'_i is a linear combination of eigenvectors involving the eigenvalue λ_j that appears in the exponential term.

The α 's in Eqs. B41 and B42 are derived from the eigenvectors of matrix A. Each eigenvalue is associated with a unique column eigenvector. These column eigenvectors are used to make up a 4x4 matrix of their own where the j th column of this new matrix is the column eigenvector associated with the j th eigenvalue. Thus, α_{ij} ($i = 1 - 4$) is the j th column eigenvector of matrix A in Eq. B35 associated with the j th eigenvalue.

In matrix form Eq. B41 is

$$\begin{pmatrix} Y'_{ha} \\ Y'_a \\ Y'_h \\ Y'_{oh} \end{pmatrix} = \begin{pmatrix} Y'_1 \\ Y'_2 \\ Y'_3 \\ Y'_4 \end{pmatrix} = \begin{pmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} & \alpha_{14} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} & \alpha_{24} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} & \alpha_{34} \\ \alpha_{41} & \alpha_{42} & \alpha_{43} & \alpha_{44} \end{pmatrix} \begin{pmatrix} e^{-\lambda_1 t} \\ e^{-\lambda_2 t} \\ e^{-\lambda_3 t} \\ e^{-\lambda_4 t} \end{pmatrix} \quad B43$$

where the columns of the α matrix are the respective column eigenvectors of matrix A associated with each eigenvalue.

Combining Eqs. B37 - B40 with Eq. B43

$$\begin{pmatrix} Y_{ha} \\ Y_a \\ Y_h \\ Y_{oh} \end{pmatrix} = \begin{pmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} & \alpha_{14} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} & \alpha_{24} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} & \alpha_{34} \\ \alpha_{41} & \alpha_{42} & \alpha_{43} & \alpha_{44} \end{pmatrix} \begin{pmatrix} e^{-\lambda_1 t} \\ e^{-\lambda_2 t} \\ e^{-\lambda_3 t} \\ e^{-\lambda_4 t} \end{pmatrix} + \begin{pmatrix} C_{ha} \\ C_a \\ C_h \\ C_{oh} \end{pmatrix} \quad \text{B44}$$

However, the eigenvectors associated with matrix A are not entirely unique. Each eigenvector can be multiplied by an arbitrary constant and still be a correct eigenvector for a given eigenvalue. To satisfy Eqs. B41 and B42, therefore, a unique multiplying constant must be found for each eigenvector. This can be done by letting α_{ij}' , $j = 1 - 4$, be the elements of the i th normalized column eigenvector of matrix A and C_{ii} be the required constant such that $\alpha_{ij} = C_{ii}\alpha_{ij}'$, where $\sum_j \alpha_{ij}' = 1$ and C_{ii} has the effect of multiplying the i th column of the α' matrix by a constant C_{ii} . Therefore,

$$\begin{pmatrix} Y_{ha} \\ Y_a \\ Y_h \\ Y_{oh} \end{pmatrix} = \begin{pmatrix} \alpha'_{11} & \alpha'_{12} & \alpha'_{13} & \alpha'_{14} \\ \alpha'_{21} & \alpha'_{22} & \alpha'_{23} & \alpha'_{24} \\ \alpha'_{31} & \alpha'_{32} & \alpha'_{33} & \alpha'_{34} \\ \alpha'_{41} & \alpha'_{42} & \alpha'_{43} & \alpha'_{44} \end{pmatrix} \begin{pmatrix} C_{11}e^{-\lambda_1 t} \\ C_{22}e^{-\lambda_2 t} \\ C_{33}e^{-\lambda_3 t} \\ C_{44}e^{-\lambda_4 t} \end{pmatrix} + \begin{pmatrix} C_{ha} \\ C_a \\ C_h \\ C_{oh} \end{pmatrix} \quad B45$$

where at $t = 0$

$$\begin{pmatrix} \alpha'_{11} & \alpha'_{21} & \alpha'_{31} & \alpha'_{41} \\ \alpha'_{21} & \alpha'_{22} & \alpha'_{23} & \alpha'_{24} \\ \alpha'_{31} & \alpha'_{32} & \alpha'_{33} & \alpha'_{34} \\ \alpha'_{41} & \alpha'_{42} & \alpha'_{43} & \alpha'_{44} \end{pmatrix} \begin{pmatrix} C_{11} \\ C_{22} \\ C_{33} \\ C_{44} \end{pmatrix} = - \begin{pmatrix} C_{ha} \\ C_a \\ C_h \\ C_{oh} \end{pmatrix} \quad B46$$

Eq. B46 can be solved for C_{ii} ($i = 1, 2, 3, 4$), since α'_{ij} is known, in a manner similar to Eq. 36.

$$C_{ii} = \frac{\det | \alpha'' |}{\det | \alpha' |} \quad B47$$

where α'' is α' where

$$- \begin{pmatrix} C_{ka} \\ C_a \\ C_h \\ C_{oh} \end{pmatrix}$$

has been substituted for the i th column. Knowing C_{ij} and each α_{ij}' derived from matrix A, each required α_{ij} for Eqs. B41 and B42 can now be calculated.

The solutions to Eq. B42 can be used in Eq. B23 to solve for du/dt and the resulting terms grouped as coefficients of the decaying eigenvalue exponentials. These coefficients can be compared to the analogous β coefficients for the same decaying eigenvalue exponentials after taking the time derivative of Eq. 33 with the result,

$$\beta_i = \frac{-C_{ia}(\alpha'_{i2} - \alpha'_{i3} + \alpha'_{i4})}{\sum_j C_{ij}(\alpha'_{j2} - \alpha'_{j3} + \alpha'_{j4})} \quad B48$$

Part III

The Conventional Class - 1 Theory

The conventional class - 1 theory was developed by Benz and McLaughlin (Benz and McLaughlin, 1983) and has become the standard model for analyzing presumed class - 1 complexes. The approach of Benz and McLaughlin differs significantly from the approach developed here.

The conventional approach does not include the OH⁻ ion. With respect to the initial differential equations describing the transmembrane transport of class - 1 uncouplers, Eqs. B3 - B10, then, Eqs. B9 and B10 are excluded. As a result, in Eqs. B3 - B6, k_D and k_{DD} must be set to zero.

The conventional approach also assumes that the reactions characterized by Eqs. B7 - B8, involving the time derivatives of N_h' and N_h'' are fast enough that $N_h' - N_h'' = 0$, that is, the pH on either side of the membrane never departs from its equilibrium value. This does not imply, however, that k_R and k_{RR} can be otherwise neglected. Thus their place in Eqs. B3 - B6 must be maintained.

Eqs. B19 and B20 then become

$$\frac{dY_{ha}}{dt} = -(2k_{ha} + k_{RR})Y_{ha} + k_R N_h^0 Y_a \quad B48$$

$$\frac{dY_a}{dt} = k_{RR} Y_{ha} - (2k_a + k_R N_h^0) Y_a - 2k_a N_a^0 u \quad B49$$

With the deletion of the equation for dY_h/dt , Eq. B21, u is no longer a dependent parameter.

$$\frac{du}{dt} = b(k_a''N_a'' - k_a'N_a'') = -bk_aY_a - bk_aN_a^0u \quad B50$$

Eqs. B48 - B50 form a complete set of differential equations characterizing the conventional class - 1 model. In place of Eq. B33 then,

$$\begin{pmatrix} \frac{dY_{ha}}{dt} \\ \frac{dY_a}{dt} \\ \frac{du}{dt} \end{pmatrix} = \begin{pmatrix} -2k_{ha} - k_{RR} & k_RN_h^0 & 0 \\ k_{RR} & -2k_a - k_RN_h^0 & -2k_aN_a^0 \\ 0 & -bk_a & -bk_aN_a^0 \end{pmatrix} \begin{pmatrix} Y_{ha} \\ Y_a \\ u \end{pmatrix} \quad B51$$

In the charge pulse method the eigenvalues, λ_i $i = 1 - 3$, of the 3x3 matrix in Eq. B51 are the reciprocal time constants of the presumed time course of u , where

$$u = u^0(\beta_1e^{-\lambda_1t} + \beta_2e^{-\lambda_2t} + \beta_3e^{-\lambda_3t}) \quad B52$$

and

$$\beta_1 + \beta_2 + \beta_3 = 1 \quad B53$$

The amplitudes can be found by first taking the time derivative of Eq. B52 and comparing it to Eq. 50 at $t = 0$,

$$\lambda_1 \beta_1 + \lambda_2 \beta_2 + \lambda_3 \beta_3 = b k_a N_a^0 \quad B54$$

Finally, the second time derivative of Eqs. B52 and B50 can be compared resulting in

$$\lambda_1^2 \beta_1 + \lambda_2^2 \beta_2 + \lambda_3^2 \beta_3 = (b k_a N_a^0)^2 + 2 b k_a^2 N_a^0 \quad B55$$

Eqs. B53 - B55 can be solved simultaneously for all the β 's.

Experimentally, it is often the case that $k_R N_h^0, k_{RR} \gg k_a, k_{ha}$, in other words, the surface reactions involving the formation or dissociation of HA are very fast compared to the movement of A^- or HA across the interior.

In this case it can be assumed that HA and A reach equilibrium on each side of the membrane quickly, that is, $N_{ha}'/N_a' = N_{ha}''/N_a'' = N_{ha}^0/N_a^0 = 10^{(pK - pH)} = \gamma$.

As a result, Eqs. B48 and B49 become dependent equations since now $Y_{ha} = \gamma Y_a$.

Let

$$v = \frac{N'_a + N'_{ha}}{N_0} - \frac{1}{2} = \frac{1 + \gamma}{N_0} N'_a - \frac{1}{2} \quad B56$$

Since $N_0 = N'_a + N'_{ha} + N''_a + N''_{ha}$ it follows immediately, given Eq. B56, that

$$N''_a = \frac{N_0}{2(1 + \gamma)} - \frac{N_0 v}{1 + \gamma}; \quad Y_a = \frac{2N_0}{1 + \gamma} v \quad B57$$

Adding Eqs. B48 and B49 and regrouping the result in terms of v it follows that

$$\begin{pmatrix} \frac{dv}{dt} \\ \frac{du}{dt} \end{pmatrix} = \begin{pmatrix} \frac{-2(k_a + \gamma k_{ha})}{1 + \gamma} & \frac{-k_a}{2(1 + \gamma)} \\ \frac{-2bk_a N_0}{1 + \gamma} & \frac{-bk_a N_0}{2(1 + \gamma)} \end{pmatrix} \begin{pmatrix} v \\ u \end{pmatrix} \quad B58$$

As before it is assumed that the eigenvalues of the 2x2 matrix in Eq. B58 are the reciprocal time constants of

$$u = u^0(\beta_1 e^{-\lambda_1 t} + \beta_2 e^{-\lambda_2 t}) \quad B59$$

where

$$\beta_1 + \beta_2 + 1 \tag{B60}$$

The time derivative of Eq. B59 can be compared to du/dt part of Eq. B58 at $t = 0$ with the result

$$\beta_1 \lambda_1 + \beta_2 \lambda_2 = \frac{bk_s N_0}{2(1 + \gamma)} \tag{B61}$$

Eqs. B60 and B61 can be used to solve for β_1 and β_2 .

APPENDIX C

CLASS - 2 UNCOUPLERS

Part I

Derivation of Fully Linearized Kinetic Equations for Class - 2 Uncouplers

The basic differential equations describing transmembrane transport of class - 2 uncouplers for a weak acid (HA) with its conjugate anion (A^-) also includes reactions involving the dimer AHA^- formed from A^- and HA.

As with the class - 1 uncoupler surface reactions with respect to H^+ and OH^- are involved. In addition to Eqs. B1 and B2, H^+ and OH^- also react with AHA^- , where again the respective rate constants are noted to the right.

Also, there is the formation of the dimer by the recombination of A^- and HA,

where k_d has units of s^{-1} and the units for k_{haha} , k_{2ha} , k_{poh} , k_{raha} , and k_f are $cm^2 s^{-1} mol^{-1}$.

The complete set of differential equations describing the membrane kinetic scheme of class - 2 uncouplers becomes, then,

$$\begin{aligned} \frac{dN'_{ha}}{dt} = & -k_{RR}N'_{ha} + k_RN'_a N'_h + k_{DD}N'_a - k_DN'_{ha}N'_{oh} + 2k_{haha}N'_hN'_{aha} - k_{2ha}N'_{ha}N'_{ha} \\ & + k_dN'_{aha} - k_fN'_{ha}N'_a + k_{ha}N''_{ha} - k_{ha}N'_{ha} \end{aligned} \quad C4$$

$$\begin{aligned} \frac{dN''_{ha}}{dt} = & -k_{RR}N''_{ha} + k_RN''_a N''_h + k_{DD}N''_a - k_DN''_{ha}N''_{oh} + 2k_{haha}N''_hN''_{aha} - k_{2ha}N''_{ha}N''_{ha} \\ & + k_dN''_{aha} - k_fN''_{ha}N''_a - k_{ha}N''_{ha} + k_{ha}N'_{ha} \end{aligned} \quad C5$$

$$\begin{aligned} \frac{dN'_a}{dt} = & k_{RR}N'_{ha} - k_RN'_a N'_h - k_{DD}N'_a + k_DN'_{ha}N'_{oh} \\ & + 2k_{poh}N'_{aha}N'_{oh} - k_{raha}N'_a N'_a + k_dN'_{aha} - k_fN'_{ha}N'_a + k''_a N''_a - k'_a N'_a \end{aligned} \quad C6$$

$$\begin{aligned} \frac{dN''_a}{dt} = & k_{RR}N''_{ha} - k_RN''_a N''_h - k_{DD}N''_a + k_DN''_{ha}N''_{oh} \\ & + 2k_{poh}N''_{aha}N''_{oh} - k_{raha}N''_a N''_a + k_dN''_{aha} - k_fN''_{ha}N''_a - k''_a N''_a + k'_a N'_a \end{aligned} \quad C7$$

$$\begin{aligned} \frac{dN'_{aha}}{dt} = & k_{2ha}N'_{ha}N'_{ha} - k_{haha}N'_{aha}N'_h + k_{raha}N'_a N'_a - k_{poh}N'_{aha}N'_{oh} \\ & + k_fN'_{ha}N'_a - k_dN'_{aha} - k'_{aha}N'_{aha} + k''_{aha}N''_{aha} \end{aligned} \quad C8$$

$$\begin{aligned} \frac{dN_{aha}''}{dt} = & k_{2ha}N_{ha}''N_{ha}'' - k_{haha}N_{aha}''N_h'' + k_{raha}N_a''N_a'' - k_{poh}N_{aha}''N_{oh}'' \\ & + k_fN_{ha}''N_a'' - k_aN_{aha}'' + k_{aha}'N_{aha}' - k_{aha}''N_{aha}'' \end{aligned} \quad C9$$

$$\frac{dN_h'}{dt} = k_{RR}N_{ha}' - k_RN_a'N_h' + k_{2ha}N_{ha}'N_{ha}' - k_{haha}N_{aha}'N_h' \quad C10$$

$$\frac{dN_h''}{dt} = k_{RR}N_{ha}'' - k_RN_a''N_h'' + k_{2ha}N_{ha}''N_{ha}'' - k_{haha}N_{aha}''N_h'' \quad C11$$

$$\frac{dN_{oh}'}{dt} = k_{DD}N_a' - k_DN_{ha}'N_{oh}' + k_{raha}N_a'N_a' - k_{poh}N_{aha}'N_{oh}' \quad C12$$

$$\frac{dN_{oh}''}{dt} = k_{DD}N_a'' - k_DN_{ha}''N_{oh}'' + k_{raha}N_a''N_a'' + k_{poh}N_{aha}''N_{oh}'' \quad C13$$

where k_{aha}' and k_{aha}'' are the rate constants (s^{-1}) associated with the movement of AHA $\cdot$ across the membrane interior and N_{aha}' , N_{aha}'' are the surface densities (in moles per unit area) of AHA $\cdot$.

The transformation Eqs. C4 to C13 into a more useful linear form proceeds in the same manner as in Appendix B with the formation of an additional parameter.

$$Y_{aha} = N_{aha}' - N_{aha}'' \quad C14$$

Let N_{aha}^0 be the equilibrium surface density AHA^- on each side of the membrane before the external electric potential is applied, where

$$N_{aha}^0 = \frac{N_{aha}^{0'} + N_{aha}^{0''}}{2} \quad C15$$

Using Eqs. C14 - C15 and Eqs. B11 - B18, Eqs. C4 - C13 are reduced to the following:

$$\begin{aligned} \frac{dY_{ha}}{dt} = & -(2k_{ha} + k_{RR} + k_D N_{oh}^0 + k_f N_a^0 + k_{2ha} N_{ha}^0) Y_{ha} + (k_R N_h^0 + k_{DD} - k_f N_{ha}^0) Y_a \\ & + (k_d + 2k_{haha} N_h^0) Y_{aha} + (k_R N_a^0 + 2k_{haha} N_{aha}^0) Y_h - k_D N_{ha}^0 Y_{oh} \end{aligned} \quad C16$$

$$\begin{aligned} \frac{dY_a}{dt} = & (k_{RR} + k_D N_{oh}^0 - k_f N_a^0) Y_{ha} - (2k_a + k_{DD} + k_R N_h^0 + k_f N_{ha}^0 + k_{raha} N_a^0) Y_a \\ & + (k_d + 2k_{kpo h} N_{oh}^0) Y_{aha} - k_R N_a^0 Y_h + (k_D N_{ha}^0 + 2k_{po h} N_{aha}^0) Y_{oh} - 2k_a N_a^0 u \end{aligned} \quad C17$$

$$\begin{aligned} \frac{dY_{aha}}{dt} = & (k_f N_a^0 + k_{2ha} N_{ha}^0) Y_{ha} + (k_f N_{ha}^0 + k_{raha} N_a^0) Y_a \\ & - (2k_{aha} + k_d + k_{po h} N_{oh}^0 + k_{haha} N_h^0) Y_{aha} - k_{haha} N_{aha}^0 Y_h - k_{po h} N_{aha}^0 Y_{oh} - 2k_{aha} N_{aha}^0 u \end{aligned} \quad C18$$

$$\frac{dY_h}{dt} = (k_{RR} + k_{2ha} N_{ha}^0) Y_{ha} - k_R N_h^0 Y_a - k_{haha} N_h^0 Y_{aha} - (k_R N_a^0 + k_{haha} N_{aha}^0) Y_h \quad C19$$

$$\frac{dY_{oh}}{dt} = -k_D N_{oh}^0 Y_{ha} + (k_{DD} + k_{raha} N_a^0) Y_a - k_{po h} N_{oh}^0 Y_{aha} - (k_D N_{ha}^0 + k_{po h} N_{aha}^0) Y_{oh} \quad C20$$

For a membrane of electric capacitance C, the decaying time course of the reduced potential for the charge pulse experimental method is

$$\frac{du}{dt} = b(k_a''N_a'' + k_{aha}''N_{aha}'' - k_a'N_a' - k_{aha}'N_{aha}') = \frac{b}{2}\left(\frac{dY_a}{dt} + \frac{dY_{aha}}{dt} + \frac{dY_{oh}}{dt} - \frac{dY_h}{dt}\right) \quad C21$$

using the above linearizations

$$\frac{du}{dt} = -bk_aY_a - bk_{aha}Y_{aha} - (bk_aN_a^0 + bk_{aha}N_{aha}^0)u \quad C22$$

where $b = F^2/CRT$.

Eq. C21 can be used to solve for u,

$$u = \frac{b}{2}Y_a + \frac{b}{2}Y_{aha} + \frac{b}{2}Y_{oh} - \frac{b}{2}Y_h + u^0 \quad C23$$

This result will be useful later to eliminate u from Eqs. C17 and C18 in terms of Y_a , Y_{aha} and the initial reduced voltage, u^0 .

Under equilibrium conditions, before the external voltage is applied across the

membrane, Eqs. C8 - C13 immediately suggest four boundary conditions. Before the external voltage is applied across the membrane, $N' = N''$, regardless of the specific surface density in question. Adding Eqs. C10 and C11, the following result is easily obtained,

$$k_{RR} = \frac{k_R N_a^0 N_h^0 + k_{haha} N_{aha}^0 N_h^0 - k_{2ha} N_{ha}^0 N_{ha}^0}{N_{ha}^0} \quad C24$$

Likewise, adding Eqs. C12 and C13,

$$k_{DD} = \frac{k_D N_{ha}^0 N_{oh}^0 + k_{poh} N_{aha}^0 N_{oh}^0 - k_{raha} N_a^0 N_a^0}{N_a^0} \quad C25$$

Adding Eqs. C8 and C9

$$N_{aha}^0 = \frac{k_f N_a^0 N_{ha}^0 + k_{rahs} N_a^0 N_a^0 + k_{2ha} N_{ha}^0 N_{ha}^0}{k_d + k_{poh} N_{oh}^0 + k_{haha} N_h^0} \quad C26$$

Adding Eqs. C4 - C9 after multiplying Eqs. C8 and C9 by 2 the result is

$$\frac{d(2N_a^0 + 4N_{aha}^0 + 2N_{ha}^0)}{dt} = 0 \quad C27$$

or if N_0 = total HA, A⁻ and AHA⁻ adsorbed at $t = 0$,

$$N_0 = 2N_a^0 + 4N_{aha}^0 + 2N_{ha}^0 \quad C28$$

Combining Eqs. B30 and C26 with C28, a useful expression for N_a^0 is found

$$N_a^0 = \frac{(1 + \gamma)}{4Z} (\sqrt{1 + 4ZN_0(1 + \gamma)^{-2}} - 1) \quad C29$$

where

$$\gamma = 10^{(pK_a - pH)}; \quad Z = \frac{k_{raha} + \gamma k_f + \gamma^2 k_{2ha}}{k_d + k_{poh} N_{oh}^0 + k_{haha} N_h^0}$$

As $t \rightarrow \infty$ Eq. C23 becomes,

$$u(\infty) = \frac{b}{2} |N'_a - N''_a|_{l=\infty} + \frac{b}{2} |N'_{aha} - N''_{aha}|_{l=\infty} \\ + \frac{b}{2} |N'_{oh} - N''_{oh}|_{l=\infty} - \frac{b}{2} |N'_h - N''_h|_{l=\infty} + u^0 \quad C30$$

To the extent u^0 is equilibrated by the above concentration gradients $u(\infty) \rightarrow 0$. Particularly for small voltages, $u^0 < 1$, there would be, at least, a large reservoir of H^+ ions. In Part II below, $u(\infty)$ is taken to be zero.

Part II

Determination Of The Rate Constants by the Charge Pulse Method

Proceeding as in Appendix B, with $u(\infty) = 0$, Eqs. C16 - C20 in the charge pulse methodology implies 5 time constants:

$$u = u^0(\beta_1 e^{-\frac{t}{\tau_1}} + \beta_2 e^{-\frac{t}{\tau_2}} + \beta_3 e^{-\frac{t}{\tau_3}} + \beta_4 e^{-\frac{t}{\tau_4}} + \beta_5 e^{-\frac{t}{\tau_5}}) \quad C31$$

$$\beta_1 + \beta_2 + \beta_3 + \beta_4 + \beta_5 = 1 \quad C32$$

The matrix equation suggested by Eqs. C16 - C20, when Eq. C23 is used to eliminate u , takes the form

$$\begin{pmatrix} \frac{dY_{ha}}{dt} \\ \frac{dY_a}{dt} \\ \frac{dY_{aha}}{dt} \\ \frac{dY_h}{dt} \\ \frac{dY_{oh}}{dt} \end{pmatrix} = A \begin{pmatrix} Y_{ha} \\ Y_a \\ Y_{aha} \\ Y_h \\ Y_{oh} \end{pmatrix} - u^0 \begin{pmatrix} 0 \\ 2k_a N_a^0 \\ 2k_{aha} N_{aha}^0 \\ 0 \\ 0 \end{pmatrix} \quad C33$$

where A is a 5x5 whose columns are shown below,

$$\begin{aligned}
& -2k_{ha} - k_{RR} - k_D N_{oh}^0 - k_f N_a^0 - k_{zha} N_{ha}^0 \\
& k_R N_h^0 + k_{DD} - k_f N_{ha}^0 \\
& k_d N_{ha}^0 + 2k_{haha} N_h^0 \\
& k_R N_a^0 + 2k_{haha} N_{aha}^0 \\
& - k_D N_{ha}^0
\end{aligned}$$

(1)

$$\begin{aligned}
& k_{RR} + k_D N_{oh}^0 - k_f N_a^0 \\
& - 2k_a - k_f N_{ha}^0 - k_R N_h^0 - k_{DD} - k_{raha} N_a^0 - bk_a N_a^0 \\
& k_d + 2k_{poh} N_{oh}^0 - bk_a N_a^0 \\
& - k_R N_a^0 + bk_a N_a^0 \\
& k_D N_{ha}^0 + 2k_{poh} N_{aha}^0 - bk_a N_a^0
\end{aligned}$$

(2)

$$\begin{aligned}
& k_f N_a + k_{zha} N_{ha}^0 \\
& k_f N_{ha}^0 + k_{raha} N_a^0 - bk_{aha} N_{aha}^0 \\
& -2k_{aha} - k_d - k_{haha} N_h^0 - k_{poh} N_{oh}^0 - bk_{aha} N_{aha}^0 \\
& -k_{haha} N_{aha}^0 + k_{aha} N_{aha}^0 \\
& -k_{poh} N_{aha}^0 - bk_{aha} N_{aha}^0
\end{aligned}$$

(3)

$$\begin{aligned}
& k_{RR}N_a^0 + k_{2ha}N_{ha}^0 \\
& - k_R N_h^0 \\
& - k_{aha}N_h^0 \\
& -k_R N_a^0 - k_{aha}N_{aha}^0 \\
& 0
\end{aligned}$$

(4)

$$\begin{aligned}
& -k_D N_{oh}^0 \\
& k_{DD} + k_{raha}N_a^0 \\
& -k_{poh}N_{oh}^0 \\
& 0 \\
& -k_D N_{ha}^0 - k_{poh}N_{aha}^0
\end{aligned}$$

(5)

The eigenvalue solutions, λ_i $i = 1-5$, to the determinant of A in C33 are, as in Appendix B, the reciprocal time constants suggested in Eq. C31, that is, $\lambda_i = \tau_i^{-1}$.

Finding the associated amplitudes follows the same analogous processes as were used in Appendix B which utilizes the eigenvectors of matrix A.

The first step is to make a change of variables such that the constant term in Eq. C33 is eliminated. This is done by solving C33 for Y_{ha} , Y_a , Y_{aha} , Y_h and Y_{oh} in the case where the LHS of the equation is set to zero. This can be done by Gaussian elimination using determinants,

$$C_i = \frac{\det | A' |}{\det | A |} \quad C34$$

where A' is matrix A with the ith column replaced by the constant term in Eq. C33, namely,

$$- \begin{pmatrix} 0 \\ 2k_a N_a^0 u^0 \\ 2k_{aha} N_{aha}^0 u^0 \\ 0 \\ 0 \end{pmatrix}$$

and i is, respectively, ha, a, aha, h and oh. The constants C_{ha} , C_a , C_h and C_{oh} imply a change of variables of the same form, but different value, of course, as found in Appendix B, Eqs. B37 - B40, respectively, and will not be repeated here. An additional change of variable is found involving C_{aha} ,

$$Y'_{aha} = Y_{aha} - C_{aha} \quad C35$$

where the primed variable is the new additional variable.

With respect to the new primed variables the constant term in Eq. C33 is

eliminated and, thus, the differential equations have a simple solution

$$Y_i' = \sum_j \alpha_{i,j} e^{-\lambda_j t} \quad \text{C36}$$

$$\frac{dY_i'}{dt} = -\sum_j \alpha_{i,j} \lambda_j e^{-\lambda_j t} = \frac{dY_i}{dt} \quad \text{C37}$$

where $i, j = ha, a, aha, h$ and oh , respectively, and the RHS of Eqs. C36 and C37 are summed over j . Thus, each Y_i' is a linear combination of eigenvectors involving the eigenvalue λ_i that appears in the exponential term.

The α 's in Eqs. C36 and C37 are derived from the eigenvectors of matrix A. Each eigenvalue is associated with a unique column eigenvector. These column eigenvectors are used to make up a 5x5 matrix of their own where the j th column of this new matrix is the column eigenvector associated with the j th eigenvalue. Thus, α_{ij} ($i = 1 - 5$) is the j th column eigenvector of matrix A in Eq. C33 associated with the j th eigenvalue.

In matrix form Eq. C36 is

$$\begin{pmatrix} Y'_{ha} \\ Y'_a \\ Y'_{aha} \\ Y'_h \\ Y'_{oh} \end{pmatrix} = \begin{pmatrix} Y'_1 \\ Y'_2 \\ Y'_3 \\ Y'_4 \\ Y'_5 \end{pmatrix} = \begin{pmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} & \alpha_{14} & \alpha_{15} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} & \alpha_{24} & \alpha_{25} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} & \alpha_{34} & \alpha_{35} \\ \alpha_{41} & \alpha_{42} & \alpha_{43} & \alpha_{44} & \alpha_{45} \\ \alpha_{51} & \alpha_{52} & \alpha_{53} & \alpha_{54} & \alpha_{55} \end{pmatrix} \begin{pmatrix} e^{-\lambda_1 t} \\ e^{-\lambda_2 t} \\ e^{-\lambda_3 t} \\ e^{-\lambda_4 t} \\ e^{-\lambda_5 t} \end{pmatrix} \quad C37$$

where the columns of the α matrix are the respective column eigenvectors of matrix A associated with each eigenvalue.

Combining Eqs. B37 - B40 and Eq. C35 with Eq. C36

$$\begin{pmatrix} Y_{ha} \\ Y_a \\ Y_{aha} \\ Y_h \\ Y_{oh} \end{pmatrix} = \begin{pmatrix} \alpha_{11} & \alpha_{12} & \alpha_{13} & \alpha_{14} & \alpha_{15} \\ \alpha_{21} & \alpha_{22} & \alpha_{23} & \alpha_{24} & \alpha_{25} \\ \alpha_{31} & \alpha_{32} & \alpha_{33} & \alpha_{34} & \alpha_{35} \\ \alpha_{41} & \alpha_{42} & \alpha_{43} & \alpha_{44} & \alpha_{45} \\ \alpha_{51} & \alpha_{52} & \alpha_{53} & \alpha_{54} & \alpha_{55} \end{pmatrix} \begin{pmatrix} e^{-\lambda_1 t} \\ e^{-\lambda_2 t} \\ e^{-\lambda_3 t} \\ e^{-\lambda_4 t} \\ e^{-\lambda_5 t} \end{pmatrix} + \begin{pmatrix} C_{ha} \\ C_a \\ C_{aha} \\ C_h \\ C_{oh} \end{pmatrix} \quad C39$$

However, the eigenvectors associated with matrix A are not entirely unique. Each eigenvector can be multiplied by an arbitrary constant and still be a correct eigenvector for a given eigenvalue. To satisfy Eqs. C36 and C37, therefore, a unique multiplying constant must be found for each eigenvector. This can be done by letting α'_{ij} , $j = 1 - 5$, be the elements of the i th normalized column eigenvector of matrix A and C_{ij} be the required constant such that $\alpha_{ij} = C_{ij}\alpha'_{ij}$, where $\sum_j \alpha'_{ij} = 1$ and C_{ij} has

the effect of multiplying the i th column of the α' matrix by a constant C_{ii} . Therefore,

$$\begin{pmatrix} Y_{ha} \\ Y_a \\ Y_{aha} \\ Y_h \\ Y_{oh} \end{pmatrix} = \begin{pmatrix} \alpha'_{11} & \alpha'_{12} & \alpha'_{13} & \alpha'_{14} & \alpha'_{15} \\ \alpha'_{21} & \alpha'_{22} & \alpha'_{23} & \alpha'_{24} & \alpha'_{25} \\ \alpha'_{31} & \alpha'_{32} & \alpha'_{33} & \alpha'_{34} & \alpha'_{35} \\ \alpha'_{41} & \alpha'_{42} & \alpha'_{43} & \alpha'_{44} & \alpha'_{45} \\ \alpha'_{51} & \alpha'_{52} & \alpha'_{53} & \alpha'_{54} & \alpha'_{55} \end{pmatrix} \begin{pmatrix} C_{11}e^{-\lambda_1 t} \\ C_{22}e^{-\lambda_2 t} \\ C_{33}e^{-\lambda_3 t} \\ C_{44}e^{-\lambda_4 t} \\ C_{55}e^{-\lambda_5 t} \end{pmatrix} + \begin{pmatrix} C_{ha} \\ C_a \\ C_{aha} \\ C_h \\ C_{oh} \end{pmatrix} \quad C40$$

where at $t = 0$

$$\begin{pmatrix} \alpha'_{11} & \alpha'_{12} & \alpha'_{13} & \alpha'_{14} & \alpha'_{15} \\ \alpha'_{21} & \alpha'_{22} & \alpha'_{23} & \alpha'_{24} & \alpha'_{25} \\ \alpha'_{31} & \alpha'_{32} & \alpha'_{33} & \alpha'_{34} & \alpha'_{35} \\ \alpha'_{41} & \alpha'_{42} & \alpha'_{43} & \alpha'_{44} & \alpha'_{45} \\ \alpha'_{51} & \alpha'_{52} & \alpha'_{53} & \alpha'_{54} & \alpha'_{55} \end{pmatrix} \begin{pmatrix} C_{11} \\ C_{22} \\ C_{33} \\ C_{44} \\ C_{55} \end{pmatrix} = - \begin{pmatrix} C_{ha} \\ C_a \\ C_{aha} \\ C_h \\ C_{oh} \end{pmatrix} \quad C41$$

Eq. C41 can be solved for C_{ii} ($i = 1, 2, 3, 4, 5$), since α'_{ij} is known, in a manner similar to Eq. C34.

$$C_{ii} = \frac{\det | \alpha'' |}{\det | \alpha' |} \quad C42$$

where α'' is α' where

$$= \begin{pmatrix} C_{hs} \\ C_a \\ C_{ahs} \\ C_h \\ C_{oh} \end{pmatrix}$$

has been substituted for the i th column. Knowing C_{ij} and each α_{ij}' derived from matrix A, each required α_{ij} for Eqs. C36 and C37 can now be calculated.

The solutions to Eq. C37 can be used in Eq. C21 to solve for du/dt and the resulting terms grouped as coefficients of the decaying eigenvalue exponentials. These coefficients can be compared to the analogous β coefficients for the same decaying eigenvalue exponentials after taking the time derivative of Eq. C31 with the result,

$$\beta_i = \frac{-C_{ii}(\alpha'_{i2} + \alpha'_{i3} - \alpha'_{i4} + \alpha'_{i5})}{\sum_j C_{ij}(\alpha'_{j2} + \alpha'_{j3} - \alpha'_{j4} + \alpha'_{j5})} \quad C43$$

APPENDIX D

A. PROTON WIRES

1. Introduction

It has been suggested (Nagle and Tristram-Nagle, 1983; Nagle, 1987) that thermal fluctuations in bilayer structure cause transient pores to form that are penetrated by the aqueous bulk phase. The pores have been called 'proton wires'. The model assumes that there is significant hydrogen bonding between the water molecules filling the pore. The protons, then, move along the hydrogen bonded chains (wires) from one side of the membrane to the other.

The following analysis does not deal with the frequency of pore formation or how long they may endure. It is only assumed that on the average they exist in sufficient number and for a sufficient time for protons to transport through the pores as they respond to either proton concentration (pH) differences and/or electrical potential differences between one side of the membrane and the other. In the case of a proton wire the interior is not hydrocarbon chains but hydrogen bonded water molecules.

A further simplification is made that the protons involved in transporting across the interior are those protons on the adsorption surface and protons diffusing onto the

membrane adsorption surface from the bulk aqueous phase are not significant. In this case, the transport of protons across the membrane interior can be characterized by two rate constants k_h' and k_h'' where

$$k_h' = k_h e^{\frac{1}{2}u} = k_h + \frac{1}{2}k_h u \quad D1$$

$$k_h'' = k_h e^{-\frac{1}{2}u} = k_h - \frac{1}{2}k_h u \quad D2$$

Integrating this concept into class - 1 and class - 2 theory two terms are added to each of the initial differential equations for dN_h'/dt and dN_h''/dt , namely, $k_h'N_h'$ and $k_h''N_h''$. Eqs. B7, B8, C10 and C11 become, respectively,

$$\frac{dN_h'}{dt} = k_{RR}N_{hs}' - k_R N_s' N_h' - k_h' N_h' + k_h'' N_h'' \quad D3$$

$$\frac{dN_h''}{dt} = k_{RR}N_{hs}'' + k_R N_s'' N_h'' - k_h'' N_h'' + k_h' N_h' \quad D4$$

$$\frac{dN_h'}{dt} = k_{RR}N_{hs}' - k_R N_s' N_h' + k_{2hs} N_{hs}' N_{hs}' - k_{hsbs} N_{hs}' N_h' - k_h' N_h' + k_h'' N_h'' \quad D5$$

$$\frac{dN_h''}{dt} = k_{RR}N_{hs}'' + k_R N_s'' N_h'' + k_{2hs} N_{hs}'' N_{hs}'' - k_{hsbs} N_{hs}'' N_h'' + k_h' N_h' - k_h'' N_h'' \quad D6$$

The analysis would continue as outlined in Part I of Appendix B and C, respectively, leading to a new formulation for matrix A, Eq. B35 and C33. This will be outlined specifically for the class 2 model below.

2. The Proton Wire Model For Class - 2 Uncouplers

For the class - 2 model, using Eqs. D1 - D2 and D5 - D6, and proceeding as in Appendix C, Eq. C19 becomes

$$\begin{aligned} \frac{dY_h}{dt} = & (k_{RR} + k_{2hs}N_{hs}^0) Y_{hs} - k_{R^0}N_h^0 Y_s - k_{hsbs}N_h^0 Y_{shs} \\ & - (2k_h + k_{R^0}N_s^0 + k_{hsbs}N_{shs}^0) Y_h - 2k_hN_h^0 u \end{aligned} \quad D7$$

The decaying time course of the reduced potential is altered and C21 is now

$$\begin{aligned} \frac{du}{dt} = & b(k_s''N_s'' + k_{shs}''N_{shs}'' + k_h'N_h' - k_s'N_s' - k_{shs}'N_{shs}' - k_h''N_h'') \\ = & \frac{b}{2} \left(\frac{dY_s}{dt} + \frac{dY_{shs}}{dt} + \frac{dY_{sh}}{dt} - \frac{dY_h}{dt} \right) \end{aligned} \quad D8$$

and in place of Eq. C22,

$$\begin{aligned} \frac{du}{dt} = & -bk_s Y_s - bk_{shs} Y_{shs} + bk_h Y_h \\ & - (bk_s N_s^0 + bk_{shs} N_{shs}^0 - bk_h N_h^0) u \end{aligned} \quad D9$$

where, still,

$$u = \frac{b}{2} Y_a + \frac{b}{2} Y_{aha} + \frac{b}{2} Y_{oh} - \frac{b}{2} Y_h + u^0 \quad D10$$

Using Eqs. D7 - D9 in place of Eqs. C19, C21 and C22, Eq. C33 is altered

$$\begin{pmatrix} \frac{dY_{ha}}{dt} \\ \frac{dY_a}{dt} \\ \frac{dY_{aha}}{dt} \\ \frac{dY_h}{dt} \\ \frac{dY_{oh}}{dt} \end{pmatrix} = A \begin{pmatrix} Y_{ha} \\ Y_a \\ Y_{aha} \\ Y_h \\ Y_{oh} \end{pmatrix} - u_0 \begin{pmatrix} 0 \\ 2k_a N_a^0 \\ 2k_{aha} N_{aha}^0 \\ -2k_h N_h^0 \\ 0 \end{pmatrix} \quad D11$$

where row 4 in matrix A, Eq. C33, has changed from

$$k_{RR} + 2k_{2ha} N_{ha}^0 \quad -k_R N_h^0 \quad -k_{haha} N_{oh}^0 \quad -k_R N_a^0 - k_{haha} N_{aha}^0 \quad 0 \quad D12$$

to

$$\begin{matrix} k_{RR} + 2k_{2ha} N_{ha}^0 & bk_h N_h^0 - k_R N_h^0 & bk_h N_h^0 - k_{haha} N_h^0 \\ -2k_h - bk_h N_h^0 - k_R N_a^0 - k_{haha} N_{aha}^0 & & bk_h N_h^0 \end{matrix} \quad D13$$

From here the analysis can proceed as in Part II, Appendix C, specifically for the charge pulse method with a new rate constant, k_h , now included.

B. INCLUSION OF $H_2O \rightleftharpoons H^+ + OH^-$ IN THE CLASS - 2 MODEL

Assume that on the adsorption surface of a lipid membrane that the reaction $H_2O \rightleftharpoons H^+ + OH^-$ is not so fast that it can be neglected. Let the reaction $H_2O \rightarrow H^+ + OH^-$ be characterized by the rate constant k_{DW} and the reverse reaction $H^+ + OH^- \rightarrow H_2O$ be characterized by the rate constant k_{HOH} .

The Eqs. C10 - C14 become, respectively,

$$\frac{dN'_h}{dt} = k_{RR}N'_{hs} - k_RN'_sN'_h + k_{2hs}N'_{hs}N'_{hs} - k_{hsbs}N'_{hs}N'_h + k_{DW}[H_2O] - k_{HOH}N'_hN'_{oh} \quad D14$$

$$\frac{dN''_h}{dt} = k_{RR}N''_{hs} + k_RN''_sN''_h + k_{2hs}N''_{hs}N''_{hs} - k_{hsbs}N''_{hs}N''_h + k_{DW}[H_2O] - k_{HOH}N''_hN''_{oh} \quad D15$$

$$\frac{dN'_{oh}}{dt} = k_{DD}N'_s - k_DN'_{hs}N'_{oh} + k_{rshs}N'_sN'_s - k_{poh}N'_{shs}N'_{oh} + k_{DW}[H_2O] - k_{HOH}N'_hN'_{oh} \quad D16$$

$$\frac{dN''_{oh}}{dt} = k_{DD}N''_s - k_DN''_{hs}N''_{oh} + k_{rshs}N''_sN''_s + k_{poh}N''_{shs}N''_{oh} + k_{DW}[H_2O] - k_{HOH}N''_hN''_{oh} \quad D17$$

where $[H_2O]$ is the bulk concentration of water and thus $k_{DW}[H_2O]$ is a constant. Eqs.

C19 and C20 become

$$\begin{aligned} \frac{dY_h}{dt} = & (k_{RR} + k_{2hs}N_{hs}^0) Y_{hs} - k_RN_h^0 Y_s - k_{hsbs}N_h^0 Y_{shs} \\ & - (k_RN_s^0 + k_{hsbs}N_{shs}^0 + k_{HOH}N_{oh}^0) Y_h - k_{HOH}N_h^0 Y_{oh} \end{aligned} \quad D18$$

$$\begin{aligned} \frac{dy_{oh}}{dt} = & -k_D N_{oh}^0 Y_{hs} + (k_{DD} + k_{rsbs} N_s^0) Y_s - k_{poh} N_{oh}^0 Y_{abs} \\ & - k_{HOH} N_{oh}^0 Y_h - (k_D N_{hs}^0 + k_{poh} N_{abs}^0 + k_{HOH} N_h^0) Y_{oh} \end{aligned} \quad D19$$

As a result columns 4 and 5 of matrix A, Eq. C33, are now, respectively,

$$\begin{aligned} & k_{RR} N_s^0 + k_{shs} N_{hs}^0 \\ & - k_R N_h^0 \\ & - k_{hsbs} N_h^0 \\ & -k_R N_s^0 - k_{hsbs} N_{abs}^0 - k_{HOH} N_{oh}^0 \\ & -k_{HOH} N_h^0 \\ & -k_D N_{oh}^0 \\ & k_{DD} N_{hs}^0 + k_{rsbs} N_s^0 \\ & -k_{poh} N_{oh}^0 \\ & -k_{HOH} N_{oh}^0 \\ & -k_D N_{hs}^0 - k_{poh} N_{abs}^0 - k_{HOH} N_h^0 \end{aligned}$$

where, again, the analysis can proceed as in Part II, Appendix C, specific to the charge pulse method with a new rate constant k_{HOH} .