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Portland State University

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THESIS APPROVAL

The abstract and thesis of Piet O. Schmidt for the Master of Science in Physics were presented July 13, 1995, and accepted by the thesis committee and the department.

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Abstract

An abstract of the thesis of Piet O. Schmidt for the Master of Science in Physics presented July 13, 1995.

Title: Origins of Effective Charge of Multivalent Ions at a Membrane/Water Interface and Distribution of 2,3,4,5-Tetrachlorophenol in a Membrane Model System

Biological cells and subcellular organelles are surrounded by membranes to form compartments performing specialized functions. Adsorption or partitioning of biologically active compounds into the membrane is the first step in the process of modification of cell function. This work is concerned with the problem of distribution of charged molecules between water and electrically charged membrane surface and between water and octanol.

Part I of this thesis is focused on the electrostatic interactions taking place between charges on the membrane and ions present in the aqueous region of the membrane/water interface. The objective was to explore theoretically the origin of anomalous behavior of Ruthenium Red (RuR), a positively charged hexavalent ion. It was discovered in studies of RuR adsorption to negatively charged membranes that within the framework of the Gouy-Chapman theory of the membrane/water

interface, RuR behaves as an ion with effective charge less than its physical charge. Moreover, the effective charge was found to be dependent on the density of electric charge at the membrane surface. Two theoretical models of the interfacial region were examined: the Rod Model and the Maximum Density Model. The Rod Model takes into account steric constraints imposed on RuR at the vicinity of the membrane surface. The Maximum Density Model attempts to account for non-ideal behavior by including repulsive interactions. These theoretical studies illustrate the consequences of finite size and ion-ion interactions of adsorption of large molecular ions to electrically charged membrane surfaces.

Part II is an experimental study whose objective was to determine the partition coefficient of the negatively charged 2,3,4,5-tetrachlorophenol (TeCP) between water and octanol. The study was based on spectrophotometric measurements of the equilibrium concentrations of TeCP in water and octanol as a function of pH. The octanol/water partition coefficient for both the non-ionized and ionized species of TeCP were determined. It was found that the partition coefficient of ionized TeCP to lipid membrane is about 400 times greater than that for octanol. This result supports the hypothesis that the octanol/water partition coefficient of ionized chlorophenols cannot be used for predicting their distribution between water and lipid-bilayer-containing elements of the environment.

ORIGINS OF EFFECTIVE CHARGE OF MULTIVALENT IONS
AT A MEMBRANE/WATER INTERFACE
AND
DISTRIBUTION OF 2,3,4,5-TETRACHLOROPHENOL
IN A MEMBRANE MODEL SYSTEM

By
Piet O. Schmidt

A thesis submitted in partial fulfillment of the
requirements for the degree of

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Für

Susi, Ute, Fritz, Jan und Annika

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Introduction to Membrane Systems

Processes at membrane surfaces are essential for biological life. Interactions of molecules with the inner and outer surface of cells and organelles trigger a large number of reactions. Adsorption of molecules to membranes is one of the major processes taking place at the membrane/water interface. Information processing in cells is carried out by messenger molecules binding on the outside of the cell and opening an ion channel or triggering the release of a second messenger into the cytoplasm of the cell. For example the hormone epinephrine binds to its receptor on the outside of liver cells triggering a series of reactions inside the cell which ultimately cause the release of glucose (Linder and Gilman, 1992). Another example is the binding of antibodies to foreign cells or to viruses which marks the beginning of the immune system's response, one of the most complex and not yet fully understood systems in higher lifeforms.

Another important function of membranes is to maintain certain ion concentration profiles across the membrane. Nerve cells, for example, maintain a potassium/sodium gradient across their membrane which gives rise to a resting potential of about -60 mV. When an electric action potential propagates along the nerve cell, potassium and sodium channels embedded in the membrane open in sequence and reverse the potential to about +30 mV for a time period of one millisecond. The

respiration chain in mitochondria establishes, via electron transport, a proton gradient across the inner mitochondrial membrane. This results in an electric potential difference which is one of the components of the driving force for the synthesis of adenosine triphosphate (ATP), the major energy source of cells (Stryer, 1981).

Greater knowledge about the processes at membrane/water interfaces aids researchers in medicine and health science to design and develop drugs.

It is the purpose of this study to get a better understanding of adsorption to membranes. The approach is twofold:

1. In Part I, the electrostatic properties of the membrane/water interface are discussed. The main interest here lies in the distribution of highly charged, large molecules in front of a charged bilayer surface. Ruthenium Red (RuR) will be considered as an example of such a molecule.
2. Part II elucidates the difference between membrane adsorption of electrically neutral and charged hydrophobic molecules, and the distribution of these species in a widely used membrane model system consisting of two immiscible phases, octanol and water.

Cells themselves are very complicated objects to study because of their inherent complexity and multifunctionality. Singer and Nicolson (1972) proposed a fluid mosaic model in which membranes consist of a ca. 70 Å thick two-dimensional solution of oriented globular proteins and lipids as indicated in Figure 1. These proteins, which are embedded into the membrane (integral proteins) or adsorbed to it (peripheral proteins), alter the electrochemical properties of the lipid bilayer and make a cell a complex system with many unknown properties.

To apply the laws of physics one has to go back to a simpler system and translate the results to the more complex biological world. Artificial liposomes consisting of charged and uncharged lipids (e.g., phosphatidylcholine or phosphatidylserine) are widely used as a practical and successful model. Figures 2b and 3 show the structure of artificial liposomes. Lipids themselves consist of a hydrophilic headgroup pointing

Figure 1: Fluid mosaic model of a membrane according to Singer and Nicolson (1972).

Figure 2: (a) Molecular structure and model of phosphatidylcholine; (b) Structure of a liposome.

to the aqueous phase and a hydrocarbon chain which forms the interior of the bilayer (see Figure 2a).

Another system extensively used as a membrane model is octanol. Octanol has a structure comparable to lipids: a hydrophobic tail (the hydrocarbon part) and a hydrophilic head (the OH group) as shown in Figure 4. It therefore exhibits similar properties as lipid material including the formation of micelles in aqueous solutions. These properties make octanol to a very successful model system for

Figure 3: SEM micrograph of an egg-phosphatidylcholine liposome prepared according to Blochel (1992), after fixation with osmium tetroxide and critical point drying (Schmidt, 1995).

Figure 4: Molecular structure of 1-octanol.

organic matter. Nonetheless it is necessary to determine the limitations of applying the octanol/water model.

Part I

Origins of Effective Charge of Multivalent Ions at a Membrane/Water Interface

Chapter 1

Introduction

Adsorption of molecules to membranes is one of the major processes taking place at the membrane/water interface. The density of adsorbed ions is proportional to their interfacial concentration which is given by a Boltzmann relation $N_{if} = N(\infty) \exp\left(-\frac{ze\psi(0)}{kT}\right)$, where $\psi(0)$ is the surface potential of the membrane and $N(\infty)$ the ion concentration in the solution. Therefore, it is of great importance to study the potential distribution at the membrane/water interface.

The most commonly used model to describe potential distribution at a membrane/electrolyte interface is the Gouy–Chapman (GC) theory. It is the objective of this work to elucidate the role of different approximations made in this approach using data on adsorption of Ruthenium Red (RuR) to artificial liposomes obtained in previous work in this lab (Völker and Smejtek, 1995). It was shown that a satisfactory fit of the experimental data to a Langmuir adsorption isotherm could only be obtained if an effective valency $z_{eff} < 6$ for the hexavalent RuR was assumed. Different surface charge densities were obtained by varying the ratio of charged/uncharged lipids. This revealed that the effective valency approaches its physical value of 6 with decreasing surface charge density.

The need to introduce an effective charge has been reported previously in studies of adsorption of melittin (Schoch and Sargent, 1980; Schwarz and Beschiaschvili,

1989; Beschiavili and Seelig, 1990) and peptides (Montich *et al.*, 1993; Mosior and McLaughlin, 1992). It is believed that this behavior is due to one or more limitations of the GC theory. To investigate these limitations, numerical calculations of the potential and of the ion distributions have been performed (i) neglecting ion–surface interactions (no adsorption), and (ii) taking into account adsorption in terms of the Langmuir adsorption isotherm. The results were compared with experimental data for RuR adsorption.

1.1 Overview of Membrane–Electrolyte Models

There are numerous current models dealing with electrostatic properties of membrane–electrolyte systems. The Gouy–Chapman (GC) theory (Gouy, 1910; Chapman, 1913) of the diffuse double layer is the most frequently used approach to describe those systems. It will be discussed in more detail in Section 2.1. The model treats the surface as infinite, smooth and uniformly charged (see Figure 5). Discreteness of charges,¹ dipole potentials arising from the structure of the lipids, mobility of charges on the surface as well as electrolyte–membrane interactions are neglected. The electrolyte is treated as being ideal² and uniform,³ which is the so called *primitive electrolyte model*. Distributed charge effects and other refinements like molecular roughness of the surface, non–coplanar charge, dielectric constant of the headgroup (Peitzsch *et al.*, 1995) or inclusion of finite membrane thickness (Duniec and Thorne, 1983), have been shown to be important only in special cases not considered here.

Despite the simplicity of the model it can claim remarkable success in predicting experimental results in a variety of systems.⁴ Nevertheless, in certain cases the deviation from experimental results is considerably high. Different approaches have

¹the charge of one lipid occupies about 0.7 nm² area of membrane surface

²ions as charged pointlike noninteracting particles

³homogeneous solvent (ϵ_r assumed to be constant throughout the aqueous phase), no hydration/solvation effects

⁴for references, see Torrie (1979) and the excellent review paper of McLaughlin (1977)

Figure 5: Model membrane surface according to the Gouy–Chapman theory.

been used to overcome the limitations of the model.

Nelson and McQuarrie (1975) introduced a discrete charge model in which the surface charges form a square lattice of fixed point charges. This model predicts deviations from the smeared charge theory that are larger for counterions than for co-ions. Winiski *et al.* (1986) performed an experimental test of the model using mono- and divalent ions which agreed much more closely with the GC theory rather than with Nelson’s and McQuarrie’s model. Discreteness of charge effects are believed to become important with increasing valency of ions (Schoch and Sargent, 1980), adsorption of hydrophilic ions (Andersen *et al.*, 1978) and for the case that the charges are buried into the membrane (McLaughlin, 1977).

Tsien and Hladky (1982) introduced a model based on virial expansion for the electrochemical potential of ions adsorbed into the membrane including ion–ion repulsion which could satisfactorily explain the findings of Andersen (1978). Stankowski (1991) used a similar approach to describe adsorption of finite size ions of large valency to lipid bilayer. With his model he could explain the effective valency of ions introduced in previous treatments (Schwarz and Beschiaschvili, 1989; Kuchinka and Seelig, 1989; Beschiaschvili and Seelig, 1990; Stankowski and Schwarz,

1990) in which the ordinary GC Model was used to fit experimental data to adsorption isotherms.

Another approach is a modification of the Poisson–Boltzmann equation (MPB) in which effects due to local ion densities are taken into account. In this framework the potential is described by a infinite set of potential equations which is truncated by a closure condition of n^{th} -order adding so called “fluctuation potentials” to the potential in the exponential of the Poisson–Boltzmann equation (Outhwaite, 1974).

Very sophisticated models like the Hypernetted Chain approximation⁵ (HNC) for bulk electrolytes which use a statistical mechanics approach on the basis of integral equations including finite size effects (hard spheres) and ion–ion correlations were applied successfully to double layer systems (Carnie *et al.*, 1981; Kjellander and Marčelja, 1985; Kjellander and Marčelja, 1988a; Kjellander and Marčelja, 1988b). Numerical solutions of this and the MPB Model predict damped oscillations (charge inversion) of the potential (Henderson *et al.*, 1979; Bhuiyan and Outhwaite, 1979). Langner *et al.* (1990) found excellent agreement of experimentally obtained potential distributions using mono- and trivalently charged lipids with the predictions of HNC.

Monte Carlo methods (MC) provide a means to verify models on a numerical basis. By simulating the diffuse double layer including the main features of the model of interest, one obtains numerical results which then can be compared to the predictions of the model calculations. Due to this totally different approach, it is more of a substitute for experimental work rather than a physical model. One has to remark that this method simulates only the behavior of the double layer within the assumptions of the respective model, rather than the “real world”. Torrie and Valleau (1979) showed that the method is applicable to the diffuse double layer problem by testing the predictions of the GC theory using conditions within which the theory is known to work correctly. Further studies (van Megen and Snook, 1980; Torrie and Valleau, 1980) applying grand canonical ensemble Monte Carlo (GCEMC) simulations

⁵which is a closure condition for the Ornstein–Zernike (Klein, 1994) integral equation

including finite size and excluded volume effects to high electrolyte concentrations and surface charge densities showed deviations from GC. Torrie and Valleau (1980) compared their results with predictions from GC, MPB and HNC and found that up to electrolyte concentrations of 0.1 M and substantial surface charge densities, GC agrees well with the simulation but HNC and MPB extend their applicability to moderate concentrations and surface charge densities. MC simulations showed that image charges have a significant effect on the ion distribution in front of the surface at low surface charge densities which were well predicted by MPB and, within the framework of the screened self-image, by HNC (Torrie *et al.*, 1982).

Chapter 2

Theory

This chapter introduces the models and their modifications which have been studied. The goal of this investigation was to keep the treatment as physically simple as possible, using the GC theory of the diffuse double layer as the basis of all models. The GC Model has been modified to account (i) for steric constraints imposed on large¹ ions in front of a membrane surface and (ii) for the finite size of ions which leads to a “volume–pressure” in regions of high ion concentration. The characteristics of these models have been studied in a simple system without ion–membrane interactions to extract the main features and have then been applied to a more realistic model which includes adsorption of ions to the surface according to a Langmuir adsorption isotherm.

2.1 The Gouy–Chapman Model and its Limitations

The GC theory is based on two fundamental equations: the Boltzmann distribution and the Poisson equation. For ion species i with valency z_i at a distance x from the

¹compared to a Debye length

surface, Boltzmann predicts a concentration of

$$N_i(x) = N_i(\infty) \exp\left(-\frac{z_i e \psi(x)}{kT}\right), \quad (1)$$

where $N_i(\infty)$ denotes the bulk concentration, k is the Boltzmann constant, T the temperature, $\psi(x)$ the potential and e the elementary charge. Poisson's equation is

$$\frac{d^2\psi(x)}{dx^2} = -\frac{\rho(x)}{\epsilon_r \epsilon_0}, \quad (2)$$

where ϵ_r is the uniform dielectric constant of the solution, ϵ_0 the permittivity of free space and $\rho(x)$ the volume charge density of the electrolyte. Combining these equations and applying the electroneutrality condition

$$\sigma_m = -\int_0^\infty \rho(x) dx. \quad (3)$$

yields the *Gouy-Chapman equation*² for the diffuse double layer

$$\sigma_m = 2\epsilon_r \epsilon_0 kT \sum_i N_i(\infty) \left[\exp\left(-\frac{z_i e \psi(0)}{kT}\right) - 1 \right]^{\frac{1}{2}}. \quad (4)$$

Eqs. 3 and 4 have to be solved numerically.³ In many applications this equation is even further simplified by assuming a 1:1 electrolyte and small surface potentials⁴ which yields an analytical solution for $\psi(x)$ (Aveyard and Haydon, 1973):

$$\psi(x) = \psi(0) e^{-\kappa x} \quad \text{with} \quad \psi(0) = \frac{\sigma_m}{\epsilon_r \epsilon_0 \kappa} \quad (5)$$

where κ is the inverse Debye length

$$\kappa = \left(\frac{2z^2 e^2 N(\infty)}{\epsilon_r \epsilon_0 kT} \right)^{\frac{1}{2}}. \quad (6)$$

Stern (1924) proposed a correction for the finite size of ions which results in a plane of closest approach⁵ at one effective ion radius,⁶ r , away from the surface.

²for a more detailed derivation see Appendix A.1

³see Appendix B.2

⁴ $\psi(0) \ll kT$

⁵the "outer Helmholtz plane" according to Grahame (Grahame, 1947)

⁶including hydration shell

Figure 6: Molecular structure of Ruthenium Red

The so modified Gouy–Chapman–Stern Model exhibits a linear potential rise in the region $0 \leq x \leq r$ which is equal to $\Delta\psi = \frac{\sigma_m}{\epsilon_r \epsilon_0} r$. This correction does not apply in our case because the potential and the ion concentrations at the outer Helmholtz plane are the same as the ones at the surface without Stern’s correction.

The GC Model is very successful in predicting potentials and (together with e.g., a Langmuir adsorption isotherm) adsorption of mono- and divalent ions to bilayer consisting of mono- and divalently charged lipids (Winiski *et al.*, 1986).⁷ However, the model is expected to give false results when dealing with ions large compared to the Debye length or ions with valency greater than two, especially at low surface charge densities.

2.2 The Rod Model

Multiply charged molecules whose dimensions are comparable to a Debye length experience steric constraints in front of a charged surface as not all orientations of the molecule are possible. Some orientations are excluded due to the proximity of the molecule to the membrane. In addition, the charge is distributed over the molecule and the molecular ion cannot be regarded to be a pointlike particle. This leads to different potential energies of the charges for different regions of the molecule as they experience different potentials. To include these effects Carnie and McLaughlin (1983) proposed a model with 2 charges on a rigid rod in which the local number density of each $+2e$ -charge on the rod is calculated separately averaging over the possible positions of the other charges. It was necessary to expand this approach for RuR (see Figure 6), which is modeled as an infinitesimal thin rod of length $a = 1.2$ nm (Carrondo *et al.*, 1980) to include three $+2e$ charges A,C at the ends and B, in

⁷see e.g. McLaughlin (1977) for a detailed review

Figure 7: Ruthenium Red Rod-Model in front of a charged surface

its center as shown in Figure 7).

Following the arguments of Carnie and McLaughlin (1983), the local density of charges type A is given by

$$N_A(r) = N_A(\infty) \exp\left(-\frac{2e\psi(r)}{kT}\right) \left\langle \exp\left(\frac{2e}{kT} [\psi(r+z/2) + \psi(r+z)]\right) \right\rangle, \quad (7)$$

where $\langle \dots \rangle$ denotes the spatial average over all positions which the other two charges can occupy. Applying the symmetry of the molecule, we obtain $N_A(r) = N_C(r)$.

Similarly, for the density of charges type B we obtain

$$N_B(x) = N_B(\infty) \exp\left(-\frac{2e\psi(x)}{kT}\right) \left\langle \exp\left(-\frac{2e}{kT} [\psi(x-z/2) + \psi(x+z/2)]\right) \right\rangle. \quad (8)$$

Expressions for the spatial averages are derived in Appendix A.2. These are

$$N_A(r) = N_A(\infty) e^{-\frac{2e\psi(r)}{kT}} \frac{1}{2a} \int_{\max[-a, -r]}^a dz e^{-\frac{2e}{kT} [\psi(r+z/2) + \psi(r+z)]} \quad (9)$$

$$N_B(x) = N_B(\infty) e^{-\frac{2e\psi(x)}{kT}} \frac{1}{2a} \int_{\max[-a, -2x]}^{\min[a, 2x]} dz e^{-\frac{2e}{kT} [\psi(x-z/2) + \psi(x+z/2)]}. \quad (10)$$

These densities together with the Poisson equation, Eq. 2, yield, after formal integration,

$$\psi(x) = \psi(0) + x \left. \frac{d\psi(x)}{dx} \right|_{x=0} - \frac{1}{\epsilon_r \epsilon_0} \int_0^x dy (x-y) \sum_i z_i e N_i(y) \quad (11)$$

This equation has to be solved numerically⁸ using the electroneutrality condition of the diffuse double layer:

$$\sigma_m = - \int_0^\infty dy \sum_i z_i e N_i(y) = - \epsilon_r \epsilon_0 \left. \frac{d\psi(x)}{dx} \right|_{x=0}. \quad (12)$$

2.3 The Maximum Density Model

The Boltzmann distribution, Eq. 1, predicts for a reasonable surface potential of -50 mV an interfacial RuR concentration of 1.2 M which corresponds to $\approx 10^{27}$ molecules/m³, assuming a bulk RuR concentration of 10^{-5} M. By comparing this number to the volume of RuR (using $a = 1.2$ nm and $r = 0.2$ nm, one finds that all free space in front of the surface would have to be occupied by RuR. In order to get a rough estimate of how this extremely high ion density could affect the potential distribution, an empirical term was introduced which inhibits a further rise of the ion density at high potentials

$$N_{MD}(x) = N(x) \exp \left(- \frac{N_{MD}(x)}{N_{max}} \right), \quad (13)$$

where N_{max} is the maximum density of ions estimated by their size and interaction radius which is assumed to be determined by the Debye length κ^{-1}

$$N_{max} = \left(\pi a \kappa^{-2} \right)^{-1} \quad (14)$$

Substituting 1.2 nm for the length of RuR and a Debye length of 2.5 nm⁹ yields a maximum concentration (converted to moles/liter) of about 0.07 M which is small

⁸see Appendix B.3

⁹determined mostly by the buffer concentration used in experiments (Völker and Smejtek, 1995)

compared to that predicted by the pure Boltzmann distribution in front of the membrane surface.

As the adsorbed ions are not completely removed from the aqueous phase one should take their presence in Eq. 13 into account. It was found that the correction is negligible within our error limits.

Numerical procedures for solving the model are given in Appendix B.4.

2.4 The Adsorption Model

In this section a brief outline of the model used by Völker and Smejtek (1995) to fit their experimental data is presented. The adsorption of RuR to lipid vesicles has been measured according to

$$R_{\text{RuR}} = \frac{[\text{RuR}]_{eq}}{[\text{RuR}]_{init}}, \quad (15)$$

where $[\text{RuR}]_{eq}$ is the equilibrium aqueous concentration of RuR in the presence of liposomes and $[\text{RuR}]_{init}$ is the initial concentration before adding the liposomes to the aqueous phase. R_{RuR} varies between 0 and 1; the smaller R_{RuR} , the greater the adsorption. The dependence of R_{RuR} on $[\text{RuR}]_{init}$ is defined as the distribution isotherm. The equilibrium concentration $[\text{RuR}]_{eq}$ depends on the adsorption of RuR to the bilayer, namely on the membrane surface density of RuR, $(\text{RuR})_m$. For bilayer prepared from two types of phospholipids: electrically neutral PC (L- α -phosphatidylcholine) and negatively charged PX,¹⁰ the balance of free and bound RuR ions, is given by

$$[\text{RuR}]_{eq} + (\text{RuR})_m A_{lip} = [\text{RuR}]_{init}. \quad (16)$$

A_{lip} , the surface area per unit volume of liposome suspension, is given by

$$A_{lip} = P_{PX}[PX] + P_{PC}[PC], \quad (17)$$

¹⁰PX represents one of the charged lipids: PS (L- α -phosphatidylserine), PI (L- α -phosphatidylinositol) or PG (L- α -phosphatidylglycerol).

where P_{PX} and P_{PC} designate the area per lipid of negatively charged and neutral lipids, and $[PX]$ and $[PC]$ are their respective concentrations in the suspension of liposomes. A similar balance equation is needed for the monovalent cations (CAT). In modeling RuR adsorption it was assumed that the concentration of monovalent cations in water did not appreciably change¹¹ $[CAT]_{eq} \approx [CAT]_{init}$.

Competitive adsorption of monovalent cations and RuR to the bilayer describable by a Langmuir adsorption isotherm is assumed. The surface density of RuR ions, $(RuR)_m$, depends therefore upon the association constant, K_{mRuR} , the concentration of RuR adjacent to the membrane, $[RuR]_{if}$, and the density of free sites available to RuR cations, f_{RuR} . A similar equation holds for monovalent cations, such as potassium:

$$(RuR)_m = K_{mRuR}[RuR]_{if}f_{RuR} \quad (CAT)_m = K_{mCAT}[CAT]_{if}f_{CAT}. \quad (18)$$

As we assume competitive adsorption to negatively charged lipids only, we have a surface density of free adsorption sites $f_{RuR} = f_{CAT} = f$, which is related to the total density of sites, Q_{PX} , by

$$(RuR)_m + (CAT)_m + f = Q_{PX}, \quad (19)$$

where Q_{PX} is determined by the concentration of negatively charged lipids,

$$Q_{PX} = \frac{[PX]}{A_{lip}}. \quad (20)$$

A Boltzmann relation is used to relate the interfacial concentrations of adsorbing species to their concentrations in the bulk solution

$$\frac{[RuR]_{if}}{[RuR]_{eq}} = \exp\left(\frac{-z_{RuR}e\psi(0)}{kT}\right) \quad \frac{[CAT]_{if}}{[CAT]_{eq}} = \exp\left(\frac{-e\psi(0)}{kT}\right). \quad (21)$$

Völker and Smejtek (1995) had to introduce an effective charge for RuR $z_{RuR,eff} < z_{RuR}$ to account for effects not included in the GC Model. It is the purpose of this study to find a model in which RuR keeps its physical valency of six.

¹¹This simplification is justifiable in view of the rather small binding constant of potassium ions to negatively charged lipids ($\approx 1 \text{ M}^{-1}$) and the experimental conditions.

The membrane surface charge density, σ_m , depends on the charge of the native PC-PX membrane, σ_0 , and on the surface densities of adsorbed cations,

$$\sigma_m = \sigma_0 + e(\text{CAT})_m + 6e(\text{RuR})_m. \quad (22)$$

The theoretical distribution isotherm R_{RuR} is obtained by solving the set of equations, Eqs. 15-22, with Eq. 4 and Eq. 3 for GC, Eq. 11 together with Eq. 12 for the Rod Model and Eq. 13 together with Eq. 14 for the Maximum Density Model. Due to its long calculation time, the Rod Model was solved using a grid of only 500 intervals for the integration. Numerical procedures are described in Appendix B.5.

Chapter 3

Results and Discussion

3.1 Electrostatic Calculations

The electrostatic calculations were performed assuming a pH of 7.3 in 0.001 M citrate/borate/phosphate (2 mM/2 mM/0.5 mM) buffer (B^{-3}). The dielectric constant of the aqueous solution was assumed to be $\epsilon_r = 78.5$.

In order to get a picture of the specific model properties, both the potential distribution (Figure 8) and the Boltzmann factors (Figure 9) have been evaluated. A higher bulk RuR concentration of 0.01 M has been chosen to exaggerate the characteristics of the models which become more evident at higher concentrations.

Exponential decay of the potential is common to all models. Characteristic for the Rod Model is the steep slope in the interfacial region and a less decaying asymptotical behavior which leads to a crossing-over with the other potentials (see arrow in Figure 8). Two opposing processes might explain this behavior: (i) the surface potential decreases almost exponentially resulting in a decrease of the Boltzmann factor, and (ii) the number of available orientations for the rod increases with distance from the surface resulting in an increase of the Boltzmann factor. This effect can clearly be seen for charge B which has no possible orientations at the surface resulting in a vanishing Boltzmann factor for $x = 0$. In the early stages of the iteration

Figure 8: Potential distributions for the three studied models. Surface charge density 1.4 e/nm^2 , RuR bulk concentration 0.01 M , $pH \text{ } 7.3$, buffer B^{-3} , Debye length 0.64 nm ; the arrow marks the crossing-over of the potential calculated by the Rod Model and of that calculated from the GC Model.

Figure 9: Boltzmann factors for the three studied models. Parameters are the same as in Figure 8.

of the surface potential, the increase of available states supercedes the decrease of the potential and the Boltzmann factor actually increases for $x \lesssim a_0$, where a_0 is equal to the length or half the length of the rod for charges type A,C or B, respectively. This might be considered as an energetically unfavorable state and therefore the slope of the potential increases to maintain the Boltzmann factor decaying monotonically. An increase in slope, on the other hand, decreases the integral number of ions in the double layer which leads to a violation of the electroneutrality condition, Eq. 3. To compensate for this, the surface potential rises in magnitude due to less efficient screening. The abrupt change of the slope for the Boltzmann factors at position a_0 arises from the fact that from this position on, the rod can occupy all available orientations no longer constrained by the membrane.

The potential distribution for the Maximum Density Model exhibits a very large surface potential but similar slope compared to GC. This can also be explained in terms of the electroneutrality condition. As the model effectively decreases RuR concentration (by at least an order of magnitude) in the wider interfacial region, the surface potential increases to maintain charge neutrality in the diffuse double layer which in turn increases the Boltzmann factor resulting in a reduced effect on $[\text{RuR}]_{if}$.

Lower bulk concentrations of RuR result in smaller deviations of the Boltzmann factors and potentials from GC (not shown).

To relate the Boltzmann factors to an effective charge for RuR, the effective valency in GC was varied until the Boltzmann factor matched the one for the Rod or the Maximum Density Model. Figure 10 shows the results for the Rod Model and Figure 11 for the Maximum Density Model for different RuR concentrations. The figures include experimentally obtained effective valencies from adsorption studies (Völker and Smejtek, 1995).

The effective valency plots have to be interpreted very carefully as they cannot be compared directly to the experimental values which were obtained from fits of the Adsorption Model employing different bulk concentrations of RuR.

Figure 10: Effective valency for Gouy–Chapman to fit the Rod Model.

Figure 11: Effective valency for Gouy–Chapman to fit the Maximum Density Model.

The figures show that both models reflect the tendency of the experimental data: decreasing surface charge density results in increasing valency with an asymptotic approach of the physical value of six.

It is important to note that the Rod Model fails for small surface charge densities. The reasons for this and the deviation from a smooth curve are still unknown. A failure of the algorithm can be excluded as calculations with increased accuracy gave the same result.

3.2 Adsorption Isotherm Calculations

The Rod and the Maximum Density Model can be applied to the Adsorption Model. The calculations for the Adsorption Model were performed with an association constant for potassium ions $K_{mCAT} = 1$, an association constant for Ruthenium Red ions $K_{mRuR} = 5.5$ and an area per lipid of 0.71 nm^2 . Figure 12 shows the adsorption isotherms for a lipid composition of PC:PS=9:1. The plots represent calculated values for the GC Model assuming a valency of 6 and 3.9 and for the Rod Model with valency 6. A plot for the Maximum Density Model has been omitted as it would be indistinguishable from the GC valency 6 plot which originates from the similarity of their interfacial RuR concentrations (Figure 14). This similarity arises from the fact that $[\text{RuR}]_{if}$ is so low (10^{-6} – 10^{-4} M) that ion interactions do not play an important role.¹

The Rod Model improves the fit to the data substantially when compared to GC with valency 6. This improvement is even better for lower surface charge densities (PC:PS=9:1) as can be seen from Figure 13 in which the effective valency for the GC fit was set to 3.1.

Nevertheless the model overestimates the RuR adsorption for lower initial bulk

¹the maximum density calculated according to Eq. 14 for our experimental conditions is about 0.07 M

Figure 12: Langmuir adsorption isotherm for RuR adsorption to PC:PS=4:1 lipid vesicles, pH 7.3, buffer B^{-3} .

Figure 13: Langmuir adsorption isotherm for RuR adsorption to PC:PS=9:1 lipid vesicles, pH 7.3, buffer B^{-3} .

Figure 14: Interfacial RuR concentration for PC:PS=4:1, pH 7.3, buffer B^{-3} .

concentrations. It is remarkable though that the difference in interfacial RuR concentration (Figure 14) is miniscule for low initial RuR concentrations and increases for higher concentrations where the model seems to predict the correct adsorption. The reason for this lies in the saturation of the adsorption isotherm² at high concentrations reducing the influence of the interfacial RuR concentration on the overall adsorption. For lower concentrations on the other hand, a minute increase in $[\text{RuR}]_{if}$ results in an increase in adsorption which leads to a depletion of the bulk phase as the surface area available for adsorption³ is around 500 m². One might argue that e.g., for the Maximum Density Model, the concentration of adsorbed ions needs to be included in the interfacial concentration as it is likely that adsorbed ions protrude into the aqueous phase from the membrane surface and therefore contribute to the maximum density. But due to the huge surface area, the concentration of adsorbed ions is negligible compared to $[\text{RuR}]_{if}$ (data not shown).

3.3 Conclusions

In this theoretical study it has been shown that steric effects play an important role in the spatial distribution of Ruthenium Red ions. Obtaining a new set of adsorption parameters using these models would be desirable but is not feasible due to the long computation times.

The results of the Maximum Density Model show that despite the failure of the model to correctly predict the adsorption isotherm, ion-ion interactions which are not included in the GC Model, are expected to be important at charged membrane surfaces.

To explore a possible way to incorporate ion-ion interactions one might consider a model in which the discreteness of the surface charge is included. Discreteness of charge gives rise to high ion concentrations in front of the charged lipids. A

²the high concentration of RuR adsorbed to the membrane decreases the surface charge density resulting in a substantial decrease of the surface potential

³ area calculated for 1 g lipid solution (MW $\approx$ 800 u) assuming a lipid area of 0.71 nm²

Figure 15: Primitive Discrete Charge Model. ψ_d discrete potential of the reduced area, ψ_0 average potential.

primitive approach to model the discreteness of the charges is to use a rectangular potential, as shown in Figure 15. Neglecting adsorption and electrostatic interactions at the areas of vanishing potential, this model simply reduces the active surface area associated with an increase in surface potential obtained by assuming a constant average potential

$$\psi_d = \frac{A_0}{A_d} \psi_0, \quad (23)$$

where ψ_d denotes the new surface potential, ψ_0 the initial average potential, A_0 the initial surface area and A_d the area remaining after subtraction of the inactive area. Assuming a ratio of $A_0:A_d=2:1^4$, leads to a doubling of the potential and a squaring of the Boltzmann factor resulting in an interfacial RuR concentration of about 75 M compared to 1 mM. At this concentration ion-ion interactions certainly become important. Applying the Maximum Density Model, one obtains a lower boundary for the concentration $N_{MD}=0.07$ M (the maximum density Eq. 14). As the surface area in reality is twice as large, the concentration halves to 0.035 M. A rough estimate might suggest that the adsorption due to the decrease of binding sites also decreases by a factor of two. But even with this assumption the adsorption will be much higher then before. In addition to that, increasing charge density should

⁴which is a reasonable assumption considering the interlipid distance on the basis of a hexagonal lattice

decrease the effect. We observe a smaller deviation for our models with an increase in surface charge density. It is therefore unlikely that discrete charge effects play an important role.

From the properties of the two models and the obtained results, conclusions for the possible characteristics of a more complete model in which RuR has its physical valency of six can be drawn. Within the framework of the smeared charge theory, steric effects are fully accounted for in the Rod Model. The failure of the Maximum Density Model is twofold: (i) the fulfillment of the charge neutrality condition leads to a substantial decrease of its effectiveness to reduce interfacial concentration, and (ii) low interfacial concentrations in the Adsorption Model suggest the absence of ion-ion interactions.

To overcome these failures one would need a much stronger nonlinear process which would alter only the ion distribution in front of the surface keeping the surface potential close to the GC result. This demands a steeper slope of the spatial distribution of the potential in the interfacial region as observed for the Rod Model.

The only way to account for these nonlinearities would be the application of one of the statistical mechanical approaches which incorporate “real” ion-ion interactions. Stankowski’s (1991) Virial Expansion Model is a promising candidate for future explorations. Integral Equation Models like HNC would certainly be the most suitable tool for the problem, but due to their difficult implementation their use in experimental studies is limited.

Part II

Distribution of

2,3,4,5–Tetrachlorophenol in a

Membrane Model System

Chapter 4

Introduction

Chlorinated phenols have been extensively employed in different industrial and agricultural applications. Pentachlorophenol (PCP) was the most widely used chlorinated phenol, but other chlorinated phenols like tetrachlorophenol (TeCP) and trichlorophenol (TrCP) are side products of chemical reactions in chlorobleaching of Kraft pulp (Paasivirta *et al.*, 1992) and evolve during the breakdown process of PCP by UV light and biodegradation (Murthy *et al.*, 1979; Mikesell and Boyd, 1986). Therefore they can be found in the environment in concentrations similar to that of PCP (Butte, 1985; Rogers *et al.*, 1992). Wood treatment was the major application of the 23 million kilograms of pentachlorophenol produced yearly in the U.S. (Cirelli, 1978; Dougherty, 1978) that ultimately end in the environment. Due to their strong oxidative character chlorinated phenols have also been extensively

Figure 16: Molecular structure of 2,3,4,5-tetrachlorophenol (TeCP).

used as biocides to control bacteria, fungi, mollusks, insects, slime, and other biota (Kaufman, 1978). They have long been known to disrupt cell function by destroying the proton gradient across the inner mitochondrial membrane and therefore uncoupling the oxidative phosphorylation of ATP (Weinbach, 1956). Partitioning of the hydrophobic chlorinated phenols into the lipid phase of the bilayer (Buff *et al.*, 1982) enables proton transport across the membrane (Jayaweera *et al.*, 1982).¹ It has also been shown that their presence decreases the fluidity of lipid bilayers (Duxbury and Thompson, 1987), the gel-to-fluid phase transition temperature (Smejtek *et al.*, 1989), the free amino acid pool in freshwater amphipod *Gammarus pseudolimnaeus* (Graney and Giesy, Jr., 1987) and the carbon assimilation by algae (Jayaweera *et al.*, 1982). For an overview of the membrane toxicity of PCP see Smejtek (1987).

Although the production of PCP has been discontinued or its use reduced in many countries, it is believed that chlorinated phenols continue to be one of the major pollutants in the environment since they are already ubiquitous.

Several studies and estimates in Germany (Geyer *et al.*, 1987), the United States (Hattemer-Frey and Travis, 1989) and the United Kingdom (Wild and Jones, 1992) showed that the food chain is the major source (90–99%) of PCP exposure to humans along with inhalation of vapors from treated wood and the consumption of drinking water, which accumulate to an estimated long-term average daily intake of about 10–48 $\mu\text{g}/\text{day}$. The average body burden of PCP varies for different countries between 548 μg and 673 μg .

Although it has been shown (Geyer *et al.*, 1987) that the half-life of PCP in humans is about 19 days, there is strong evidence for chronic effects due to long term exposure (Crosby, 1981) especially in organs with high bioconcentration factors (BCF) which are liver (BCF=5.7) and brain (BCF=3.3) (Geyer *et al.*, 1987). The

¹there are two possible mechanisms: (i) A^- penetrates through the membrane, binds to a H^+ , diffuses back and releases the proton to the outside (Terada, 1990) or (ii) an AHA^- complex is formed at the outer surface, permeates through the membrane, binds a proton which results in a dissociation of the dimer, AH travels back to the outer surface of the membrane and releases the proton (Barstad *et al.*, 1993)

high hydrophobicity of chlorinated phenols leads to a strong affinity to lipid material such as cell membranes or organic material in soils to which they adsorb.

Kerler and Schönherr (1988), among others, showed that the ability of substances to adsorb to organic material is closely related to their octanol/water partition coefficient, P_{ow} .

P_{ow} is most commonly obtained by the shake flask method as proposed by OECD (OECD, 1981). In this approach a compound X is injected into a two-phase system consisting of known volumes of octanol and water. After shaking the solution and separating the phases, samples from either the water phase and/or the octanol phase are taken and the concentration of the compound is determined. The octanol/water partition coefficient P_{ow} is defined by

$$P_{ow} = \frac{[X]_o}{[X]_w}, \quad (24)$$

where the subscript “O” stands for the octanol and “W” for the aqueous phase. As the procedure is very laborious,² other methods of obtaining P_{ow} have been explored. One of them is the generator column method invented by Wasik *et al.* (1981) and improved for $\log P_{ow}$ values > 6 by Woodburn (1984). In the slow stirring method the solution is not shaken but slowly stirred under close temperature control to avoid emulsion formation (Brooke *et al.*, 1986). It is especially designed for very high values of P_{ow} but requires long stirring times (2–3 days) to reach equilibrium.

There are several indirect methods, experimental as well as numerical, to estimate P_{ow} . The most frequently used is the estimation of P_{ow} based on reversed-phase high-performance liquid chromatography (RP-HPLC) retention times in which a linear relationship between retention times of known compounds and their partition coefficient is used to predict P_{ow} of a unknown compound (Weber, Jr. *et al.*, 1986). Probably the least accurate estimate for the partition coefficient can be obtained from the water solubility of a compound (Miller and Wasik, 1985) using the

²each sample point requires about 4 hours

relation

$$\log P_{ow} = A - BV - \log C_L^S \quad \text{for liquids and} \quad (25)$$

$$\log P_{ow} = A - BV - \log C_s^S + \log F \quad \text{for solids,} \quad (26)$$

where V is the molar volume, F the fugacity ratio given by $F \approx \exp[6.8(1 - T_M/T)]$ with the melting temperature T_M , C_L^S and C_s^S are the water solubilities for liquid and solid compounds, respectively, and A and B are correlation parameters obtained from experimental data.

Several numerical estimation methods for the partitioning of compounds into organic matter using the octanol/water partition coefficient have been established. Quantitative structure activity relationships (QSAR) are the most accurate approach in which characteristics of the compound like charge density distribution, number of different atoms and functional groups are accounted for in simple regression equations of the form

$$\log P_{ow} = \text{const.} + \sum_i n_i P_i + \sum_i P'_i q_i + \sum_i P''_i q_i^2 + \sum_m F_m, \quad (27)$$

where P_i , P'_i , P''_i are parameters characteristic of a given atom i , n_i is the number of occurrences of such atoms and q_i is the charge density on i . F_m represents the presence or absence of functional groups (Leo *et al.*, 1971; Klopman *et al.*, 1985). Extensions including solvatochromic parameters,³ molecular surface, and volume (Kamlet *et al.*, 1988; De Bruijn and Hermens, 1990) improved the correlation of data. Similar models have been proposed to calculate the partition coefficient for a new solute on the basis of a known coefficient. This opens the possibility for obtaining a set of parameters which relates structural and/or functional properties of molecules to their toxicity (Leo *et al.*, 1971), bioconcentration (Mackay, 1982; van Gestel *et al.*, 1985) and presence in soils and sediments (Schellenberg *et al.*, 1984; Lagas, 1988). For a recent review of different QSAR methods see Sabljic (1991).

³solute polarizability, hydrogen bond acceptor basicity, and hydrogen bond donor acidity

Although the octanol/water model is successful in predictions of partitioning of neutral molecules in biological tissue, the situation is different if ions are present in the system. This holds in particular for hydrophobic ionizable organic compounds (HIOC's) like chlorinated phenols (HA) with a pK_a of between 3.7 and 8.3 for which ionized forms (A^-) need most definitely to be taken into account at ambient pH values. In this case two partition coefficients determine the distribution of the compound, one for the neutral form, γ_{HA} , and one for the ionized form, γ_A .

Kaiser (1982) studied the dependence of the apparent P_{ow} of PCP as a function of pH and determined significant anomalies which were believed to be partly caused by the varying ionic strength of the solution. Westall (1985) performed a detailed study of the effect of ionic strength and pH on the distribution of chlorinated phenols in octanol/water. Smejtek and Wang (1993) compared partitioning of PCP in the octanol/water system with adsorption to phospholipid vesicles and found substantial differences for $pH > pK_a$.

The present study is in line with Smejtek's and Wang's studies. Our goal is the determination of the partition coefficient for 2,3,4,5-tetrachlorophenol and its phenolate, simultaneously, by means of the shake flask method. Different approaches for the theoretical description of the partitioning process are outlined and discussed. The obtained data are compared to results from adsorption studies for the same compound under the same experimental conditions (Blochel, 1992). The applicability of the octanol/water partition coefficient to predict biological significance in this particular case is questioned.

Chapter 5

Theory

5.1 Partitioning Theory

The partitioning of a substance between two or more phases is described by the laws of thermodynamics. To derive a description for the state of a solute particle, one assumes an ideal gas (Adam *et al.*, 1988) whose thermodynamic state is determined by its chemical potential, μ , defined by¹

$$\mu = \left(\frac{\partial G}{\partial N} \right)_{T,V} \quad (28)$$

where N is the number of particles, T the temperature, V the volume and G the free energy of the system, defined by

$$G(N, V, T) = U + pV - TS = H - TS. \quad (29)$$

In this equation S denotes the entropy, U the internal energy and p the pressure of the system. The latter quantities have been combined to the enthalpy $H = U + pV$. For a pure substance at constant temperature and volume, Eq. 28 reduces to

$$\mu = \frac{G}{N}. \quad (30)$$

¹the subscripts denote the quantities held constant

An infinitesimal change dG at constant temperature results in a change $d\mu$ of the chemical potential which can be written as

$$d\mu = \frac{dG}{N} = \frac{V}{N}dp = RT\frac{dp}{p}, \quad (31)$$

where R is the ideal gas constant and the relation $pV = nRT$ for the ideal gas has been used. Integration of Eq. 31 assuming an initial pressure p_0 and initial chemical potential² μ_0^* yields

$$\mu = \mu_0^* + RT \ln \frac{p}{p_0} = \mu_0^* + RT \ln p, \quad (32)$$

where the initial pressure p_0 has been assumed to be 1 bar in the last step. μ_0^* is a constant determined by the initial pressure p_0 , the temperature and the nature of the gas.

This result can easily be transferred to dilute solutions in which the interaction between the solute particles can be neglected as it was done for the ideal gas. Substituting the pressure p in Eq. 32 by

$$p = \frac{N}{V}RT = cRT, \quad (33)$$

where c is the molar concentration, gives the chemical potential of a single solute

$$\mu = \mu_0^* + RT \ln(RT) + RT \ln c = \mu_0 + RT \ln c. \quad (34)$$

It is known from thermodynamic theory that chemical equilibrium is reached when $dG = 0$. For the case of partitioning of a substance between two phases, dG is given by the sum of the individual free energies of the solute in the two phases, dG' and dG'' , which equals

$$dG = dG' + dG'' = \mu'dN' + \mu''dN'' = (\mu' - \mu'')dN', \quad (35)$$

where a closed system³ has been assumed. The equilibrium condition $dG = 0$ can only be fulfilled for $\mu' = \mu''$ leading to

$$\frac{(\mu'_0 - \mu''_0)}{RT} = \ln c'' - \ln c' = \ln \frac{c''}{c'}. \quad (36)$$

²often called the "standard chemical potential"

³conservation of particles: $dN' = -dN''$

This equation can be rewritten as

$$P = \frac{c''}{c'} = e^{(\mu'_0 - \mu''_0)/RT} = e^{-\Delta G_0/RT}, \quad (37)$$

where the partition coefficient P and the free energy of transfer, ΔG_0 , have been introduced.

The theoretical determination of the partition coefficient has now been reduced to find an expression for the standard energy of transfer which is the energy needed to bring one mole of a substance from one phase to the other. According to Eq. 29 the free energy of transfer can be divided into an enthalpy term, ΔH , and an entropy term, $T\Delta S$.

This general theory holds for partitioning of a substance in octanol *and* sorption to membranes. Differences arise from the fact that a membrane is a thin (≈ 70 Å) bilayer with a large surface area⁴ in contact with the surrounding solution, whereas octanol forms a bulk phase.

Partitioning into membranes is often described by the total energy model invented by Flewelling and Hubbell (1986). In this model the total energy of transfer⁵ is composed of the Born, image, dipole and neutral energy terms. The Born energy is the sum of energies needed to discharge a sphere in one dielectric, transfer it to the other phase and recharge it there. The image energy arises from a polarization of the membrane which can mathematically be described by image charges located a certain distance away from the interface in the region of the membrane. The dipole term accounts for the contribution of the dipole moments of lipid headgroups to the overall surface potential. As this interaction is hard to describe due to the discreteness of surface charge and variations in the dielectric constant near the surface, only a first order approximation can be made. Similar considerations hold for the neutral energy which is (apart from electrical contributions) the energy of transfer for a hydrophobic ion from one phase to the other including van der Waals, hydrophobic and steric factors.

⁴500 m² calculated for 1 g lipid solution (MW $\approx$ 800 u) assuming a lipid area of 0.71 nm²

⁵which equals the enthalpy of transfer, ΔH

Figure 17: Reactions at the octanol/water interface involving TeCP in its neutral (HA) and ionized (A^-) forms, with proton (H^+) or potassium (K^+) counterions.

The total energy according to this model exhibits a potential well for positively as well as for negatively charged ions close to the membrane/water interface. Therefore the negative surface charge does not hinder partitioning of anions into the membrane. This model successfully predicts experimental values for ΔG_0 for the binding of tetraphenylphosphonium (TPP^+) and tetraphenylboron (TPB^-) (Flewelling and Hubbell, 1986).

A similar model might be applicable to the partitioning of ions into octanol by neglecting the energy terms related to surface interactions which are the dipole and image energies. Another important difference is the fact that electroneutrality of the phases needs to be satisfied. Therefore partitioning of A^- into the octanol phase implicates the simultaneous partitioning of counterions. This co-partitioning of counterions is the limiting factor for γ_A and is the origin of the strong dependence on the ionic strength of the solution (Westall *et al.*, 1985). Westall suggested the existence of free ions in the octanol phase on the basis of Bjerrum's model (Harned and Owen, 1958).

Figure 17 shows possible reactions at the octanol/water interface involving HA

and A^- . The individual partition coefficients are the sums of the partition coefficients for A^- implying simultaneous partitioning of H^+ or K^+ .

$$\gamma_{HA} = \gamma_{HA} + \gamma_{KA} \quad \text{and} \quad \gamma_A = \gamma'_A + \gamma''_A. \quad (38)$$

Recent computer simulations of ion transport across a 1,2-dichloroethane/water interface (Benjamin, 1993) show that the dynamics of the interface on a picosecond scale plays an important role in the transfer of Cl^- ions.

5.2 Distribution Ratio and Partition Coefficients

Adsorption of charged molecules to biological membranes can adequately be modelled by the Gouy–Chapman theory for the diffuse double layer including a Langmuir adsorption isotherm, which has been described in detail in Section 2.4. From this model the linear partition coefficient, β_A^m , can be obtained

$$\beta_x^m = \frac{(X)_m}{[X]_{aq}}. \quad (39)$$

The relation between the linear partition coefficient and the bulk partition coefficient, γ_x^m , is given by

$$\gamma_x^m = \frac{\langle [X]_m \rangle}{[X]_{aq}} \quad \text{with} \quad (40)$$

$$\langle [X]_m \rangle = \frac{\int_0^t [X(z)]_m \cdot dz}{t}, \quad (41)$$

where $[X(z)]_m$ denotes the local concentration of the compound inside the membrane at a distance z from the surface (Smejtek and Wang, 1993).

The theoretical description for partitioning of compounds (charged or uncharged) between bulk phases is based on simple chemical equilibrium equations employing partition coefficients. For the determination of individual partition coefficients for TeCP and its ionized form, $\gamma_{HA} = \frac{[HA]_O}{[HA]_W}$ and $\gamma_A = \frac{[A^-]_O}{[A^-]_W}$ respectively, a relationship

between these parameters and a measurable quantity needs to be developed. This quantity is the distribution ratio, D , defined by

$$D = \frac{[\text{HA}]_o + [\text{A}^-]_o}{[\text{HA}]_w + [\text{A}^-]_w}, \quad (42)$$

which can be obtained experimentally. The pK_a of the compound in water is given by

$$pK_a = -\log \frac{[\text{A}^-]_w [\text{H}^+]}{[\text{HA}]_w} \quad (43)$$

which yields together with $pH = -\log[\text{H}^+]$

$$[\text{A}^-]_w = [\text{HA}]_w 10^{pH-pK_a}. \quad (44)$$

Substituting Eq. 44 into Eq. 42 results in

$$D = \frac{\gamma_{\text{HA}} + \gamma_{\text{A}} 10^{pH-pK_a}}{1 + 10^{pH-pK_a}}. \quad (45)$$

This equation relates γ_{HA} and γ_{A} to D and pH as parameters which can be directly obtained from experimental data. A nonlinear fit of value pairs D and pH to Eq. 45 yields γ_{HA} and γ_{A} .

5.3 Absorption Spectroscopy

It was assumed that TeCP (HA) and its ionized form A^- obey Beer's Law which relates the concentration of a substance to the intensity $I(x)$ of a beam of light traveling through a solution containing the substance. This can be expressed by

$$I(x) = I_0 e^{-A} \quad \text{with} \quad A = \epsilon xc, \quad (46)$$

where I_0 is the incident light intensity, A the absorbance, x the path length the light has traveled through the solution, c the concentration and ϵ the extinction coefficient of the compound in the specific solvent.

The mechanism behind this law is based on quantum physics. Molecules have

Figure 18: (a) Electronic and vibrational energy states of a molecule, E_0 : ground state, E_1 : excited state; (b) solvent shift of energy transition (bathochromic shift), E_1^* : temporary excited state before reordering of solvent molecules

different energy states which are of electronic, rotational or vibrational origin. By absorbing any form of energy (e.g., kinetic energy or light) a molecule can undergo a transition from its ground state to an excited state. The energy E of a photon is related to its wavelength λ by

$$E = \frac{hc_m}{\lambda}, \quad (47)$$

where h is Planck's constant and c_m the velocity of light in the medium. If a photon with an energy equal to the energy of a possible transition⁶ hits a molecule, the molecule undergoes a transition to an excited state by absorbing the photon. This results in a decrease of photon flux through the sample at that particular wavelength. Eq. 46 reflects this effect in the form of a classical relationship where the extinction coefficient incorporates the quantum mechanical properties of the specific molecule.

The energy gap between two electronic states is usually of the order of an electron volt (eV) which corresponds to the energy of visible or ultraviolet (UV) light. Rotational and vibrational energy gaps are much smaller and can therefore be excited by infrared (IR) light or microwave radiation. As the energy of these transitions is relatively low, some molecules are in vibrational or rotational excited states at room

⁶the transitions are restricted by the quantum mechanical selection rules

temperature due to thermal excitation. This results in a splitting of the electronic energy states as vibrational levels are superimposed (see Figure 18a). Molecules can therefore undergo electronic and vibrational/rotational transitions simultaneously which manifests itself as a broadening of the absorption peak.

Changes in the energetic behavior of the molecule like conformational changes, group substitutions, solvent interactions, etc., alter the extinction coefficient. Of particular importance in our case are solvent effects as we use buffer (water) and octanol as solvents in the experiment. A shift in the absorbance wavelength can be caused by the properties of the solvent. Most molecules are more polar in the excited state than in the ground state. This leads to a different ordering of the polar water molecules around the solute molecule, depending on its state. The water molecules cannot rearrange during the very short transition process (≈ 1 fs) from the ground to the excited state which results in a temporary energy rise of the excited state. Therefore a higher excitation energy is needed which manifests itself in a shorter wavelength of the absorbed light (see Figure 18b). This effect is the origin for the shift in the absorbance wavelength for the neutral form of TeCP from $\lambda = 298$ nm in water (as a polar solvent) to $\lambda = 301.5$ nm in octanol (as a nonpolar solvent).

Chapter 6

Materials and Methods

6.1 Chemicals

Potassium chloride was bought from EM Science, Cherry Hill, NJ. Boric acid and potassium citrate were both from Matheson, Coleman & Bell Manufacturing Chemists, Norwood, Ohio. 2,3,4,5-tetrachlorophenol (98% pure) and 1-octanol (spectroscopic grade) were obtained from Aldrich Chemical Company, Milwaukee, WI. Chemicals were used without further purification. All solutions were prepared with deionized water.

6.2 Spectrophotometric Measurements

The spectrophotometer used in all experiments was Model DU-7 from Beckman Instruments, Irvine, CA. For the buffer phase 10 mm (Pyrocell Manufacturing Co., Inc., Westwood, NJ), 50 mm and 1 mm cuvettes (Spectrocell Corp., Orland, PA) were used. The octanol phase was measured in 10 mm cuvettes from Spectrocell. According to the specifications the spectrophotometer has ± 0.5 nm wavelength accuracy, $\pm 0.5\%$ photometric accuracy and a baseline drift below 0.003 A/hr. A tungsten lamp is employed for the visible region (700–315 nm) and a deuterium

Figure 19: Block diagram of the optical path in the spectrophotometer (Beckman, 1982).

lamp for the UV region (315–200 nm). A microprocessor controlled mirror selects the lamp for the wavelength region being scanned. The offset at the 315 nm switching point was repeatedly checked to be within specifications. The light beam is narrowed by the slit assembly and focussed on the grating by the collimator mirror as shown in Figure 19. The grating breaks the light beam into its spectrum. It is controlled by a stepper motor to adjust the wavelength being reflected back onto the collimator mirror. From here the light is focussed through a set of microprocessor-controlled blocking filters which provide constant energy throughout the spectrum. Two transfer mirrors position the beam path so that it can pass through the sample compartment. The detector mirror focusses the beam onto the photomultiplier tube which produces an electrical signal that is amplified and then analysed by a microprocessor.

A scan speed of 600 nm/min at a resolution of 0.5 nm was used throughout the experiment. The spectrophotometer was controlled by an IBM compatible PC running a program designed for that purpose. The software is described in Appendix C.

To perform a measurement, a background solution and a sample solution is needed. The background has the same consistency as the sample only without the compound to be measured. Therefore, two cuvettes are needed which were rinsed with methanol and acetone, air dried and carefully cleaned with lens tissue before each experiment. The absorbance difference between both cuvettes¹ was not allowed to exceed 0.002 A. During the experiment the cuvettes were rinsed at least three times with the new solutions before a measurement was performed.

6.3 Phase Preparation

In all experiments citrate/borate/phosphate buffer (2 mM/2 mM/0.5 mM), B⁻³, with a salt concentration of 0.03 M KCl was used as the aqueous phase. The addition of the salt was necessary to provide the same experimental conditions as in the membrane adsorption measurements (Blochel, 1992) and to maintain a constant K⁺ concentration² as the partitioning depends strongly on this parameter (Westall *et al.*, 1985). Buffer solutions were prepared by diluting a B⁻¹ stock solution and adding the necessary volume of a ≈ 2 M KCl solution.

To reduce the effects³ of water solvation in octanol and vice versa,⁴ presaturated phases have been used for all experiments. Octanol and buffer solutions were combined at a ratio 1:5 in 250 mL polypropylene centrifuge bottles. The bottles were shaken for one hour on a Wrist-Action shaker model BB from Burell Corp., Pittsburgh, PA, and then centrifuged at 1500 g in a IEC DPR-6000 centrifuge.

Extreme care has to be taken when separating the two phases. The octanol phase was removed from the top of the aqueous phase with a syringe. The syringe was fixed on a holder to facilitate one-hand operation. An octanol layer of approximately 3

¹the cuvettes were filled with the respective background since measurements with dry cuvettes gave false results

²the addition of KCl and KOH during titration is negligible

³change in volume and spectroscopic properties

⁴water has a high solubility (2.3 M) in octanol, whereas octanol is poorly soluble in water (4.5 mM)

Figure 20: (a) Spectrum of the combined aqueous phase used for the partitioning experiment after saturation with octanol, measured against pure buffer; (b) Spectrum of the combined octanol phase used for the partitioning experiment after saturation with buffer, measured against pure octanol.

mm on top of the aqueous phase was always discarded to eliminate interfacial effects of the mixing process. The aqueous phase was moved to a stock container with a 150 mL pipet, again discarding the last 5 mm. For the partitioning experiment the phases of two saturation batches consisting of six bottles each were combined.

The “quality” of saturation was examined spectrophotometrically using unsaturated solutions as background. The difference in absorbance for the saturated buffer phase in the region of interest (300–330 nm) had to be below 0.005 A (see Figure 20a). The saturated octanol phase exhibited a peak at around 275 nm whose origin is unknown (see Figure 20b). During several saturation batches the main features of the spectrum were extracted and served as criteria for the success of the saturation.

The formation of octanol micelles in the aqueous phase and vice versa is very likely to occur as it is reported for various oil/water emulsions (Cazabat and Langevin, 1980; Smit *et al.*, 1990). The size of these micelles is of the order of a wavelength (≈ 200 nm) and therefor causes scattering of light in the UV region. This results in an increase in the measured absorbance and might be the reason for the peak at

275 nm in saturated octanol. The possible effects of micelle or emulsion formation has been neglected in this treatment.

6.4 Extinction Coefficients

Extinction coefficients for TeCP in both the aqueous and the octanol phase were needed for the interpretation of the partitioning data. For the aqueous phase only the extinction coefficient for the ionized form was determined as all samples in the partitioning experiment were titrated to a high pH for the spectroscopic determination of TeCP concentration.

TeCP sample solutions for the aqueous phase were prepared from stock solutions ranging between 1 mM and 5 mM due to the limited solubility of TeCP (see Table 3). To increase the solubility of TeCP, KOH was added in small amounts for the preparation of the 5 mM stock solution. It was not possible to dissolve TeCP in saturated buffer as aggregates of TeCP and octanol seem to form. All stock solutions were therefore prepared from unsaturated buffer but diluted with saturated one. The aqueous solution was titrated to a pH well above the pK_a of 6.35 (Schellenberg *et al.*, 1984). As TeCP has a high solubility in octanol, the stock solutions had concentrations of 5 mM and 17 mM. It was found that the extinction coefficient for the saturated and the pure solutions are the same within our measurement accuracy. Sample concentrations ranged from 0.1 mM to 0.4 mM for both solvents.

6.5 Partitioning

The samples for the partitioning experiment were prepared from a 20.0 mM stock solution of TeCP dissolved in saturated octanol and pH -adjusted saturated buffer. We used 25 mL of buffer and 5 mL of octanol keeping the octanol:water ratio 1:5, which was the same as used in the phase-saturation procedure. The initial pH of the buffer phase ranged between 3.23 and 12.3 resulting in a final pH between 3.42 and

11.9 measured with a Model 6072 microcomputer pH meter from Jenco Electronics, Taipei, Taiwan.

The experiment was performed using the same preparation methods as described for the presaturation of the phases. For each pH value two 50 mL glass centrifuge tubes were prepared, one containing octanol with TeCP and the other just presaturated octanol which was used as background in the spectrophotometric measurements. Attention has to be paid to the position of the tubes on the shaker to assure the mixing of the whole solution. Centrifuging at 1500 g was done in a Safeguard centrifuge from Clay-Adams Inc., New York, NY.

Samples were always taken from the aqueous phase. In three cases samples were also taken from the octanol phase to determine the mass balance of TeCP which was found to be well within our error limits. At high final pH , dilution of the sample⁵ was necessary as the concentration of TeCP in the buffer phase exceeded the absorbance limit of the instrument. Low values of the final pH resulted in concentrations of the order of the detection limit of the spectrophotometer. For the lowest pH a cuvette with a path length of 5 cm was used.

The pH of the sample from the aqueous phase was measured immediately after the sample was taken to prevent a drop in pH caused by dissolution of CO_2 from air. Samples with a final pH lower than 9.4 were titrated to a pH well above the pK_a to transform the major part of TeCP into its ionized form. This procedure was chosen since only information about the total TeCP concentration was needed, rather than the concentration of the neutral and the ionized species separately.

⁵alternatively a cuvette with 1 mm path length was used instead of 1 cm

Chapter 7

Results and Discussion

7.1 Extinction Coefficients

The extinction coefficient for 2,3,4,5-tetrachlorophenol in buffer and octanol has been measured for several concentrations using three different stock solutions. Table 1 lists the sample concentrations and the obtained values for the extinction coefficients. All concentration determinations for the partitioning experiment are based

Figure 21: (a) Spectrum for 0.423 mM 2,3,4,5-tetrachlorophenol in presaturated 1-octanol; (b) Spectrum for 0.269 mM 2,3,4,5-tetrachlorophenolate in buffer B⁻³ at pH 10.8.

solution	stock conc. [mM]	sample conc. [mM]	ext. coeff. $\left[\frac{1}{\text{M}\cdot\text{cm}}\right]$	average $\left[\frac{1}{\text{M}\cdot\text{cm}}\right]$
$\text{B}^{-3}+\text{KOH}$	5.00	0.200	4400	4460 ± 40 ($\lambda = 314.5 \text{ nm}$)
		0.100	4450	
B^{-3}	1.06	0.264	4500	
		0.265	4470	
B^{-3*}	1.09	0.107	4460	
		0.298	4430	
Octanol	5.48	0.329	3000	3020 ± 60 ($\lambda = 301.5 \text{ nm}$)
		0.329	2940	
		0.439	3000	
		0.110	3080	
Octanol*	5.28	0.105	3120	
		0.423	3040	
Octanol*	17.2	0.345	2980	
		0.207	3030	

Table 1: Extinction coefficients for TeCP in buffer and in octanol; the star “*” marks presaturated phases.

on the average values of $\epsilon = 4460 \pm 40$ ($\lambda = 314.5 \text{ nm}$) for the buffer phase and $\epsilon = 3020 \pm 60$ ($\lambda = 301.5 \text{ nm}$) for the octanol phase.

7.2 Partitioning

The partitioning experiment was performed for 15 values of the initial pH of the buffer phase. Table 2 shows a summary of the obtained data. The distribution ratio Eq. 42 was calculated from the spectrophotometrically obtained aqueous concentrations of TeCP applying the mass-balance equation for the system.

Figure 22 shows the distribution ratio between octanol and buffer for the measured pH values. Solid curves illustrate the distribution ratio predicted from Eq.

initial pH	final pH	$[HA]_w + [A^-]_w$ [mM]	$[HA]_o + [A^-]_o$ [mM]	$\log D$
12.3	11.9	2.05	9.77	0.689
11.8	11.0	1.79	11.1	0.792
11.4	10.0	0.678	16.6	1.39
11.4	10.0	0.872	15.6	1.25
11.2	9.76	0.678	16.6	1.39
10.7	9.40	0.360	18.2	1.70
10.4	9.35	0.295	18.5	1.80
10.8	9.14	0.231	18.8	1.91
9.99	9.07	0.182	19.1	2.02
9.00	8.41	0.0710	19.7	2.44
7.77	7.81	0.0215	19.9	2.97
7.47	7.48	0.0107	20.0	3.27
6.94	7.10	0.0052	20.0	3.58
6.40	6.62	0.0025	20.0	3.90
3.23	3.42	0.0012	20.0	4.22

Table 2: Experimental data from octanol/water partitioning of TeCP.

Figure 22: pH -dependence of the distribution ratio of TeCP in octanol and water; gray lines are the standard deviation, the dotted line marks the pK_a

Figure 23: pH -dependence of the concentration of TeCP in octanol and water; gray lines are the standard deviation, the dotted line marks the pK_a

45 employing $\gamma_A = 7.4 \pm 1.8$ and $\gamma_{HA} = 33000 \pm 4000$. These values were a non-linear least square fit of the experimental data to Eq. 45. Gray lines show the standard deviation of the predicted distribution ratio. As expected for very high and very low values of the pH , the distribution ratio saturates and corresponds to that for the neutral and ionized TeCP, γ_{HA} and γ_A respectively. The value obtained for $\log \gamma_{HA} = 4.52 \pm 0.05$ is in good agreement with the literature values that are ranging from $\log P_{ow} = 4.21$ to 4.87, depending on the method used (Veith and Mekenyan, 1993; Bryant and Schultz, 1994; Schellenberg *et al.*, 1984).

The experiment was designed to produce a strong pH dependence of the concentration profiles (Figure 23). Volume ratio and initial TeCP concentration were chosen to get a maximum concentration variation in the aqueous phase utilizing the widest range of measurable absorbances. The high aqueous concentration of TeCP for $pH - pK_a > 2$ reflects upon the value of the octanol/water partition coefficient of the ionized species.

7.3 Conclusions

The knowledge of the partition coefficient for the negatively charged species of 2,3,4,5-tetrachlorophenolate, γ_A , makes it possible to compare it with the value for the linear partition coefficient for adsorption to phospholipid membrane, β_A^m (by means of Eq. 40). Assuming a membrane thickness of $t = 38 \text{ \AA}$ (McIntosh and Simon, 1986) one obtains a membrane bulk partition coefficient $\gamma_A^m = 3000$. This value is 400 times greater than that predicted by the octanol/water partition coefficient. Smejtek and Wang (1993) found analogous behavior for PCP using similar experimental methods. A summary of relevant properties and results is given in Table 3.

The major result of this study is that the octanol/water partition coefficient is not a suitable means for the prediction of distribution of the ionized form of

MW (u)	pK_a	C^s (mg/L)	$\log P_{ow}$
231.89	6.35 ± 0.02^a	67.4	4.87 ± 0.08^a
$\log \beta_A^m$	$\log \gamma_A^m$	$\log \gamma_A$	$\log \gamma_{HA}$
-5.246 ± 0.015^b	3.475 ± 0.015^b	0.87 ± 0.10	4.52 ± 0.05

Table 3: Selected properties of 2,3,4,5-tetrachlorophenol.

^aSchellenberg (1984)^bBlochel (1992)

2,3,4,5-tetrachlorophenol between water and lipid membranes and that P_{ow} is expected to seriously underestimate the concentration of ionized TeCP in biological cell membranes.

The rationale for the difference can be based on the physical properties of the two model systems. Octanol, as a bulk phase, requires maintenance of electroneutrality and therefore co-partitioning of counterions which requires additional free energy. In contrast TeCP ions in the lipid membrane are expected to be stabilized by a deep potential energy minimum close to the lipid/water interface (Flewelling and Hubbell, 1986). These ions are effectively screened by the counterions from the aqueous phase. This interaction is expected to lower the electrostatic energy of the membrane system compared to that in bulk octanol phase.

Appendix A

Derivations

A.1 Gouy–Chapman Theory

Consider an infinite uniformly charged surface in contact with an infinite homogeneous electrolyte. Ions in an electric field obey a Boltzmann distribution Eq. 1 which in return gives rise to a potential originating from the spatial density

$$\rho(x) = \sum_i z_i e N_i(x). \quad (48)$$

Combining this equation with Poisson's equation, Eq. 2, yields the Poisson–Boltzmann equation

$$\frac{d^2\psi(x)}{dx^2} = -\frac{1}{\epsilon_r \epsilon_0} \sum_i z_i e N_i(\infty) \exp\left(-\frac{z_i e \psi(x)}{kT}\right) \quad (49)$$

which can be integrated using the boundary conditions

$$\lim_{x \rightarrow \infty} \psi(x) = 0 \quad \text{and} \quad \lim_{x \rightarrow \infty} x \frac{d\psi(x)}{dx} = 0 \quad (50)$$

and the identity

$$2 \frac{d^2\psi(x)}{dx^2} = \frac{d}{dx} \left(\frac{d\psi(x)}{dx} \right)^2. \quad (51)$$

One obtains

$$\left(\frac{d\psi(x)}{dx} \right)^2 = \frac{2kT}{\epsilon_r \epsilon_0} \sum_i N_i(\infty) \left[\exp\left(-\frac{z_i e \psi(x)}{kT}\right) - 1 \right]. \quad (52)$$

Applying the electroneutrality condition¹ to the double layer gives

$$\sigma_m = - \int_0^\infty \rho(z) dz. \quad (53)$$

Substituting Eqs. 2 and 52 into Eq. 53 gives the Gouy-Chapman equation, Eq. 4.

$$\begin{aligned} \sigma_m &= \int_0^\infty \epsilon_r \epsilon_0 \frac{d^2 \psi(x)}{dx^2} dx = -\epsilon_r \epsilon_0 \left(\frac{d\psi(x)}{dx} \right)_{x=0} \\ &= 2\epsilon_r \epsilon_0 kT \sum_i N_i(\infty) \left[\exp \left(-\frac{z_i e \psi(0)}{kT} \right) - 1 \right]^{\frac{1}{2}}. \end{aligned} \quad (54)$$

A.2 Rod Model

The charge density of RuR in this model is found by extending the Boltzmann distribution Eq. 1 to three charges type A, C at the ends and type B, at the center of a rod each with distributions N_A , N_C and N_B . They account for the position of the respective charge and a spatial average over all possible positions of the other two charges which are restricted by the membrane. One obtains

$$N_A(r) = N_A(\infty) \frac{\int_0^{2\pi} d\phi \int_0^{\min[\pi, \cos^{-1}(-r/a)]} d\theta \sin \theta g(r + 0.5a \cos \theta, \theta)}{\int_0^{2\pi} d\phi \int_0^\pi d\theta \sin \theta}, \quad (55)$$

where

$$g(r, \theta) = \exp \left(-\frac{2e}{kT} [\psi(r) + \psi(r - 0.5a \cos \theta) + \psi(r + 0.5a \cos \theta)] \right) \quad (56)$$

is the probability of finding a molecule² at a distance r from the surface making an angle θ with the normal (see Figure 7). The maximum angle of the molecule without penetrating the membrane is given by $\cos^{-1}(-r/a)$. Substituting $z = a \cos \theta$ and Eq. 56 into Eq. 55 and performing the $d\phi$ integration one obtains

$$N_A(r) = N_A(\infty) e^{-\frac{2e\psi(r)}{kT}} \frac{1}{2a} \int_{\max[-a, -r]}^a dz e^{-\frac{2e}{kT} [\psi(r+z/2) + \psi(r+z)]}. \quad (57)$$

¹the surface charge density σ_m is balanced by the net charge in the aqueous diffuse double layer

²the position of the molecule is determined by the position of its center

Similar considerations for charges type B yield a density of

$$\begin{aligned}
 N_B(x) &= N_B(\infty) \frac{\int_0^{2\pi} d\phi \int_{\max[0, \cos^{-1}(2x/a)]}^{\min[\pi, \cos^{-1}(-2x/a)]} d\theta \sin \theta g(x, \theta)}{\int_0^{2\pi} d\phi \int_0^{\pi} d\theta \sin \theta} \\
 &= N_B(\infty) e^{-\frac{2e\psi(x)}{kT}} \frac{1}{2a} \int_{\max[-a, -2x]}^{\min[a, 2x]} dz e^{-\frac{2e}{kT}[\psi(x-z/2) + \psi(x+z/2)]}. \quad (58)
 \end{aligned}$$

These densities together with Eq. 48 and the Poisson equation, Eq. 2, yield³

$$\frac{d^2\psi(x)}{dx^2} = -\frac{\sum_i z_i e N_i}{\epsilon_r \epsilon_0}. \quad (59)$$

A first formal integration using the boundary conditions, Eq. 50, yields

$$\frac{d\psi(x)}{dx} = \left. \frac{d\psi(x)}{dx} \right|_{x=0} - \frac{1}{\epsilon_r \epsilon_0} \int_0^x dy \sum_i z_i e N_i(y). \quad (60)$$

Integrating by parts and considering the boundary conditions gives the identity

$$\psi(x) = \psi(0) + x \frac{d\psi(x)}{dx} - \int_0^x dy (y) \frac{d^2\psi(y)}{dy^2} \quad (61)$$

Substitution of Eqs. 59 and 60 into Eq. 61 yields

$$\psi(x) = \psi(0) + x \left. \frac{d\psi(x)}{dx} \right|_{x=0} - \frac{1}{\epsilon_r \epsilon_0} \int_0^x dy (x-y) \sum_i z_i e N_i(y) \quad (62)$$

which is identical to Eq. 11.

³ $z_A = z_B = z_C = 2$ as N_A , N_C and N_B represent the densities of the 2e-charges at the ends and the center of the rod, respectively

Appendix B

Numerical Methods

The algorithms for solving the equations were implemented in Visual Basic (VB) 3.0 professional edition for Windows®. This software development tool was used as it provides a fast way to write programs due to its extended basic language set with dynamic memory management and interactive debugging features. The latter is extremely helpful as one can perform code changes while the program is running. The price for this advantage is a comparably slow performance due to the fact that the program is interpreted¹ rather than compiled. It would be desirable to have both: an interpreter for program development and a compiler for the final executable file. Program development was done on a IBM compatible 486/33, the final calculations were performed on four IBM compatible Pentium®/90 systems. Computation times for the Rod Model (the most time intensive) ranged from 12 to 24 hours for the electrostatic part and between 2 and 3 days for the adsorption calculations.² An estimated 10000 integrations have to be performed accumulating to about one billion floating point operations.

¹interpreted "p-code"

²due to the fact, that for each iteration step in the Adsorption Model the complete Rod Model has to be solved

B.1 Integration

All numerical integrations were performed using a combination of Simpson's and the trapezoid rule (Davis and Rabinowitz, 1984). Simpson's rule in compound form assumes an even number of equally spaced subintervals $a = x_0 < x_1 < \dots < x_{2n-1} < x_{2n} = b$ with $x_{i+1} - x_i = h$. Using the notation $f_i = f(x_i)$, Simpson's rule can be written as

$$\int_{x_0}^{x_{2n}} f(x) dx = \frac{h}{3} [f_0 + 4(f_1 + f_3 + \dots + f_{2n-1}) + 2(f_2 + f_4 + \dots + f_{2n-2}) + f_{2n}] + E_n,$$

where E_n is the upper limit of the error

$$E_n = -\frac{(b-a)^5}{180N^4} f^{(4)}(\zeta), \quad a < \zeta < b \quad \text{and} \quad N = 2n.$$

As one can see from the definition of E_n , Simpson's rule is exact for polynomials of degree less than 4. The application of the method is restricted to $N \geq 2$ and to an even number of subintervals. For $N = 1$ the trapezoid rule

$$\int_{x_0}^{x_1} f(x) dx = \frac{h}{2} (f_0 + f_1)$$

is used. For an odd number of subintervals the integration is performed according to Simpson's rule without the last subinterval which is accounted for by an additional term calculated with the trapezoid rule.

Tests showed that a grid of at least 1000 intervals was required for the first four Debye lengths to keep the error well below 1%. For reasonable computation times an interpolation for another four Debye lengths was performed assuming exponential decay, which was found to produce correct results within our error limits.

B.2 Numerical Solution of the GC Model

The Gouy–Chapman equation, Eq. 4, together with the electroneutrality condition, Eq. 3, must be solved in order to obtain $\psi(x)$. This was done using the formal

solution of the Poisson equation, Eq. 11,

$$\psi(x) = \psi(0) + x \left. \frac{d\psi(x)}{dx} \right|_{x=0} - \frac{1}{\epsilon_r \epsilon_0} \int_0^x dy (x-y) \sum_i z_i e N_i(y) \quad (63)$$

which has been derived in Section A.2. For $N_i(y)$ the regular Boltzmann distribution, Eq. 1, was used. To perform the integration, $\psi(0)$ and $\left. \frac{d\psi(x)}{dx} \right|_{x=0}$ are needed. Eq. 4 is solved iteratively for $\psi(0)$ with a root-finding subroutine adopted from a Fortran-77 subroutine package using successive bisectioning and inverse parabolic interpolation (Digital Equipment Corporation, 1982). The magnitude of the surface electric field $\left. \frac{d\psi(x)}{dx} \right|_{x=0}$ can be obtained from the surface charge density σ_m of the membrane using the charge neutrality condition, Eq. 12. This procedure reduces the solution for the potential $\psi(x)$ to a simple integration of Eq. 63, as the integrand involves potentials with $y < x$ only.³

For the implementation on a computer, it was necessary to go a different way as the errors introduced by the finite grid size accumulate and cause a violation of the boundary conditions

$$\psi(x) < 0 \quad \text{for all } x \quad \text{and} \quad |\psi(x_i)| > |\psi(x_{i+1})| \quad \text{for } x_i > x_{i+1}. \quad (64)$$

for large x . To enforce these boundary conditions, an iterative method called “shooting” (Davis and Rabinowitz, 1984) was implemented and optimized for our problem. For the GC Model the simplest version of this algorithm shown in Figure 24 was used. As we know, in principle the surface potential to any accuracy, the surface electric field is being varied in order to get a complete solution. Starting from the initial values, calculated as described above, the integration was performed until Eq. 64 was violated. By changing the initial slope of the potential, a solution satisfying the boundary conditions could be found. Numerical tests of the algorithm showed that the deviation of total charge density in the diffuse double layer, σ_{DL} , obtained by integration and by iteration⁴ from the surface charge density was well below 1%

³note that for $y = x$ the integrand vanishes

⁴ σ_{DL} can be obtained from the shooting method (“slope”) using the right hand side of Eq. 12

Figure 24: Flowchart for the simple “shooting” algorithm used for solving the GC Model; σ_m : surface charge density on membrane, σ_{DL} : total charge density in diffuse double layer.

when a grid of 1000 intervals for the first four Debye lengths and interpolation to eight Debye lengths was used. These parameters were kept throughout all calculations except for the adsorption part with the Rod Model where 500 intervals were used instead.

B.3 Numerical Solution of the Rod Model

The Rod Model, Eqs. 9–11, are very hard to solve as they contain advanced and retarded potentials in x . We have two unknowns, the potential and the densities, which each must be determined by iteration. As we deal with integrals over exponentials, a slight change in the potential causes a big change in the densities and vice versa. This leads to an oscillatory behaviour of the solutions. To prevent this from occurring, mixing of iterates (see below) and maximum accuracy for the integration was necessary.

The model equations were solved by extending the procedure for the GC Model. This was necessary, as we have no information on either the surface potential or the surface electric field. As in the case described in Section B.2, the potential is being integrated in an inner loop until the boundary conditions, Eq. 64, are violated. By changing the slope ($\frac{d\psi(x)}{dx}\big|_{x=0}$) iteratively, a solution fulfilling the boundary conditions can be found.⁵ After having calculated $\psi(x)$ for a value of $\psi(0)$, the densities for the different charges on RuR, N_{AC} and N_B were evaluated on the basis of this potential. It was necessary to mix the iterates for the density and the potential according to Croxton and McQuarrie (1979) using a mixing parameter $\alpha = 0.2$ with

$$N^{i,new} = \alpha N^i + (1 - \alpha)N^{i-1} \quad \text{and} \quad \psi^{i,new} = \alpha \psi^i + (1 - \alpha)\psi^{i-1}, \quad (65)$$

where i denotes the number of the iterate. The difference between iterates $i - 1$ and

⁵a solution satisfying the boundary conditions does not exist for all $\psi(0)$; in which case the precision limit of the number representation is reached and the routine quits

Figure 25: Flowchart for the algorithm used for solving the Rod Model; σ_m : surface charge density on membrane, σ_{DL} : total charge density in diffuse double layer, δ : relative difference from iteration i to $i + 1$, error: a constant determining the maximum error.

i, new for the density was calculated using

$$\delta = \frac{1}{j_{max}} \left(\sum_j^{j_{max}} \left(N^{i-1}(j \cdot dx) - N^{i,new}(j \cdot dx) \right)^2 \right)^{\frac{1}{2}} \quad (66)$$

and compared to the maximum error⁶ allowed.

In an outer loop the offset ($\psi(0)$) is varied until the $\psi(x)$ satisfies the second boundary condition, Eq. 12. The loop for finding the surface potential consisted of the subroutine mentioned in Section B.2 which calculated the root of Eq. 12.

B.4 Numerical Solution of the Maximum Density Model

The potential for the Maximum Density Model was solved by using the same algorithm as for the Rod Model substituting the densities N_{AC} and N_B by N_{MD} . This density was obtained by applying to Eq. 13 the root-finding routine mentioned in Section B.2. As no advanced or retarded potentials are involved, the density could be calculated during the potential integration without any additional iteration. The algorithm is outlined in Figure 26.

B.5 Numerical Solution of the Adsorption Model

The solution for the Adsorption Model is obtained by iterating the surface potential $\psi(0)$ and the final bulk concentration of RuR, $[RuR]_{aq,0}$. This was done by using the root-finding routine mentioned in Section B.2 twice. For each value of these parameters the Adsorption Model is solved which gives (i) a new value for the surface charge density on the membrane, σ_m , the total charge in the double layer, σ_{DL} , and (ii) a new bulk concentration for RuR. For a correct solution in order to satisfy all equations, σ_m must balance σ_{DL} and the assumed final bulk concentration

⁶ $\delta < 10^{-3}$ was found to be sufficient

Figure 26: Flowchart for the algorithm used for solving the Maximum Density Model; σ_m : surface charge density on membrane, σ_{DL} : total charge density in diffuse double layer, error: a constant determining the maximum error.

Figure 27: Flowchart for the solution of the Adsorption Model; σ_m : surface charge density on membrane, σ_{DL} : total charge density in diffuse double layer, $[RuR]_{aq,0}$: assumed final bulk RuR concentration, $[RuR]_{aq}$: RuR bulk concentration obtained from Model, $[RuR]_{if}$: interfacial RuR concentration, error: a constant determining the maximum error.

$[RuR]_{aq,0}$ must match the calculated concentration $[RuR]_{aq}$ within the error limits. Solving the Adsorption Model involves the interfacial concentration of RuR which is calculated according to our models.⁷ Figure 27 outlines the numerical procedure.

⁷this involves for the Rod and the Maximum Density Model a complete evaluation of $\psi(x)$ as we need the total charge density in diffuse double layer

Appendix C

Spectrophotometer Control Software

The main goal for the development of the control software was to achieve an easy management of the spectra and to establish a basis for further data processing of the spectra. This goal was reached by converting the raw data from the spectrophotometer into an ASCII file in which each absorption point corresponds to a wavelength. The file included creation date and time as well as up to 50 comment lines describing the experimental conditions of the obtained data. To automate the data acquisition procedure, data used for a batch of similar measurements can be stored in a “profile”. Table 4 lists all parameters stored in a profile and a scan file. The scan additionally includes a [Data] section in which the scan data are listed using the tab character¹ as delimiter. All files are regular ASCII text files for readability reasons and to facilitate the import of data into other programs for further data processing or display.

The program was written in Visual Basic 3.0 for Windows®. It provides a fast and easy way to develop user friendly software using the Windows graphics interface. A button bar gives fast access to the most frequently used functions. The scan data

¹ASCII (9)

Mode	String * 1	"S": Scan, "T": Timedrive			
swLen	Integer	wavelength: 190...900 nm (integer) ×10 or Timedrive: 0...1200 s (float) ×10			
ewLen	Integer				
glMax	Integer	wavelength 190...900 nm (integer) ×10 to be displayed in graph			
glMin	Integer				
smm	Integer	Gelscan start in mm			
emm	Integer	Gelscan end in mm			
ULim	Long	Absorption: −4.5...4.5 (float) ×1000 Transmission: 0...200.0 (float) ×1000			
LLim	Long				
Func	String*1	"A": Absorption, "T": Transmission, "G": Gelscan, "K": Kinetics			
Speed	String * 1	value	Abs./Trans.	Gelscan	Kinetics
		0	600 nm/min	1 rd/mm	10 sec/rd
		1	300 nm/min	2 rd/mm	15 sec/rd
		2	120 nm/min	5 rd/mm	20 sec/rd
		3	60 nm/min	10 rd/mm	30 sec/rd
		4	1200 nm/min	20 rd/mm	1 min/rd
		5	n/a	60 rd/mm	5 min/rd
Calc	String * 1	value	Abs.		Trans.
		1	Abs		%T
		2	peak pick		peak pick
		3	1st deriv		n/a
		4	2nd deriv		n/a
		5	Log Abs		n/a
Nr	Integer	Scan number 0...99			
Cell	Integer	Cell number 0...7			
TotTime	Integer	Timedrive total time: 0...1200 (integer)			
SipTime	Integer	Sippump-time ×10			
wLenRead	Integer	wavelength for reading: 190...900 nm (integer) ×10			
Reading	Single	current reading			
dl	Single	Δλ of data, depending on speed			
Date	Double	Date and Time of Scan			
FileName	String	Profile/Scan-Name			
Comments	String	description of experimental parameters			
MaxData	Integer	number of data points; 0 indicates profile			
Data	String	Scan data (stored only in a scan file)			

Table 4: List of parameters stored in a profile and a scan file.

Figure 28: The user interface of the spectrophotometer control software.

is graphically displayed in an extra window which can be interactively positioned and resized. Figure 28 shows the user interface of the program.

The software implements all commands that can be performed on the spectrophotometer via remote control (Beckman, 1984) including the transmission of scan data from and to the spectrophotometer.

Communication between software and spectrophotometer is established by standard serial connection (RS-232).² As the interface for the spectrophotometer does not support hardware handshaking, it was necessary to slow down the transmission rate by sending character by character to the spectrophotometer rather than a whole

²using 19200 baud, no parity, 8 data bits and 1 stop bit

command string at once. Characters were received from the spectrophotometer using the polling method³.

³the input port was checked every 100 ms for incoming data during active communication and every 500 ms during idle

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