

**VIOLOGEN-MEDIATED ELECTRON TRANSFER ACROSS
DIHEXADECYLPHOSPHATE BILAYER MEMBRANES**

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DEDICATION

This dissertation is dedicated to all of those who have influenced and helped me throughout my academic career. I especially wish to thank my mother, Beverly Pasqual, and my step-father, Frank Pasqual, for emotional and financial support. And I would like to dedicate this work to my fiancée, Denise, whom I love dearly. Also my sister and brother-in-law, Tara and Todd, as well as my other friends from the great city of Issaquah, Washington for providing unerring friendship whenever needed. I also wish to express my gratitude to Eberhardt, Hoa, Larry, and Gubby for their love and free meals during my stay here at OGI. This is also dedicated to my beautiful daughter, Heather. I also owe a lot to my deceased father, James Patterson, who instilled in me the strong desire to accomplish things and the confidence to know that I can do it.

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LIST OF ABBREVIATIONS AND SYMBOLS

CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
C_4MV^{2+}	1-methyl-1'-(C_4 alkyl) - 4,4'-bipyridinium
DHP	Dihexadecylphosphate
DNP	2,4-dinitrophenol
DODAC	Diocetadecyldimethylammonium chloride
DPPC	Dipalmitoylphosphatidylcholine
EDTA	Ethylenediaminetetraacetic acid
MMB ⁺	1-methyl-4,4'-bipyridinium
MSPB ⁺	1-methyl-1'-sulfonato-n-propyl-4,4'-bipyridinium
MV^{2+}	1,1'-dimethyl-4,4'-bipyridinium
PC	Phosphatidylcholine
PMB ⁴⁺	1-1"-propanediylbis(1'-methyl-4,4'-bipyridinium)
R_b	Viologen binding ratio (See Eq. 4-1)
SDS	Sodium dodecylsulfate
TPP ⁺	Tetraphenylphosphonium ion
Tris	Tris(hydroxymethyl)aminomethane
V_0	Photomultiplier voltage at 0% transmittance
V_i	Photomultiplier voltage at time t
V_{inf}	Photomultiplier voltage at reaction end
V_L	Photomultiplier voltage at 100% transmittance
V_0	Photomultiplier voltage at t=0
V_{off}	Offset voltage
ZnTPPS ⁴⁻	[5,10,15,20-tetrakis-(4-sulfonatophenyl)porphinato] zinc(II) ion
ZnTPyP ⁴⁺	[5,10,15,20-tetrakis-(N-methylpyridyl)porphinato] zinc(II) ion
α	See Eq. 4-11
$\Delta\psi$	The transmembrane potential

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ABSTRACT

Viologen-Mediated Electron Transfer Across Dihexadecylphosphate Bilayer Membranes

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Oregon Graduate Institute, 1990

Supervising Professor: James K. Hurst

The ability of alkyl viologens (C_nMV^{2+}) to mediate electron transfer across dihexadecylphosphate (DHP) bilayers was studied. DHP vesicles were prepared in weakly alkaline buffers and reduction of viologen molecules adsorbed on the inner and/or outer vesicle surface(s) was monitored after the anaerobic addition of dithionite ($S_2O_4^{2-}$) ion. In the absence of external viologen the reduction of inner viologen was extremely slow, with half-lives on the order of hours. In the presence of external C_nMV^{2+} the reduction of inner viologen proceeded over the course of minutes with the general trend that second-order rates decreased as n increased. For methyl viologen ($MV^{2+}, n=1$) the reduced product was spectroscopically observed to be characteristic of dimers in cases where the initial concentration of viologen inside the vesicles exceeded that outside. It was also determined that under these conditions most of the reduced viologen had migrated into the vesicles. These phenomena were observed to lesser extents as the viologen alkyl chain length increased.

It is hypothesized that electron transfer, as well as viologen migration, is controlled in part by transmembrane electrical potentials. Transmembrane oxidation-reduction leads to polarization of the membrane. The migration of viologen cations inward compensates for this charge buildup. The addition of other lipophilic ions was studied to help clarify this notion. Various other transmembrane redox systems were briefly studied.

The charge recombination rates of a photochemical system containing ZnTPPS^{4-} and alkyl viologens adsorbed to DHP vesicles were studied using laser flash photolysis. Conditions were varied to determine which factors affected the rates. The recombination reaction was found to be first-order and possible mechanisms are proposed.

CHAPTER 1

INTRODUCTION

The bilayer membrane which constitutes small unilamellar vesicles is capable of drastically altering the reaction kinetics between molecules which are adsorbed at its aqueous interface or solubilized within its hydrophobic core and molecules dissolved in bulk solution⁽¹⁾. One property which may influence the kinetics is the electrostatic charge at the interface which is dictated by the headgroup charge of the surfactant which makes up the bilayer. For example, dihexadecylphosphate molecules will produce vesicles with a negative surface charge. Similarly, dioctadecyldimethylammonium chloride and phosphatidylcholine form vesicles with overall positive and neutral surface charge respectively. The ability to choose the electrostatic charge at the interface allows for selective adsorption or repulsion of other charged molecules. The hydrophobic core of the bilayer membrane also plays a role in determining the adsorption of molecules to the vesicle. Charged molecules with large hydrophobic regions will interact strongly with the hydrophobic interior of the bilayer which will increase their probability of binding. Molecules which are highly hydrophobic will become "dissolved" in the bilayer.

The hydrophobic core of the bilayer also plays another key role in determining the physical properties of the membrane. It provides a very unstable environment for small charged ions and therefore these ions are

relatively impermeable across the barrier. In biology this fact is used to great advantage as an energy storage system in cells where large ion gradients can be maintained across cell membranes. The discovery of this phenomenon has led to much study in the field of bioenergetics⁽²⁾. In synthetic vesicle systems this means that transmembrane potentials which might develop are not easily dissipated by movement of simple ions in solution. Therefore when equilibrium constants for reactions across the membrane are calculated for charged species the transmembrane potential must be taken into account as part of the overall energetics. For example, we can look at simple diffusion of a charged ion across a membrane starting at a nonequilibrium position. If no other forces were present, as would be the case for an uncharged molecule that doesn't interact with the membrane, ion flow across the membrane would continue until the free energy change became zero according to equation 1-1.

$$\Delta G_c = 2.303RT \log([c_1]/[c_2]) \quad (1-1)$$

This implies equal concentrations on either side of the membrane at equilibrium. But if there is no other ion flow in the system, a transmembrane electrical potential will begin to develop which opposes diffusion of the ion along its concentration gradient. The free energy of the electrical potential is given in equation 1-2.

$$\Delta G_e = mF\Delta\psi \quad (1-2)$$

where m is the charge of the ion, F is the Faraday constant, and $\Delta\psi$ is the transmembrane potential, usually expressed in volts. So in this case net ion flow across the membrane will cease when $\Delta G = \Delta G_c + \Delta G_e =$

0; i.e. when the electrical gradient exactly opposes the concentration gradient.

Another important property when discussing ion diffusion is the inherent diffusion rate of the molecule in the absence of any external forces. The diffusion rates of small ions across bilayer membranes have been studied extensively⁽³⁾. These rates depend on properties of the ion such as size, charge, and degree of solvation. They also depend on properties of the membrane such as surface charge, dielectric constant of the hydrophobic region, and fluidity. These properties are determined by the surfactant which makes up the bilayer and the temperature. Bilayer membranes are known to undergo a major phase transition upon cooling which takes the membrane from a relatively fluid phase where the alkyl "tails" of the surfactant are considered to be highly disordered, to a more rigid phase where the alkyl chains are more ordered and lateral diffusion of the surfactant molecules is significantly slowed. This phase transition, known as the gel-to-liquid crystalline transition, is of sufficient energy to be easily observed using differential scanning calorimetry on vesicle suspensions. The lipid composition and method of vesicle preparation can have profound effects on the transition temperature⁽⁴⁾.

Vesicle bilayer membranes provide a barrier for microcompartmentalization of reactants across which chemical reactions may occur. Similar to the way in which cells store energy, chemical potential energy can be stored across the vesicle bilayer if a chemical reaction leads to accumulation of a given chemical species on one side of the membrane. This leads to a chemical, and possibly an electrical,

gradient if net charge transfer has taken place as part of the reaction. These gradients represent stored energy. For this stored energy to be useful from a practical point of view there must be some way for this energy to be utilized. One solution to this problem would be to couple the products formed on either side of the membrane to secondary reactions whose products are useful in devices or other reactions.

For example, one could envision a reaction scheme where solar energy is converted into useable chemical energy. If the transmembrane reaction were net electron transfer from a photosensitizer on one side of the membrane to an appropriate water-reducing catalyst on the other, then, with the aid of a water-oxidizing catalyst on the photosensitizer side, one might observe production of O_2 on one side of the membrane and H_2 on the other side. Since these can be recombined in a combustion process they represent useable chemical energy.

It is for reasons such as these that transmembrane electron transfer has been a much-studied subject. Both chemical⁽⁵⁾ and photochemical⁽⁶⁾ reactions have been studied. Several controversial questions persistently arise during these types of studies. One is whether or not transmembrane electron transfer has actually occurred. Sometimes it is found that loss of compartmentalization has occurred and the redox reaction has taken place on the same side of the membrane instead of across it⁽⁷⁾. Another controversial question is whether transmembrane electron transfer has occurred via quantum mechanical tunneling or transmembrane diffusion of the reductant. A third possibility is that some molecule is present in the membrane which is able to mediate electron transfer between reactants. In most cases

these questions have not yet been unequivocally answered.

A good example is a study done by Dannhauser et al⁽⁸⁾. Here manganese porphyrins were linked to large poly(ethylenimine) anchors and the length of the linkage varied. It was calculated that when the porphyrins were able to come within 4 angstroms of each other from opposite sides of the membrane, transmembrane electron transfer occurred. This implies electron tunneling through a hydrophobic media over a distance of 4 angstroms. However, since this is such a small distance it is difficult to rule out variations in membrane thickness and transient intercalation of the anchoring group which would allow electron transfer by direct contact of the porphyrins.

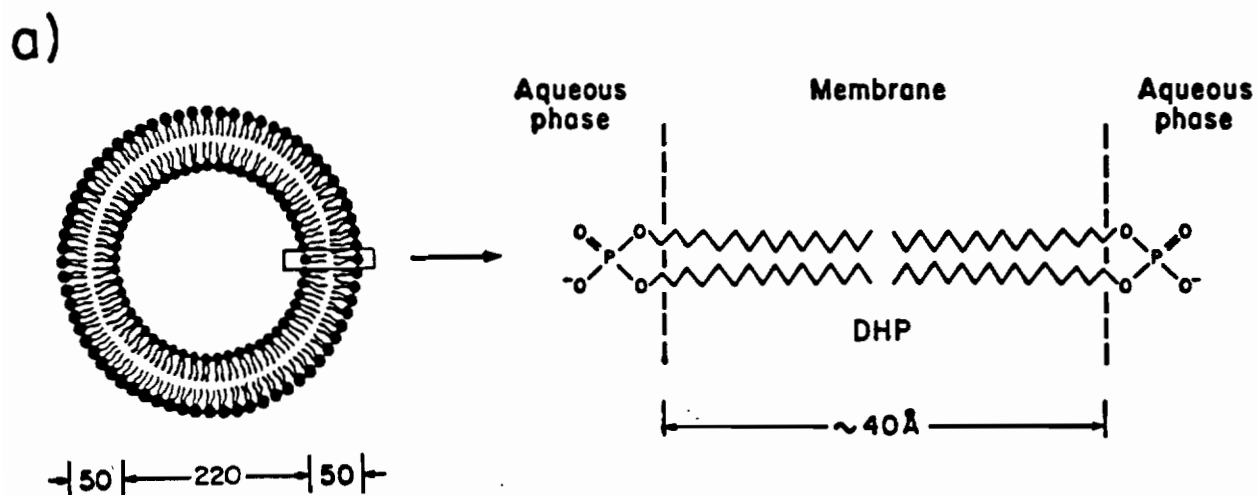
The most obvious way to distinguish between diffusional and tunneling mechanisms would be to systematically vary the membrane thickness since tunneling has an exponential distance dependence while diffusion would have a linear distance dependence. But in practice it is difficult to change the membrane thickness without changing its physical properties which would affect diffusional rates. Though not conclusive, observation of redistribution of reactants tends to support diffusional mechanisms, since diffusion has taken place, while lack of temperature dependence would support tunneling mechanisms. It is easy to see that this is a very complicated question and its answer may require more sophisticated techniques.

Another point to consider when studying transmembrane electron transfer is that it is electrogenic. That is, electron transfer leads to membrane polarization. Therefore ion flow must accompany directional electron transfer if it is to continue unimpeded. For electron

transfer, the ion flow must consist of either cation flow in the same direction or anion flow in the opposite direction. When studying electron transfer rates one must be careful that the electron exchange is rate-limiting and not the attending ion flow. In order to ensure that this is the case, ionophores can be added to the system.

Ionophores are hydrophobic ions which are capable of traversing the bilayer membrane⁽⁹⁾. Well-known examples include valinomycin, which transports potassium ion across the membrane, and CCCP which translocates a proton. Others researchers have tried polarizing the membrane beforehand such that electron transfer is promoted⁽¹⁰⁾.

In the studies reported in this dissertation the bilayer membrane used almost exclusively is composed of dihexadecylphosphate (DHP) molecules. Under the influence of ultrasound these molecules aggregate to form unilamellar vesicles containing approximately 10,000 monomer units and having an outer hydrodynamic radius of 140-160 angstroms (Fig. 1-1a). The vesicle bilayer thickness is on the order of 40-50 angstroms⁽⁵⁾. The transmembrane electron transfer event observed is from dithionite ion ($S_2O_4^{2-}$) in bulk solution to an alkyl viologen localized on the inner vesicle surface. Alkyl viologens, C_nMV^{2+} , are redox active dyes (Fig. 1-1b) which form clear aqueous solutions in the oxidized state and blue-violet solutions in the reduced state. Dithionite ion and reduced viologens react rapidly with molecular oxygen and thus must be kept anaerobic. Under favorable conditions, such as high concentration, viologen radical tends to form dimers and/or multimers⁽¹¹⁾. This phenomenon is easily observed spectroscopically in the visible region.



b)

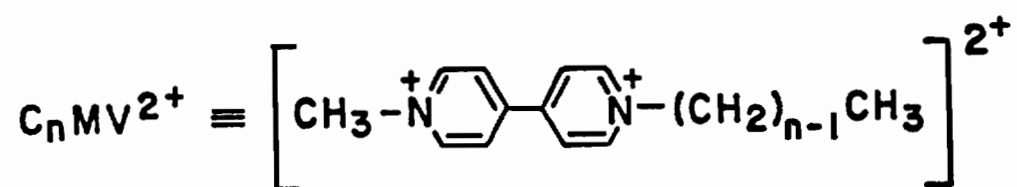


Figure 1-1: Molecules used in transmembrane redox studies. a) Dihexadecylphosphate molecule and its incorporation into small unilamellar vesicles. b) Structure of alkyl viologens. From reference 5.

In this work $C_p MV^{2+}$ is distributed on both sides of the vesicle bilayer and dithionite ion is rapidly added using an anaerobic stopped-flow apparatus. Formation of reduced viologen is then monitored spectroscopically at the monomer-dimer isosbestic point. There are two kinetically distinct steps which can then be isolated. One is reduction of viologen on the outer vesicle surface and the other is reduction of viologen on the inner surface. This second step involves some type of transmembrane oxidation-reduction.

The reduction of viologen distributed exclusively on the outer vesicle surface has been well-studied in this lab^(1a). It was found to be a pseudo-first-order reaction when the reductant was in large excess. For viologen alkyl chain lengths with $n \geq 12$ the reduction became biphasic which was explained by postulating two distinct binding geometries for surface-bound viologens. The kinetic results from these studies are shown in Figure 1-2. The major portion of this dissertation is devoted to the study of the transmembrane step; both its kinetics and how it is controlled by the transmembrane potential which develops. The observed behavior indicates that external viologen molecules were able to mediate transmembrane oxidation-reduction across vesicle bilayers and the reduced viologen functioned as the lipophilic ion needed to neutralize the transmembrane potential. The exact mechanism of the electron transfer process is still unclear.

The remaining portion of the dissertation will be devoted to continued studies of a photochemical charge separation-recombination reaction sequence⁽¹²⁾. Many researchers have used the interfacial properties of micelles to enhance charge separation yields in

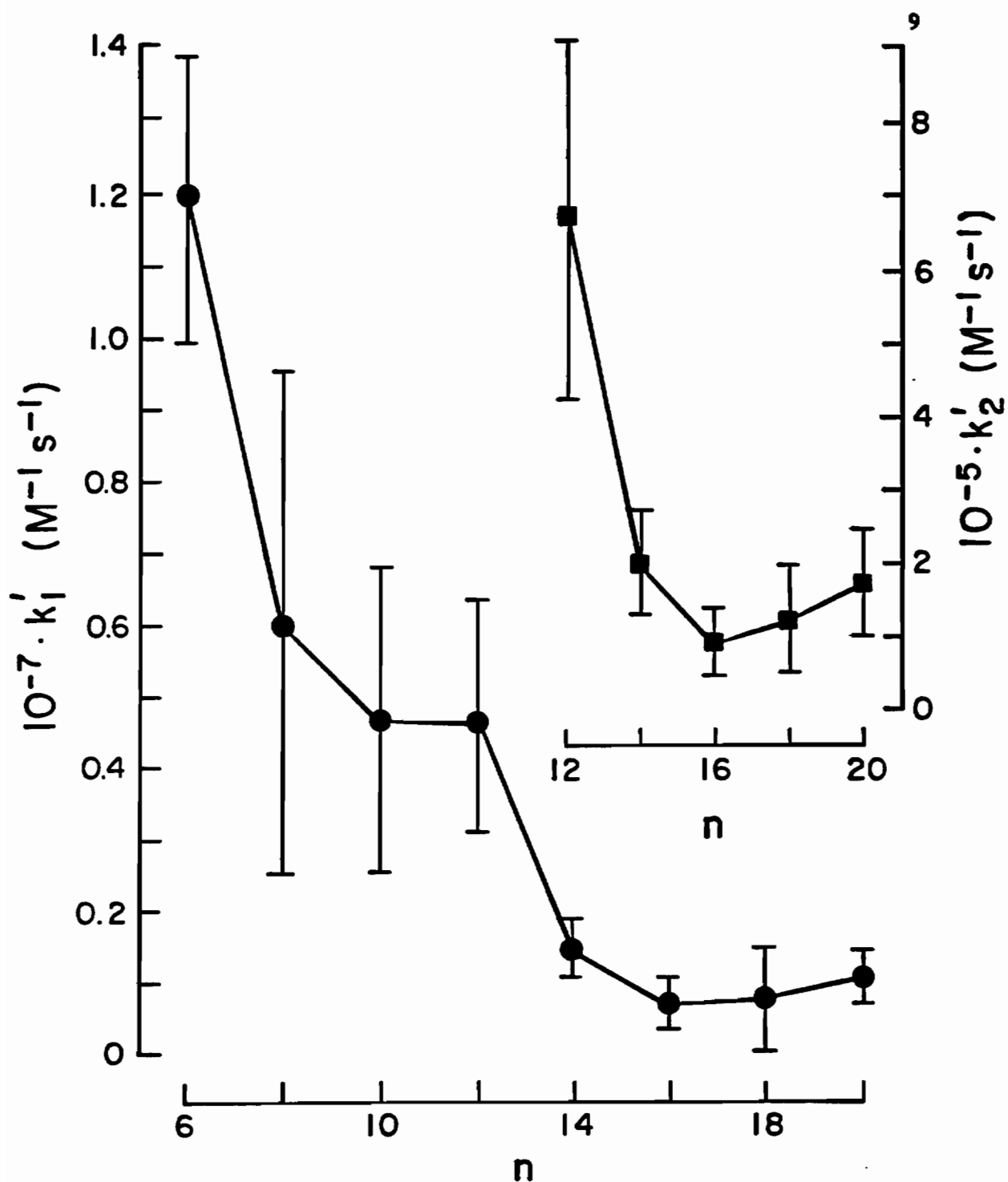


Figure 1-2: Rate constants for dithionite reduction of alkyl viologens adsorbed to DHP vesicles in 20 mM Tris-HCl, pH 8.0. Reaction is biphasic for alkyl chain lengths greater than ten carbons. From reference 1a.

photochemical systems⁽¹³⁾. In our studies we take advantage of the negative surface charge of DHP vesicles to repel the oxidized sensitizer anion, ZnTPPS^{3-} π -cation, from reduced viologen bound to the vesicle. This increases the lifetime of the charge-separated species. Using laser flash photolysis techniques we have studied the recombination rate dependence on factors such as viologen alkyl chain length, viologen loading, sensitizer concentration, and ionic strength.

CHAPTER 2

EXPERIMENTAL

Materials: Dihexadecylphosphate, methyl viologen, and imidazole were obtained from Aldrich. Tris (Tris(hydroxymethyl)aminomethane) was obtained from Sigma. These chemicals were used as received and were reported to be >99% pure. Critical experiments were checked using dihexadecylphosphate which had been twice recrystallized from methanol. Sodium dithionite came from various suppliers and was >95% pure. All alkyl viologens except methyl viologen were synthesized in this lab according to procedures previously described⁽¹⁴⁾ and tested pure according to NMR and TLC on silica plates using a 3:1:1 methanol:H₂O:50% aqueous ethylammonium chloride mobile phase. All alkyl viologens were used as the chloride salt unless otherwise specified. Sephadex G-50 Fine gel was obtained from Sigma and Chelex 100 ion exchange resin was obtained from Bio-Rad. All other materials were used as received. Water was purified with a Corning Megapure deionization distillation system.

Vesicle Preparation: Most vesicle suspensions prepared for this work were composed of the synthetic lipid, dihexadecylphosphate. The procedure for other surfactants was identical except for egg phosphatidylcholine which had to be sonicated in an ice bath to prevent degradation. The basic procedure is described below:

The appropriate mass of DHP (M.W. 546.86) was weighed into a glass beaker. Usually 4 or 8 mM DHP solutions were prepared in buffered solutions. In these experiments the buffers were usually either Tris-HCl, pH 8.0 or imidazole-HCl, pH 7.5. The vesicles were then formed by ultrasonic dispersal with the use of a 0.5 inch flat tip on a conventional horn energized by a Heat Systems-Ultrasonics W185 sonicator operated at a power setting of 4.0. Usually two 10 minute pulses separated by 5 minutes were sufficient to obtain clear solutions. If some turbidity remained, another 10 minute pulse was used. The resulting opalescent solution was then filtered through 0.8 and 0.22 micron pore size cellulose nitrate membrane filters. The solution was then centrifuged at 20 °C in a TY65 rotor at 36,000 RPM (approx. 100,000 g) on a Beckman L5-65 centrifuge to remove multilamellar vesicles, undispersed lipid, and titanium particles from the sonication horn. After centrifuging for 90 minutes the supernatant from the top 80% of the tube was carefully decanted and the resulting vesicles used within 6 hours.

If it was desired to have an equilibrium distribution of viologen on both the inner and outer vesicle surfaces then the appropriate alkyl viologen could be added before sonication from a 10-100 mM stock solution. The bulk viologen concentration in the vesicle suspension could then be determined after the centrifugation step using optical absorption spectroscopy. For this measurement unloaded vesicles were used as the reference solution and the viologen concentration was calculated using a MV^{2+} molar absorptivity of $2.19 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ at

approximately 260 nm¹.

If it was desired to have viologen localized only on the outer vesicle surface then the vesicle solution was mixed 50:50 with a viologen solution made up in the identical buffer system. This was accomplished with the use of a tangential-flow mixer which was fed by two syringes containing equal volumes of solution which were expelled at equal rates through the mixer. The mixing process occurred right before the centrifugation step. Again, the exact viologen concentration could be determined spectrophotometrically.

Column Chromatography: When a nonequilibrium or asymmetric distribution of reactants was desired, chromatography of the vesicle suspension was required. Two types of chromatography were employed; gel exclusion and cation exchange. Gel exclusion chromatography could be used for reactants which were not tightly bound to the outer surface such as MV²⁺ or Fe(CN)₆³⁻. Cation exchange chromatography was used to remove viologen which was tightly bound to the surface. These included monoalkyl viologens with chain lengths up to 10 carbons. Viologens with n≥12 could not be removed even with cation exchange chromatography.

The gel exclusion chromatography was performed using Sephadex G-50 Fine packing material. The gel was hydrated for 3 hours in the buffer to be used and then poured into a 2.6 x 45 cm column. The void volume

* Molar absorptivities of alkyl viologens bound to DHP vesicles have been determined in this lab (See ref. 1a). The peak maximum in the UV undergoes a bathochromic shift compared to aqueous solutions of 3-7 nm upon binding. The greater shifts are associated with the longer alkyl chains and thus tighter binding. The molar absorptivity used represents an approximate average (+/- 0.28 x 10⁴ M⁻¹cm⁻¹) of the values for all alkyl chain lengths and was universally used.

was then calculated by running through a concentrated solution of Blue Dextran. The vesicles were prepared in the presence of the reactant which was desired to be entrapped inside. For example, if $\text{Fe}(\text{CN})_6^{3-}$ was to be entrapped the vesicles would be cosonicated in the presence of 20 mM $\text{K}_3\text{Fe}(\text{CN})_6$. Since $\text{Fe}(\text{CN})_6^{3-}$ does not bind to the vesicle surface this high concentration is necessary to achieve any observable entrapment. If MV^{2+} , which does bind to the membrane, was being entrapped then only about 200–500 μM would need to be present during sonication. The vesicle solution was then loaded onto the column and eluted with the same buffer in which the vesicles were prepared. After the void volume had eluted, the effluent was collected up to the same volume that was being chromatographed. The amount of entrapped reactant could then be checked spectrophotometrically. The completeness of removal from the outer surface could be checked for $\text{Fe}(\text{CN})_6^{3-}$ and MV^{2+} by adding $\text{S}_2\text{O}_4^{2-}$ under anaerobic conditions and analyzing for any immediate decrease in the peaks at 420 nm and 260 nm respectively. Phosphate analysis showed that the vesicle concentration was diluted approximately two-fold by the chromatographic removal of external ions.

The cation exchange chromatography was performed using Chelex 100 chelating ion exchange resin. The resin itself consists of diacetic acid amine groups attached to a styrene divinylbenzene copolymer matrix⁽¹⁵⁾. The bidentate nature of the chelating group is ideal for ion-pair formation with the dipositive viologen cation headgroup. Even so, only viologens with single alkyl chain lengths up to 10 carbons could be removed from DHP vesicles using this method. To entrap viologen, the vesicles were prepared in the presence of approximately 4

times the concentration of viologen desired in the chromatographed suspension. The resin was regenerated before each use by the following procedure:

First, 2 bed volumes of 1N HCl was run through, then 5 bed volumes of water, then 2 bed volumes of 1N NaOH, then 5 bed volumes of water. Then one bed volume of 0.5M buffer made up of the buffer ion to be used during chromatography which is adjusted to a pH equal to its pK_a was run through. Finally, 5 bed volumes of the buffer to be used was eluted.

The resin was packed into a 1.5 x 20 cm column with a glass frit at the bottom. The void volume was determined using a concentrated solution of Blue Dextran. After the buffer level in the column was brought down to the top of the resin bed, the vesicle suspension was slowly added while eluting at a rate of one drop per second. After the void volume was eluted a volume of effluent equal to the sample volume was collected. Residual viologen on the outer surface was checked for by adding $S_2O_4^{2-}$ under anaerobic conditions and checking for a decrease in absorbance at 260 nm or an increase in absorbance at 604 nm. Phosphate analysis showed that the vesicle concentration decreased by approximately 20% after chromatography.

Viologen Synthesis: The syntheses for two different viologen derivatives will be described. These are 1,1"-propanediylbis(1'-methyl-4,4'-bipyridinium) (PMB^{4+}) and 1-methyl-1'-sulfonato-n-propyl-4,4'-bipyridinium ($MSPB^+$). These are shown as the final products in Figures 2-1 and 2-2 respectively. PMB^{4+} was prepared as the chloride salt while $MSPB^+$ was prepared as the iodide salt.

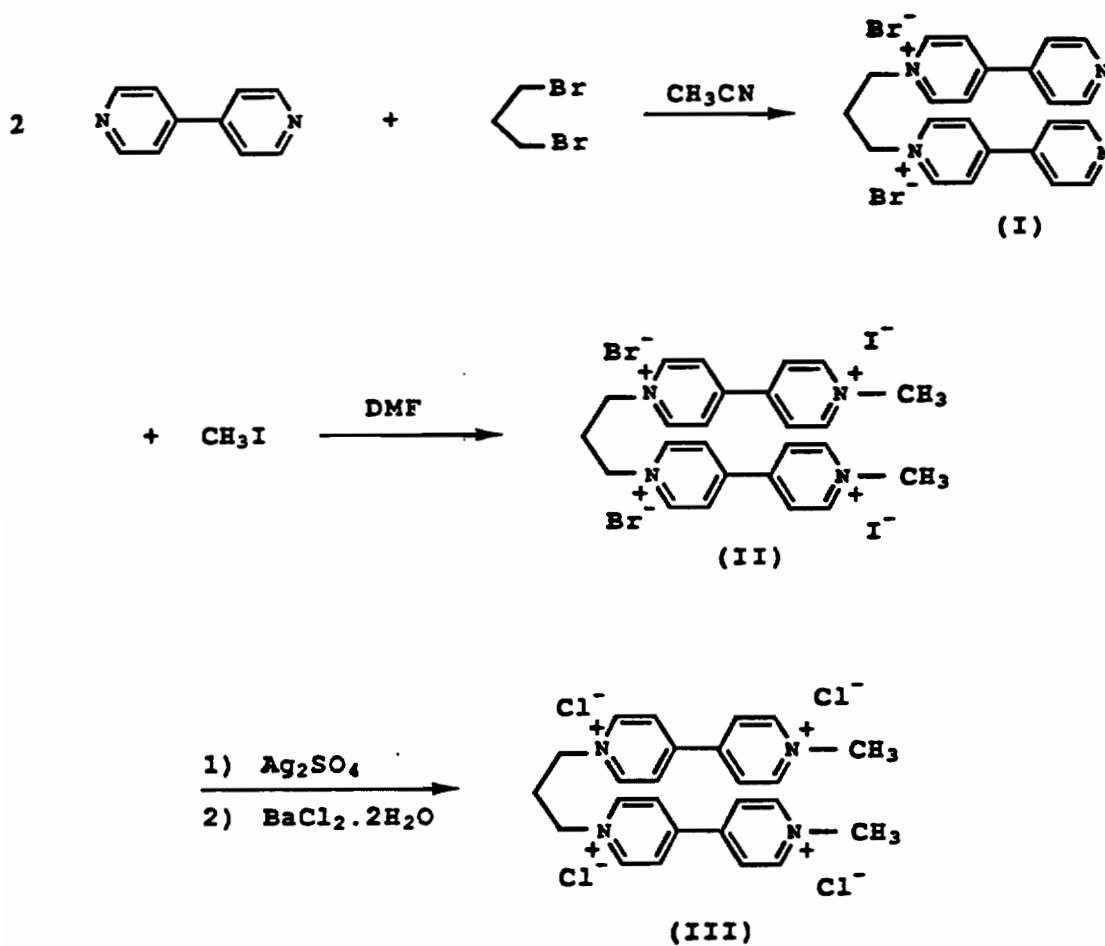


Figure 2-1: Schematic procedure for synthesis of 1-1'-propanediylbis (1'-methyl-4,4'-bipyridinium). See text for details.

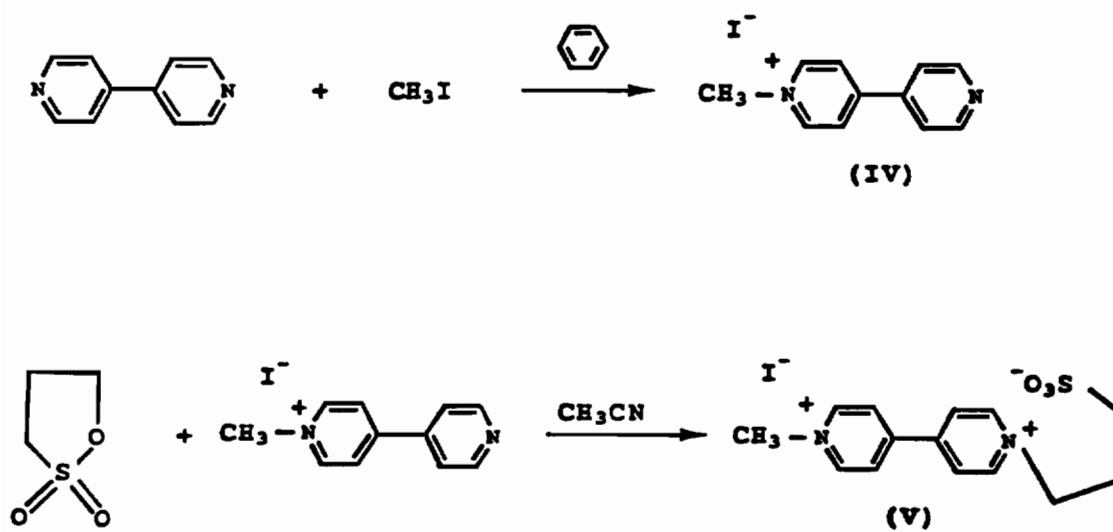


Figure 2-2: Schematic procedure for synthesis of 1-methyl-1'-sulfonato-n-propyl-4,4'-bipyridinium. See text for details.

The synthesis of PMB^{4+} is shown schematically in Figure 2-1.

Initially a 3-fold excess of bipyridine (4.52g) was added to freshly distilled 1,3-dibromopropane (1.58g). This was done by adding 50 ml of acetonitrile to the bipyridine in a roundbottom flask. The 1,3-dibromopropane was also diluted with 50 ml of acetonitrile and was slowly added over a 12 hour period with heating. The reaction mixture was then refluxed for an additional 6 hours, then cooled and filtered. The solid was dissolved in a hot propanol/ H_2O mixture resulting in a clear yellow solution with a dark brown precipitate. Hot filtration removed the precipitate and cooling led to the formation of light yellow-brown needles. The solid was then recrystallized from an ethanol/acetone mixture to produce the yellow intermediate (I). Next, 1.20g of I was placed in a roundbottom flask with 35 ml of DMF. Then 0.25 ml methyl iodide was added changing the yellow solution to orange-brown. This mixture was refluxed overnight. A second aliquot of 0.5 ml methyl iodide was added and the mixture was allowed to reflux for 22 hours. This resulted in 1.69g of crude product (II). Then 1.34g of Ag_2SO_4 was added to an aqueous solution of the product to remove the Br^- and I^- . After filtering, 1.03g of BaCl_2 was added to remove SO_4^{2-} and exchange for Cl^- . The crude chloride salt was then recrystallized from ethanol to obtain 0.25g pure material (III). This showed one spot on a silica TLC plate using a 3:3:1 methanol: H_2O :50% ethylammonium chloride solvent system. NMR spectroscopy indicated that the desired product had been synthesized. The proton shifts relative to TMS and their assignments are as follows: δ 2.95 ppm (t, protons α to N on propyl bridge); δ 3.60 ppm (m, protons β to N on propyl bridge); δ 4.54 ppm (s,

-CH₃); δ 8.68 ppm (m, aromatic protons meta to N); δ 9.20 ppm (t, aromatic protons ortho to N-CH₃); δ 9.43 ppm (t, aromatic protons ortho to N-propyl).

The reaction scheme for MSPB[†] is shown in Figure 2-2. First the 1-methyl-4,4'-bipyridine (MMB[†]) iodide salt was synthesized^(14a). To start, 12.5g of 4,4'-bipyridine was weighed into a 250 ml roundbottom flask. Then 100 ml benzene was added with stirring until the bipyridine dissolved. The methyl iodide was then added to 25 ml benzene in an addition funnel and thoroughly mixed. This solution was then added dropwise to the bipyridine solution over 1.5 hours. The mixture was stirred at room temperature for 24 hours. Another 100 ml benzene was added and the resulting slurry was stirred for another 24 hours. The solid was filtered and placed in a Soxhlet extractor. The solid was then extracted with toluene for 48 hours to remove any residual bipyridine. The yellow-orange product was then recrystallized twice from methanol after hot filtration. An orange methanol-insoluble impurity was removed by this process.

The following is a modification of a previous procedure for a similar compound⁽¹⁶⁾. 0.80g 1-methyl-4,4'-bipyridine iodide salt was dissolved in 40 ml acetonitrile with heating. To this 2.0g propane sultone was added and the mixture refluxed for 18 hours. The solvent was stripped off using a rotary evaporator under vacuum. The solid was dissolved in less than 10 ml warm water. The product was then precipitated with the addition of ethanol. The solid product was then filtered and washed with ethanol. This yielded 0.49g of bright orange product (V), a 43% yield. Thin layer chromatography on silica plates

using a 3:3:1 methanol:H₂O:50% ethylammonium chloride solvent showed a single component. Also, the NMR spectrum was consistent with the desired product. The median proton shifts relative to sodium 2,2-dimethyl-2-silapentane-5-sulfonate were as follows: δ 2.2 ppm (m, protons β to N-propyl); δ 2.8 ppm (t, protons γ to N-propyl); δ 4.4 ppm (s, -CH₃); δ 4.8 ppm (t, protons α to N-propyl); δ 9.0 ppm (t, aromatic protons meta to N); δ 9.6 ppm (d, aromatic protons ortho to N-propyl); δ 9.7 ppm (d, aromatic protons ortho to N-CH₃).

Reducing Agents: The primary reducing agent used for these studies was sodium dithionite, Na₂S₂O₄. This is a relatively mild, widely used reducing agent although its exact chemistry is not very well understood. In order to test that our observed results were not exclusive to reduction by dithionite, the anionic reductant Cr^{II}EDTA was also prepared.

All dithionite solutions were prepared fresh daily in anaerobic solutions from solid Na₂S₂O₄ and used within 4 hours of preparation. These stock solutions were constantly purged with argon gas before and after addition of the solid Na₂S₂O₄ since dithionite ion reacts rapidly with O₂⁽¹⁷⁾. Because S₂O₄²⁻ degradation is relatively rapid at acidic pH⁽¹⁸⁾, all stock solutions were prepared at pH 8.0, usually in the same buffer that was used in the experiment. Solid Na₂S₂O₄ was stored desiccated under vacuum. Exact S₂O₄²⁻ concentrations were determined spectrophotometrically at 315 nm ($\epsilon = 6900 \text{ M}^{-1}\text{cm}^{-1}$) prior to experiments⁽¹⁷⁾.

Anaerobic solutions of $\text{Cr}^{\text{II}}(\text{EDTA})^{2-}$ were also prepared for the purpose of viologen reduction. This was accomplished by first preparing the substitution labile Cr^{2+} and then adding the ethylenediamine-tetraacetic acid ligand. First a Zn/Hg amalgam was prepared. Zinc granules were covered with 1N HCl for one minute to activate the surface. The acid was then decanted off and the granules were covered with a 0.25 M aqueous solution of HgCl_2 . This was stirred vigorously for 3 minutes with a glass rod. This solution was decanted and the granules were washed several times with water. The Zn/Hg granules were then stored immersed in a slightly acidic HCl solution (pH = 2-3).

Stock solutions of $\text{Cr}^{\text{II}}(\text{EDTA})^{2-}$ were prepared just prior to use. A solution containing 10 mM $\text{Cr}(\text{ClO}_4)_3$ in 20 mM KCl and 1 mM HCl was deaerated by bubbling with argon. Then 8-10 granules of Zn/Hg amalgam were added and the solution was allowed to bubble for 2-3 hours. The appearance of a light blue color signified formation of Cr^{2+} . Finally, an anaerobic solution of 10 mM sodium EDTA was added in a 2:1 EDTA to Cr^{2+} volume ratio to give an approximately 3.3 mM $\text{Cr}^{\text{II}}(\text{EDTA})^{2-}$ solution.

Equilibrium Dialysis: Binding ratios for viologens bound to DHP vesicles were determined using equilibrium dialysis. The dialysis cells consisted of two 5 ml chambers separated by a Scienceware #40299 cellulose membrane (6,000 M.W. cutoff). A typical experiment would involve filling one chamber with an unloaded vesicle solution and the other with a viologen solution in the same buffer. The cell was then placed on a slow-moving shaker and the compartmentalized solutions were allowed to equilibrate overnight. Studies with alkyl viologens in

buffer solutions showed that equilibration was complete within eight hours. The concentration of viologen in each chamber was determined spectrophotometrically and thus the amount of viologen bound to the vesicles and free in solutions was calculated. It was also found that 5-10% of the added viologen adhered to the dialysis membrane.

Phosphate Analysis: Lipid concentrations were independently determined by measuring the phosphorous concentration in the solution. This was done by first mineralizing the lipid under strongly oxidizing conditions and then analyzing for inorganic phosphate concentration using a modification of a published procedure⁽¹⁹⁾.

One hundred microliters of the vesicle solution was placed in the bottom of a Pyrex test tube and was evaporated to dryness over a Bunsen burner. Next, 0.4 ml of concentrated sulfuric acid was added and the mixture was boiled until the sample was completely charred. The sample was allowed to cool for one minute. Then one drop of 30% H_2O_2 was added and the solution was boiled for 30 seconds. If the solution was not clear another drop of H_2O_2 was added and boiled until clear. The sample was again allowed to cool for one minute, then diluted to 25 ml with water.

The sample was then tested to determine inorganic phosphate concentration. First, two different reagents had to be prepared. Reagent A consisted of a solution of 2.86×10^{-2} M ammonium heptamolybdate. Reagent B was prepared by dissolving one gram of polyvinyl alcohol in 100 ml boiling water. This solution was allowed to boil for one hour while adding water to maintain constant volume. After

cooling, 18.5 mg of malachite green hydrochloride (M.W. 365) was added. Reagent B had to be stored in the dark and was prepared fresh every 4 weeks.

Analysis was made on a 3 ml aliquot of the diluted sample by adding sequentially 150 μ l of Reagent A and 0.3 ml of Reagent B followed by thorough mixing. After standing for 10 minutes, the absorbance at 623 nm was recorded and compared to a calibration curve such as that shown in Figure 2-3 to determine the phosphate concentration.

Stopped-Flow Procedure: The stopped-flow apparatus is described in Chapter 3. About 30 ml of the vesicle system to be studied was poured into one of the stopped-flow reservoirs. The other reservoir was filled with 29 ml of the appropriate buffer. Both reservoirs were then bubbled with argon for 30-45 minutes. Meanwhile, a stock solution of $\text{Na}_2\text{S}_2\text{O}_4$ was prepared by adding solid sodium dithionite to 5 ml of deaerated buffer to achieve a dithionite ion concentration 30 times that desired in the stopped flow reservoir. Just prior to starting the experiment one ml of the dithionite stock solution was added to the reservoir containing the deaerated buffer with care to avoid admitting oxygen to the system. Then, using the transfer syringe, a small aliquot of the reservoir solution was injected into a 1 mm pathlength cell which had been deaerated. The exact concentration of dithionite ion in the reservoir was then determined by recording the absorbance at 315 nm. The reactant solutions were drawn into the drive syringes and forced through the observation chamber by the plunger under approximately 15 psi gas pressure.

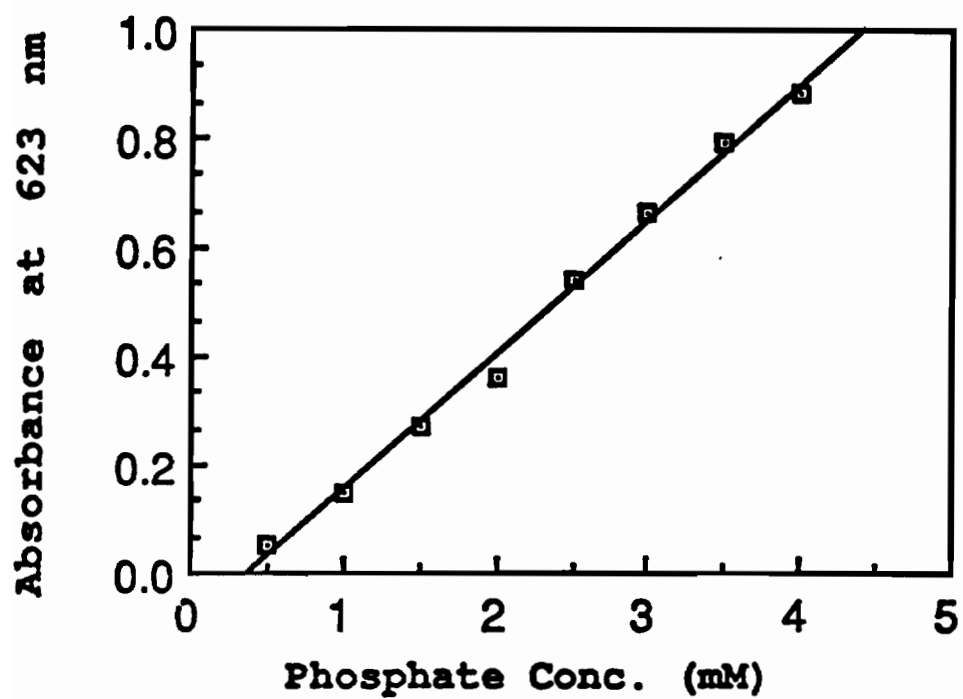


Figure 2-3: Experimental calibration curve for phosphate analysis.

Photochemical Systems: These experiments were performed at the Solar Energy Research Institute in Golden, Colorado in collaboration with Dr. John S. Connolly. The laser flash photolysis system is described in Chapter 3. The samples consisted of DHP vesicles prepared with alkyl viologens loaded on the outer surface as described above. Approximately 10 mM stock solutions of tetrasodium [5,10,15,20-tetrakis-(4-sulfonatophenyl)porphinato] zinc(II) (ZnTPPS^{4-} ion) were prepared daily and were stored at 4° C in the dark when not being used. The ZnTPPS^{4-} ion was then added to the vesicle suspension from the concentrated stock solution. The exact concentration of ZnTPPS^{4-} was determined optically at 556 nm ($\epsilon = 2.21 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$)⁽²⁰⁾ and the sample was placed in a long-neck 1 cm pathlength fluorescence cell. The cell was deaerated by blowing argon or nitrogen through the sample with a long needle for at least 10 minutes. The needle was removed and the rubber septum was wrapped with Parafilm. Finally the sample was inserted into the cell holder and subsequently subjected to laser flashes (fwhm 15 ns) at 605.6 nm.

CHAPTER 3

INSTRUMENTATION AND ANALYSIS

Spectrophotometers: The fluorescence spectrometer used was a Perkin-Elmer MPF-66. This instrument has an excitation and emission range of 200-900 nm utilizing a xenon lamp source and a standard photomultiplier tube. Two different UV/Vis spectrophotometers were used. One was a Perkin-Elmer Lambda 9 which is a continuous scanning, double beam, double monochromator instrument. It has a wavelength range of 185-3200 nm, thus covering the UV to near IR regions of the spectrum. The Lambda 9 was interfaced to a PE 7500 computer for data manipulation. The other UV/Vis spectrophotometer used was a Hewlett-Packard 8452A diode array optical bench interfaced to an HP UV/Vis ChemStation data system computer. The optical bench has a spectral range of 190-820 nm with 2 nm resolution. It is capable of acquiring spectra over the entire spectral range at a maximal rate of one every 0.6 seconds.

Stopped-Flow Instrumentation: The anaerobic stopped-flow apparatus used could conveniently measure reaction rates for processes having half-lives from 100 milliseconds to a couple of hours. Usually the first 30 to 40 msec of the waveform obtained showed signs of residual turbulence from the mixing process. A simplified block diagram for the apparatus is shown in Figure 3-1. Though not shown in the diagram, the system

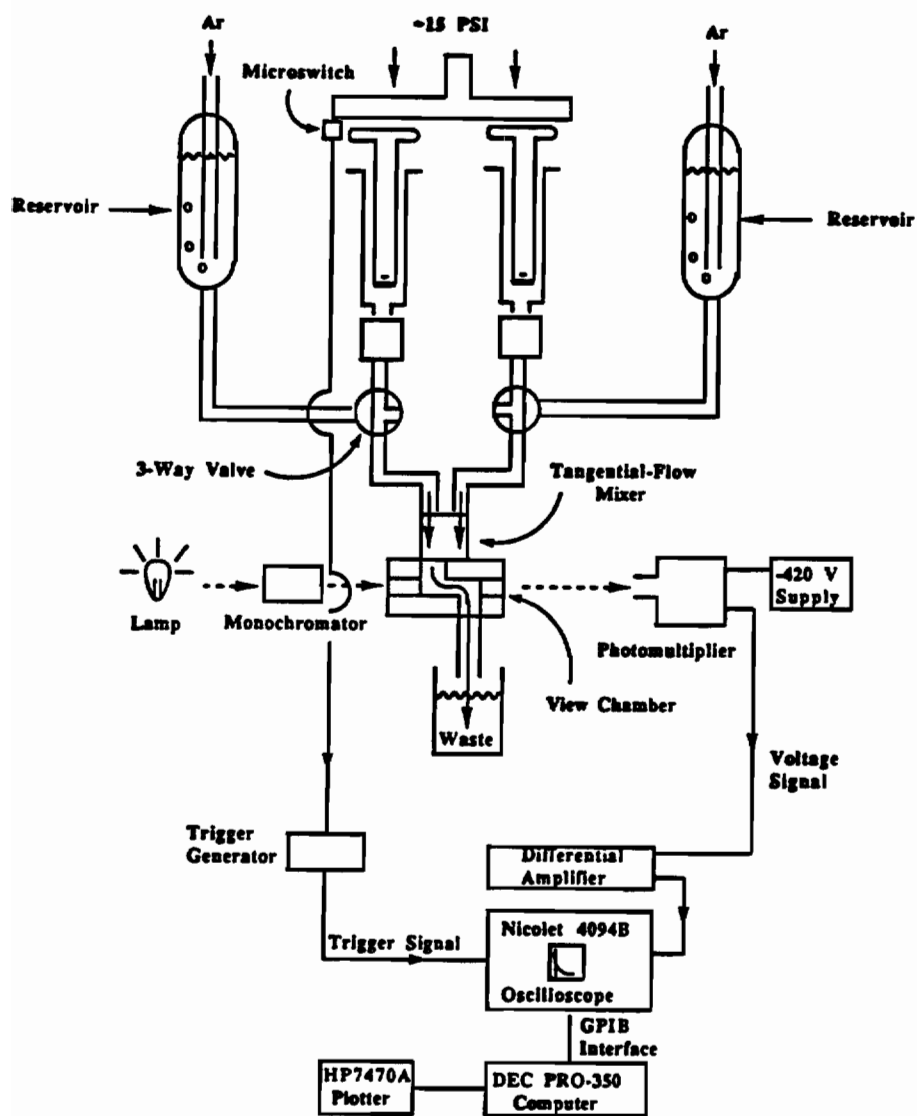


Figure 3-1: Schematic of stopped-flow apparatus.

also had the capability of temperature control by running water through the lucite holders around the syringes and the view chamber from a constant temperature bath.

The "wet" part of the apparatus consisted of two glass reservoir chambers to hold the solutions to be mixed. They were fitted with rubber septa and allowed oxygen purging and direct loading of the drive syringes via Hamilton 3-way valves. Also not shown in Figure 3-1 is another 3-way valve located at the base of the reservoir which allowed deoxygenated water or buffer to be drawn into the drive syringes instead of the reactants.

After the drive syringes were loaded the 3-way valves were adjusted such that the reactant mixtures were forced through a tangential-flow mixer and then through the view chamber. The drive syringes were driven by a plunger which was propelled by approximately 15 psi of argon gas. When the plunger stopped moving it tripped a microswitch which in turn triggered the oscilloscope to initiate data acquisition.

The reaction was monitored spectrophotometrically through the view chamber which had a 1 cm pathlength. White light from a tungsten lamp was passed through a Bausch & Lomb #33-86-07 monochromator equipped with continuously variable entrance and exit slits. The narrow band of diffracted light was directed through the view chamber and into a photomultiplier set at a potential of -420 volts. The voltage output from the photomultiplier was then fed into a Tektronix AM 502 differential amplifier. The amplified signal was then input into a Nicolet 4562 plug-in residing in a 4094B oscilloscope. This oscilloscope collects waveforms consisting of 3968 data points at a rate

of from 200 seconds to 0.5 microseconds per point. The digitized data was then output to a DEC PRO-350 computer via an IEEE-488 GPIB bus using the software listed in Appendix A.

Stopped-Flow Analysis: The relationship between voltage, V_i , recorded from the photomultiplier and solution absorbance is given by:

$$A_i = -\log \frac{V_i - V_d}{V_L - V_d} \quad (3-1)$$

where V_d is the dark voltage (= 0% transmittance) and V_L is the voltage with water or buffer in the view chamber (= 100% transmittance).

Experimentally, V_d was set to zero volts by blocking the light beam and adjusting the voltage to zero with the differential amplifier. V_L was then set to one volt by drawing water or buffer into the view chamber and adjusting the light intensity by varying the monochromator entrance and exit slit widths. The entrance slit width was kept at approximately twice the exit slit width to minimize the wavelength band width. Then the reactants were introduced into the view chamber and allowed to react completely. The voltage was then adjusted back to zero volts using the differential amplifier to allow for a lower voltage range setting on the oscilloscope and therefore greater resolution. The magnitude of the voltage shift was called the offset voltage, V_{off} . It had to be recorded and added back into the voltages read off the oscilloscope before doing the analysis. A graphical representation of these voltages applied to a typical waveform is shown in Figure 3-2.

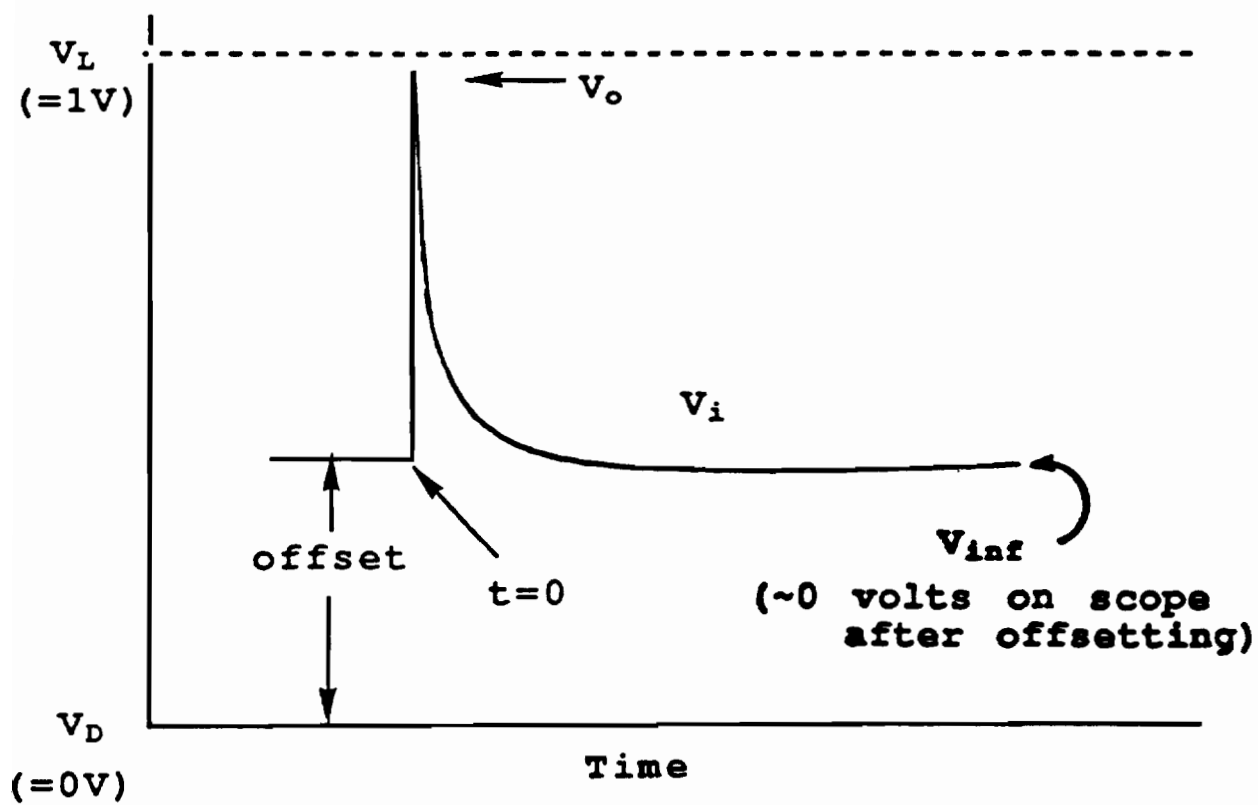


Figure 3-2: Voltages associated with a sample waveform acquired from the stopped-flow apparatus.

Some of the stopped-flow data was analyzed using the analysis program listed in Appendix B which fits sums of first-order reactions. Since the reactions are first-order, the voltages were converted to c/c_0 in the analysis program according to Equation 3-2.

$$\frac{c}{c_0} = \frac{A_i - A_{\text{inf}}}{A_0 - A_{\text{inf}}} = \frac{\log V_i - \log V_{\text{inf}}}{\log V_0 - \log V_{\text{inf}}} = \frac{\log V_i/V_{\text{inf}}}{\log V_0/V_{\text{inf}}} \quad (3-2)$$

Here V_0 is the voltage before any reaction has taken place and V_{inf} is the voltage at infinite reaction time. V_{off} must be added back to all voltages read off the oscilloscope. This simplified equation arises as a consequence of the fact that V_j equals zero volts and V_L equals one volt; thus from Equation 3-1 we have $A_j = -\log V_j$. Once converted to c/c_0 the data were fit to Equation 3-3 using the program listed in Appendix B.

$$\frac{c}{c_0} = A_0 + A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t} \quad (3-3)$$

Theoretically, if the reaction consists only of first-order processes, A_0 should be zero and the sum of the other A_i factors should be equal to one. These checks can be used to assess the acceptability of the data. Of course, A_2 and/or A_3 can be set equal to zero depending on the complexity of the reaction. The fit quality was checked by observing a random residual for the best-fit line and the actual data.

It soon became apparent when studying systems with equal concentrations of MV^{2+} inside and outside of DHP vesicles that the transmembrane step was not first-order. It was hypothesized that the

reaction was electron exchange between a MV^+ outside and a MV^{2+} inside. For equimolar MV^{2+} inside and outside this implies a simple second-order reaction which should follow the rate equation given in Equation 3-4.

$$\frac{1}{c} - \frac{1}{c_0} = kt \quad (3-4)$$

This leads to Equation 3-5.

$$\frac{c_0}{c} = c_0 kt + 1 \quad (3-5)$$

Then we define the following,

$$X \equiv -\log(V_0/V_{inf}) \quad (3-6)$$

$$Y \equiv -\log(V_i/V_{inf}) \quad (3-7)$$

These can be inserted into Equation 3-5 by utilizing Equation 3-2 giving Equation 3-8.

$$\frac{1}{Y} = \frac{c_0 k}{X} t + \frac{1}{X} \quad (3-8)$$

Using Equation 3-8, plots of $1/Y$ versus time were used to calculate the second-order rate constant, k . Since the outer reaction is over 99% complete after 500 milliseconds under typical conditions, the tail end of the waveform represents only the transmembrane step. The value of X was obtained from the y-intercept of the plot and c_0 is just equal to one-half of the initial MV^{2+} concentration for equimolar inside and

outside systems.

So a typical analysis consisted of fitting the very rapid initial reduction to a first-order curve and the slower tail of the waveform to a second-order form. The longer alkyl chain viologen systems were more complicated and could not be analyzed using this simple method. For these systems simulated mechanisms were used. These were generated by the program listed in Appendix C. This method was also used to confirm the simple two-step mechanism implied by the analysis given here for the MV^{2+} system under experimental conditions where inside and outside viologen concentrations were unequal.

Laser Flash Instrumentation: The laser flash apparatus used was housed at the Solar Energy Research Institute in Golden, Colorado in the laboratory of Dr. John S. Connolly. A simplified schematic of the components used for acquiring the laser flash data is shown in Figure 3-3. The appearance and decay of viologen radical was monitored at 605.6 nm where the absorption by ground and triplet excited state $ZnTPPS^{4-}$ ion is isosbestic⁽¹²⁾. This was done by monitoring changes in transmission of the continuous light provided by a 450 W Xe arc lamp. The light source was found to be most stable when the lamp current was set to about 20 amps. This light was passed through two Spex monochromators, one before and one after the sample, and the output detected by a Hamamatsu R955 photomultiplier with its dynode chain truncated at the fifth stage. The signal was amplified approximately 80 times by a Pacific Instruments wide-band (240 MHz) amplifier and the output voltage recorded on a Nicolet 4180 plug-in housed in a Nicolet 4094B

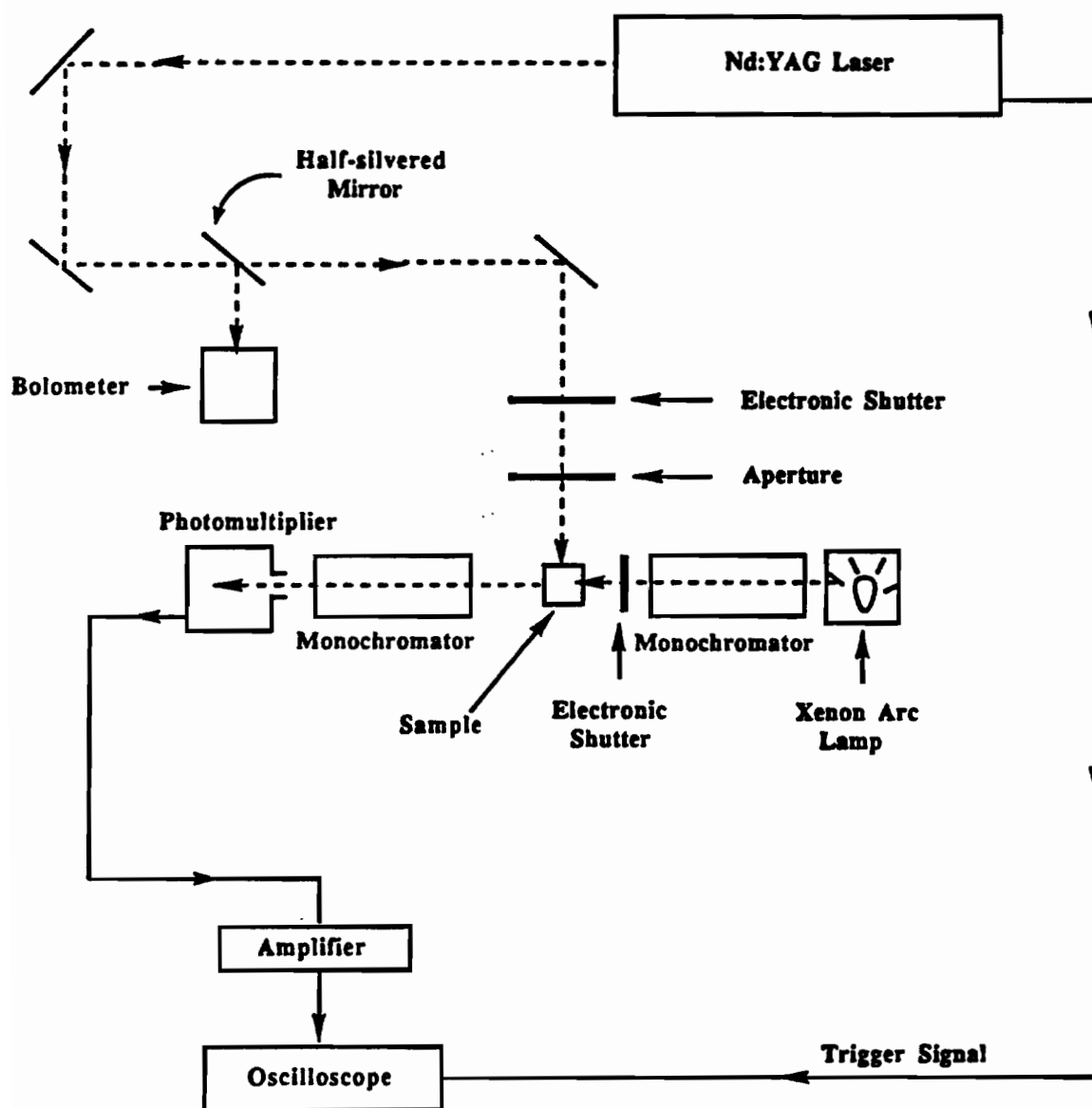


Figure 3-3: Schematic of laser flash photolysis system.

oscilloscope. The trigger signal was synchronized with the 5 Hz repetition rate of the laser but was timed appropriately to obtain information slightly before the laser flash occurred. Typically 8 to 264 flashes were averaged in the oscilloscope for each waveform depending on the intensity of the signal. The opening and closing of the various shutters and the determination of the number of flashes to run were all under control of a Tektronix CP 1164 computer. This system has been previously described⁽¹²⁾.

Laser Flash Analysis: The laser flash data were analyzed for first and second-order behavior. Modified versions of the program in Appendix B were used to do these analyses. Each program manipulated the voltage data differently before fitting. Similar to the stopped-flow analysis, voltages were obtained from a photomultiplier and converted to a transmittance scale using V_j (0% transmittance) and V_l (100% transmittance). In this case though these could not be set to 0 and 1 volt respectively. Thus these voltages appear in the equations used.

For first-order fits the approach was to convert the data to c/c_0 and allow the program to fit to an exponential equation. The conversion was performed using Equation 3-9.

$$\frac{c}{c_0} = \frac{-\log(\%T)}{-\log(\%T_0)} = \frac{\log((V_j - V_l)/(V_0 - V_l))}{\log((V_j - V_0)/(V_0 - V_l))} \quad (3-9)$$

where the voltage variables have the same meanings as for the stopped-flow analysis. The data were then fit to Equation 3-3 and the fit quality evaluated by looking at the residual.

The program for second-order fits converted the data into a form that would be linear in time if the reaction was simple second-order; i.e. if it behaved according to Equation 3-4. This, along with Equation 3-10, allows the derivation of the general result given in Equation 3-11.

$$A_i - A_{inf} = \epsilon bc \quad (3-10)$$

where ϵ is the molar absorptivity of the reactants and the products have ϵ equal to zero. This gives

$$\frac{\epsilon b}{A_i - A_{inf}} = kt + \frac{1}{c_0} \quad (3-11)$$

Then, using Equation 3-1 with b equal to one centimeter, Equation 3-11 becomes

$$\frac{1}{\log\left(\frac{V_{inf} - V_0}{V_L - V_0}\right) - \log\left(\frac{V_i - V_0}{V_L - V_0}\right)} = \frac{k}{\epsilon} t + \frac{1}{c_0 \epsilon} \quad (3-12)$$

For these experiments V_{inf} was equal to V_L since V_L was determined in the presence of the sample which had minimal absorbance at 605.6 nm.

Therefore, Equation 3-12 reduces to Equation 3-13

$$\frac{1}{\log((V_{inf} - V_0)/(V_i - V_0))} = \frac{k}{\epsilon} t + \frac{1}{c_0 \epsilon} \quad (3-13)$$

In the program the data were converted according to the left-hand side of Equation 3-12 and fitted to a straight line using a weighted linear least squares algorithm. The slope of the line would then give the second-order rate constant since the molar absorptivity was known.

CHAPTER 4

RESULTS AND DISCUSSION

I) Interaction of Alkyl Viologens with Dihexadecylphosphate Vesicles.

I.a) Equilibrium Dialysis: The binding of small molecules to a condensed phase is easily described theoretically when the binding sites are identical and independent. In these cases the binding curve at a constant temperature is described by the Langmuir adsorption isotherm equation given in Equation 4-1.

$$\frac{\delta}{1-\delta} = K[A] \quad (4-1)$$

The parameter δ is the fraction of sites occupied by the adsorbing species A, and K is the equilibrium constant for the binding of that species⁽²¹⁾.

But the binding of a viologen molecule to the surface of a DHP vesicle is affected by viologen molecules already bound. The binding of viologen cations reduces the negative surface charge on the vesicle making subsequently bound viologens less thermodynamically stable. This is an example of anticooperative binding for which no binding constant can be obtained and is supported by the absence of a fit to the Scatchard equation or Langmuir adsorption isotherm for viologen binding to PC vesicles⁽²²⁾. Therefore, in this work, the extent of viologen

binding will be described in terms of a binding ratio which will be dependent on the viologen-to-DHP ratio.

The method of equilibrium dialysis was used to determine the binding of some of the viologens to DHP vesicles in 20 mM Tris-HCl, pH 8.0. After equilibration, the binding ratio, R_b , was calculated for each sample according to

$$R_b = \frac{[V^{2+}]_{\text{bound}}}{[V^{2+}]_{\text{unbound}}} \quad (4-2)$$

The data are compiled in Table 4-1. The total viologen concentration is given because the binding is concentration dependent. The greater the concentration of bound viologen the less negative is the vesicle surface charge due to neutralization by the viologen dication.

In one experiment where MV^{2+} was cosonicated with DHP during vesicle formation and a binding ratio of 3.22 was assumed for external MV^{2+} , it was calculated from the concentrations of bound and unbound MV^{2+} that 31% of the viologen was entrapped in the vesicle interior and thus was not accessible to the bulk solution.

Other experiments included dialyzing vesicles containing only internal MV^{2+} against Tris buffer and observing no accumulation of MV^{2+} in the buffer chamber, showing complete removal of external viologen by gel chromatography. DHP vesicles with equimolar MV^{2+} inside and outside were reduced with a 5-fold excess of dithionite, oxygenated, and then dialyzed against Tris buffer. This system also showed no accumulation of MV^{2+} in the buffer chamber indicating migration of all MV^{2+} into the vesicle (see section III.c).

Table 4-1
Viologen Binding Ratios for DHP Vesicles ^a

Viologen	[V ²⁺] _{total}	[DHP]	R ₁
MV ²⁺	52 uM	2 mM	3.79
	210 uM	2 mM	0.75
	178 uM	4 mM	3.43
	164 uM	4 mM	4.07
	174 uM	4 mM	3.22
C ₆ MV ²⁺	87 uM	2 mM	3.23
	248 uM	2 mM	1.22
C ₈ MV ²⁺	87 uM	2 mM	11.1
	345 uM	2 mM	5.20
C ₁₀ MV ²⁺	92 uM	2 mM	>100
	229 uM	2 mM	30.3
C ₁₂ MV ²⁺	82 uM	2 mM	>100
C ₁₆ MV ²⁺	36 uM ^b	2 mM	>100
PMB ⁴⁺	47 uM	2 mM	>100
MSPB ⁺	101 uM	2 mM	<0.01

^a 20 mM Tris-HCl, pH 8.0. All expts. at room temperature.

^b Two-thirds of viologen bound to dialysis membrane.

There appears to be profound buffer and pH effects on the binding of MV^{2+} to the surface of DHP vesicles. The pK_a of the phosphate headgroup in DHP vesicles has been estimated at approximately 6⁽²³⁾ and the degree of protonation of the headgroup would certainly affect the adsorption of cations. Under similar conditions it was found that the binding ratio for MV^{2+} to DHP vesicles went from 2.71 in 20 mM Tricine, pH 8.1 to 6.23 in 20 mM Glycine, pH 9.5. Also, it was concluded in an earlier reported study that MV^{2+} binding to DHP vesicles was essentially 100%⁽²⁴⁾. But in this case the vesicles were prepared in water using sodium hydroxide to neutralize the DHP phosphate headgroup. In this system the low ionic strength may have screened the DHP headgroup less effectively so that vesicles prepared this way had a greater effective negative surface charge density. In this medium the viologen dication would be more strongly attracted to the vesicle surface. The binding of MV^{2+} is particularly sensitive to these effects as can be seen in the variability of R_b in Table 4-1. It was not possible to obtain reproducible values of R_b for this system due to this sensitivity.

I.b) Cation Exchange Chromatography: Cation exchange chromatography was employed in order to determine the equilibrium distribution of viologens inside and outside of DHP vesicles after cosonication of the components. It also served as a qualitative measure of the strength of viologen binding to the vesicle surface since all but the most tightly bound viologens could be removed using the Chelex 100 cation exchange resin. Monoalkyl viologens could be removed only if the alkyl chain contained 10 or fewer carbon atoms. For viologens that could be

efficiently removed, the equilibrium distribution in DHP vesicles could be calculated by measuring the viologen concentration before and after chromatography, the latter corrected for vesicle dilution as determined by phosphate analysis. Residual viologen on the outer surface after chromatography could be quantitatively determined by adding dithionite ion to anaerobic solutions. The results from these experiments are displayed in Table 4-2.

It can be seen that for C_{MV}^{2+} with $n \leq 10$ the equilibrium distribution of viologen is approximately one-third inside and two-thirds outside. Vesicles with an outer hydrodynamic radius of 160 angstroms and a bilayer thickness of 50 angstroms would have 32% of their total surface area on the inside and 68% on the outside. As mentioned in Chapter 1 these dimensions are typical for DHP vesicles. This implies that the viologens are evenly distributed on the vesicle surfaces and the inside/outside distribution is determined by the relative inner and outer surface areas. Monoalkyl viologens with $n \geq 12$ as well as PMB^{4+} could not be efficiently removed using the Chelex 100 cation exchange resin, even after 24 hour exposure, and therefore the equilibrium distribution of these viologens could not be calculated using this method.

I.c) Viologen Redistribution Between Vesicles: In micellar systems, two different mechanisms have been proposed for migration of solubilizates from one micelle to another. One involves dissociation of the molecule into bulk solution and then subsequent adsorption onto the other micelle⁽²⁵⁾. The other mechanism assumes that exchange of adsorbed

Table 4-2
Equilibrium Distributions of Alkyl Viologens Across Vesicle Bilayers ^a

Viologen	[V ²⁺] (uM)	In:Out distrib. after Chelex 100	DHP concen. Before:After Chelex 100 ^b	Calculated In:Out distribution when cosonicated
MV ²⁺	368	96:4%	8.2:6.0 mM	30:70%
C ₆ MV ²⁺	367	100:0%	6.2:5.7 mM	32:68%
C ₈ MV ²⁺	290	97:3%	6.4:5.1 mM	34:66%
C ₁₀ MV ²⁺	278	84:16%	7.5:5.8 mM	32:68%
C ₁₂ MV ²⁺	227	<5% removed	7.4:5.6 mM	^c

^a 20 mM Tris-HCl, pH 8.0. Viologen cosonicated with DHP during vesicle formation.

^b Concentration of DHP as determined by phosphate analysis before and after chromatography.

^c Equilibrium distribution could not be calculated due to poor viologen removal.

molecules occurs during the collision time of two micelles⁽²⁶⁾. Either of these mechanisms could be applicable to the redistribution of viologens between DHP vesicles.

The viologen binding ratios for DHP vesicles would be expected to indicate the relative rates of viologen redistribution between vesicles. If a given viologen is more tightly bound to a vesicle, its redistribution rate would be expected to be slower because of longer residence times on the vesicle surface. For MV^{2+} and C_6MV^{2+} , where relatively large percentages of the viologen are not bound, rapid redistribution of these viologens between vesicles would be expected. It was found that if these viologens were added to vesicle suspensions in a bolus from a concentrated stock solution the physical characteristics of the system were always identical with solutions where the viologen had been evenly distributed over the vesicle population by means of a tangential-flow mixer. On the other hand, if $C_{16}MV^{2+}$ was added to a vesicle suspension from a concentrated stock solution the physical properties of the resulting system were distinguishable from those for a similar flow-mixed system (see below). Furthermore, if a large quantity of a concentrated $C_{16}MV^{2+}$ solution was added, the vesicles would partially precipitate.

One physical property of these types of systems which can be used to monitor viologen redistribution is viologen dimerization upon reduction (see section II.b for more information on viologen dimers). In general, for a given viologen, the greater the surface concentration of viologen on the vesicles the greater the degree of dimerization. This observation gave the first indication that $C_{16}MV^{2+}$ does not rapidly

redistribute among DHP vesicles. It was noted that if approximately 10 μM $\text{C}_{16}\text{MV}^{2+}$ was added to a 4 mM vesicle suspension from a concentrated stock solution, reduction of the system yielded approximately 65% dimer. If the viologen was flow-mixed 50:50 with 8 mM DHP vesicles the reduced product showed about 25% dimer. It appeared as though only those vesicles in contact with the droplets of the stock solution being added were picking up the viologen and it was not redistributing to the unloaded vesicles on the timescale of the experiment. Thus the vesicles which did contain the viologen had a higher viologen surface concentration as compared to the vesicle system where the viologen was flow-mixed evenly throughout the vesicle volume.

These data appeared inconsistent with results from this lab on a similar system⁽¹²⁾. In that case $\text{C}_{16}\text{MV}^{2+}$ loaded on DHP vesicles in the presence of ZnTPPS^{4-} was found to redistribute to unloaded vesicles within two minutes, the shortest accessible sampling time. The physical property being monitored in this case was the photoexcited porphyrin quenching rate which was dependent upon the viologen surface concentration. In order to determine whether or not these two systems were fundamentally different, a systematic series of experiments was designed as described below.

DHP vesicle suspensions were prepared with approximately 25 and 100 μM $\text{C}_{16}\text{MV}^{2+}$ evenly distributed by flow-mixing. The higher concentration contained about 20% more dimer when reduced. Then an aliquot of the 100 μM sample was diluted 4-fold with a vesicle suspension of the same DHP concentration. If the viologen redistributed homogeneously among all of the vesicles in the system we would expect the same percentage of dimer

as in the 25 μM standard upon reduction. If no redistribution occurred then the amount of dimer formed upon reduction should be the same as the 100 μM standard since the surface concentration would be unchanged. ZnTPPS^{4-} or other ions were subsequently added to a similar system to see if they would affect the redistribution rate.

The results from these experiments are given in Table 4-3. They show that redistribution of $\text{C}_{16}\text{MV}^{2+}$ between vesicles is normally very slow, with a half-life of over 30 minutes in this case. With addition of ZnTPPS^{4-} this process becomes much more rapid, with a half-life of less than two minutes. It is possible that this porphyrin, with its high negative charge, ion pairs the viologen dication headgroup, facilitating its removal from the vesicle surface. The viologen can then redistribute to another vesicle. This must be a dynamic system since it is known that very few porphyrin-viologen ion pairs exist at any given time. This comes from the observation that there is no shift in the porphyrin visible spectrum in these vesicle systems, unlike mixtures in buffer solutions. Ion pairing is extensive if the viologen cannot preferentially adsorb to the negatively charged vesicle surface.

It appears, therefore, that ZnTPPS^{4-} ion is acting as a "viologen shuttle" removing a viologen molecule from one vesicle surface and redistributing it to another. When EDTA^{4-} ion was added to this system in place of ZnTPPS^{4-} there was some initial redistribution of the viologen but then no further changes with time. This may be due to DHP vesicle destabilization by EDTA^{4-} . Initially, some of the vesicles may have dissociated and the viologen redistributed to unloaded vesicles. But the EDTA^{4-} ion did not shuttle viologen from one vesicle to another;

Table 4-3
Redistribution of $C_{16}MV^{2+}$ to Unloaded DHP Vesicles ^a

System	Incubation ^b Time	% Dimer ^c	Comments
25 μ M Standard	-	30%	Represents complete redist.
100 μ M Standard	-	50%	Represents no redistribution
No added ions	5 min.	46%	Almost no redistribution
No added ions	40 min.	39%	About 55% redistribution
50 μ M ZnTPPS ⁴⁻	2 min.	38%	About 60% redistribution
50 μ M ZnTPPS ⁴⁻	5 min.	34%	About 80% redistribution
50 μ M EDTA ⁴⁻	5 min.	43%	{Taken together these show no redistribution caused by EDTA ⁴⁻ ion}
50 μ M EDTA ⁴⁻	15 min.	44%	
50 μ M MMB ⁺	5 min.	45%	Almost no redistribution

^a Except for standards all experiments consisted of combining 25% 100 μ M $C_{16}MV^{2+}$ on 2 mM DHP vesicles with 75% 2 mM DHP vesicles plus any additional ions if indicated. All solutions made in 20 mM Tris-HCl, pH 8.0.

^b The time between mixing of components and reduction by dithionite ion.

^c See section II.b this chapter on how to calculate percent dimer.

otherwise it would have continued to do so over time. As expected the positively charged 1-methyl-4,4'-bipyridinium ion (MMB⁺) also had no effect since it should not form ion pairs with viologen molecules.

I.d) Viologen Entrapment in Vesicles: In order to observe transmembrane electron transfer events between viologen molecules one must be able to entrap significant amounts of viologen inside the vesicles. Entrapment is done by cosonicate the viologen with the vesicle-forming lipid and then removing external viologen by chromatography. In order to entrap significant quantities of a molecule it must have a fairly high binding ratio, R_b . In the limit of R_b approaching zero (i.e. no binding) the amount of entrapped molecules would be proportional to the entrapped volume of the vesicle relative to the bulk solution. For example, a 4 mM DHP solution of vesicles with outer radii of 160 angstroms and bilayers 50 angstroms thick would entrap about 1.3 ml of solution per liter. These are typical dimensions of small unilamellar vesicles made up of about 10,000 lipid molecules (Chapter 1). Therefore, about 0.13% of the added material would be entrapped.

On the other hand, if R_b approaches infinity (i.e. complete binding) then the amount of entrapped viologen should be proportional to the inner surface area relative to the total surface area both inside and outside. As was just calculated in section I.b this is about 33% inside and 67% outside. In this case about 33% of the added material would be entrapped. These represent the two extremes and entrapment of most molecules would be expected to lie between these bounds. The

viologen dications have a high affinity for DHP vesicles and have entrapments near the maximum, as can be seen for some of the monoalkyl viologens in Table 4-2. However MSPB⁺ with its very low R_f shows almost no entrapment. This is probably due to the fact that it has only one positive charge overall and therefore has less electrostatic attraction to the negative vesicle surface.

In egg phosphatidylcholine vesicles MV²⁺ shows very little entrapment. This is because the egg PC headgroup is zwitterionic and therefore has no overall charge. In this case there is minimal electrostatic attraction between the viologen and the bilayer surface⁽²²⁾ unlike in the case of DHP vesicles. However, if hydrophobic forces of attraction exist between dopant and vesicle, e.g. from an n-alkyl chain, significant entrapment is observed. For instance, C₁₆MV²⁺ can be entrapped in egg PC vesicles.

Entrapment of cationic molecules in DHP vesicles has been claimed to be pH dependent. Tricot et al⁽²³⁾ have shown that entrapment of MV²⁺ and Ru(bpy)₃²⁺ falls off to almost zero above pH 7 or 8. The explanation was leakage of cations out of the vesicles at high pH during the chromatography step. Obviously, there are more factors involved than just pH though since very high entrapment was observed in Tris buffer at pH 8.0. A high R_f would be required for significant entrapment but alone is not sufficient to guarantee it. If indeed the leakiness of the vesicles can be increased then the transmembrane potential caused by the concentration gradient which develops during chromatography could result in zero observed entrapment. This is because the cations would continually run down this concentration gradient as external ions are

removed by the chromatographic process until the vesicles were emptied.

In order to determine the reason for low entrapment in a given system both the intrinsic leakage rate for the viologen and its R_f value must be known. For example DHP vesicles prepared in 20 mM glycine buffer at pH 9.5 showed no entrapment of MV^{2+} . As reported in section I.a of this chapter, this system should have a high R_f so the explanation for the lack of observed entrapment would be that the vesicles are very leaky. This was supported by the fact that DHP vesicles prepared in this buffer tend to be unstable over time. Alternatively, vesicles prepared in 20 mM ethanolamine at pH 10.7 showed about 10% entrapment. If dithionite was added to the chromatographed vesicles no radical appeared even after 10 minutes so these vesicles show no signs of leakiness. But the R_f value calculated was 0.60 under these conditions, which is quite small, so low intrinsic entrapment is expected. If the vesicles are not leaky the percent entrapment can be estimated by multiplying the relative inner surface area by the amount of viologen associated with the vesicle. This is expressed in Equation 4-3 for DHP vesicles.

$$\% \text{ entrapment} \approx 0.33 \frac{R_f}{R_f + 1} \quad (4-3)$$

If Equation 4-3 is applied to the ethanolamine example the calculated entrapment of 12% compares well with the experimentally observed value of 10%.

For all experiments done in Tris or imidazole buffer, entrapment was no problem for all viologens carrying a positive charge of two or

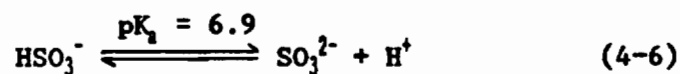
higher. Of course asymmetric systems containing different viologens inside and outside were not always possible since the viologen on the outer surface sometimes could not be removed. But leakiness was never a problem in either one of these buffer systems. In fact MV^{2+} entrapped within DHP vesicles prepared in 20 mM Tris buffer showed less than 5% leakage over a 24 hour period at room temperature.

II) Reduction of Alkyl Viologens Adsorbed to the Outer Surface of Dihexadecylphosphate Vesicles.

II.a) General: Dithionite ion reduces alkyl viologens completely in weakly alkaline solutions. The oxidation potential of dithionite in this case appears to be pH dependent since it will no longer reduce significant amounts of viologen at pH 5 even though its characteristic peak at 316 nm is still present. The exact mechanism of this reaction is not well understood and is often not discussed in studies involving dithionite. Kinetic studies of viologen reduction by dithionite in aqueous solution have shown that rates have a half-order dependence on the concentration of dithionite⁽²⁷⁾. The conclusion drawn was that SO_2^- ion was the actual reductant, which is in equilibrium with $\text{S}_2\text{O}_4^{2-}$ according to Equation 4-4.



This dissociation reaction has an equilibrium constant equal to $1.4 \times 10^{-9} \text{ M}^{(28)}$. One of the more complete studies of the dithionite ion redox potential suggests the following equations for the reduction step⁽²⁹⁾.



The midpoint potential was determined to be -0.66 V at pH 7 and 25 °C. Since the acid dissociation constant of (bi)sulfite is in this range the change in the midpoint potential is -59 mV below the pK_a and -118 mV above the pK_a for each pH unit increase. This mechanism would

qualitatively explain the observed pH dependence of viologen reduction. This is consistent with dithionite losing its ability to reduce MV^{2+} at around pH 5 since the reduction potential for MV^{2+} is about -450 mV and is pH independent within a physiologically relevant pH range⁽²⁹⁾.

MV^{2+} is reduced in strongly alkaline solutions without the addition of any other reductant⁽³⁰⁾. The mechanism involves the formation of a viologen pseudo-base which subsequently reacts with another MV^{2+} to form one methyl viologen radical and one pyridone molecule. Under certain conditions viologen radical can also be fairly unstable in aqueous solution. Initial decomposition rates of up to 2% per minute were observed for $C_{16}MV^+$ on DHP vesicles. The decomposition led to the formation of at least one fluorescent product; MV^{2+} is nonfluorescent. This fluorescent decomposition product has been previously observed and was reported to be an efficient photosensitizer for the reduction of MV^{2+} to MV^+ (31). The decomposition rate increased with increasing viologen alkyl chain length, but was independent of dithionite concentration and the percentage of monomer produced upon reduction. Representative data are given in Table 4-4.

The major decomposition product was characterized by TLC and fluorescence spectroscopy. TLC was run on silica plates using a 3:2:1 solvent system of butanol:water:acetic acid. The fluorescent decomposition product ran with an R_f of approximately 0.20. The fluorescence was green and showed a major peak at 520-524 nm in the emission spectrum. Typical emission and excitation spectra are shown in Figure 4-1.

Table 4-4
Viologen Decomposition Rates Under Various Conditions ^a

Viologen	[DHP]	[C ₁₆ MV ²⁺]	[S ₂ O ₄ ²⁻]	Initial Decomposition Rate
C ₁₆ MV ²⁺	4 mM	25 uM	250 uM	0.5% /min.
C ₆ MV ²⁺	"	"	"	0.11% /min.
MV ²⁺	"	"	"	0.05% /min.
C ₁₆ MV ²⁺	1 mM ^b	50 uM	100 uM	1.0% /min.
"	"	"	250 uM	1.3% /min.
"	"	"	500 uM	1.4% /min.
"	"	"	1 mM	1.3% /min.
C ₁₆ MV ²⁺	4 mM ^c	50 uM	100 uM	1.0% /min.
"	"	"	250 uM	1.3% /min.
"	"	"	500 uM	1.2% /min.
"	"	"	1 mM	1.1% /min.

^a In 20 mM Tris-HCl, pH 8.0. Viologens were flow-mixed onto outer vesicle surface.

^b This DHP concentration resulted in 43% viologen radical dimer.

^c This DHP concentration resulted in 23% viologen radical dimer.

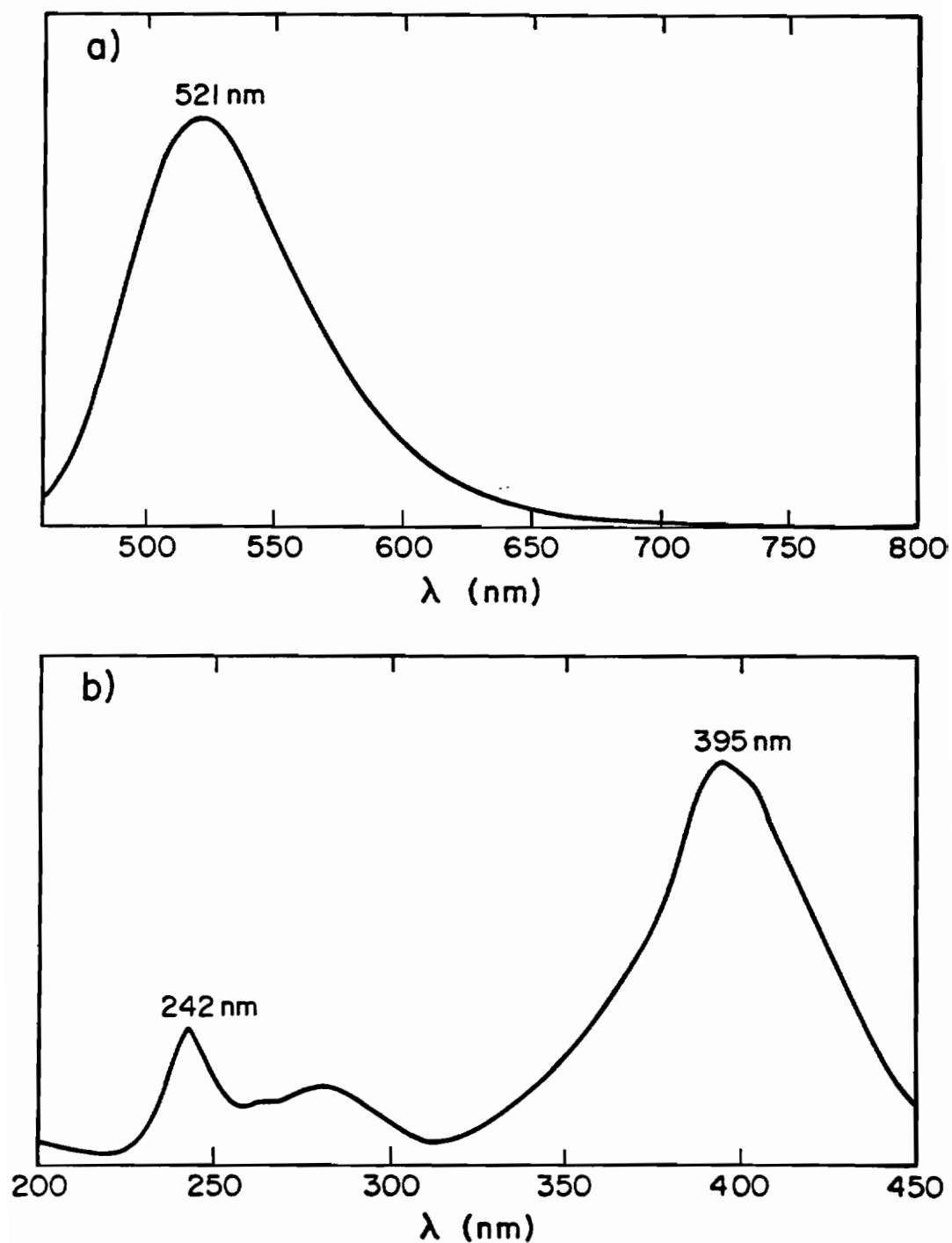


Figure 4-1: Fluorescence spectroscopy of C_6MV^{2+} decomposition product in 20 mM Tris-HCl, pH 8.0. a) Emission spectrum, excited at 395 nm. b) Excitation spectrum, monitored at 525 nm.

Large quantities of the decomposition product could be generated by allowing an anaerobic solution of MV^{2+} or C_6MV^{2+} to stand for 24 hours in the presence of dithionite ion. The solution could then be oxygenated and the fluorescent product was observed. Upon standing for another 24 hours after oxygenation, the fluorescent compound decomposed further to an unknown product which was not fluorescent.

By following the loss of viologen radical at 552 nm it was found that the decomposition reaction approximately fit homogeneous second-order kinetics. But it seemed to show slightly less than first-order dependence on the viologen concentration. This implies the existence of another reactant species at approximately the same concentration; otherwise pseudo-first-order kinetics would be observed. The other reactant is still unknown but appears to be related to molecular oxygen. Decomposition rates were enhanced when solutions were incompletely deoxygenated before addition of dithionite ion or if air was injected after dithionite was added. Methyl viologen radical reacts with O_2 in aqueous solution to rapidly form H_2O_2 which slowly decomposes to form H_2O ⁽³²⁾. It was found by fluorescence spectroscopy that if H_2O_2 was added directly to a reduced solution of C_6MV^{2+} on DHP vesicles, large amounts of the decomposition product were formed immediately. It is therefore possible that the other reactant is hydrogen peroxide. It has been hypothesized⁽³¹⁾ that the primary fluorescent product is a pyridone compound, which would support the assertion that the decomposition reaction is oxidative in nature. The pyridone formed⁽³¹⁾ had a fluorescence emission maximum at 520 nm, which is nearly identical to the decomposition product.

Although the decomposition reaction was not completely characterized, conditions had to be found for minimizing its rate. Unfortunately $C_{16}MV^{2+}$, which had the fastest decomposition rate, also had the slowest transmembrane electron transfer rate. This made it difficult to accurately determine the reaction curve for this system since the decomposition rate was of the same order of magnitude as the reaction rate toward the end of the reaction. Buffer and pH changes were explored in order to find conditions where the decomposition rate was manageable.

As can be seen from the data in Table 4-5 the viologen radical decomposition rate is extremely sensitive to the buffer composition. In the absence of DHP the reaction rate increases with pH. This would be consistent with the decomposition product being pyridone since the initial step in its formation is believed to be hydroxylation of the bipyridine ring system⁽³¹⁾. The pH dependence could also arise if the reaction with H_2O_2 previously discussed was due to perhydroxylation by HO_2^- ; H_2O_2 has a pK_a of 11.8⁽³³⁾. Thus the concentration of HO_2^- would increase with pH. There could even exist two different pathways leading to the same end product since the solutions without DHP decomposed significantly slower than those which contained DHP (Scheme I).

If DHP vesicles are present in solution this pH dependence no longer holds. This is not totally surprising since the reduced $C_{16}MV^{2+}$ viologen is expected to be strongly associated with the vesicle bilayer and thus not exposed to bulk solution. Also, hydroxide ion would be expected to be repelled from the negatively charged vesicle surface, so that the interfacial pH is lower than that in bulk solution.

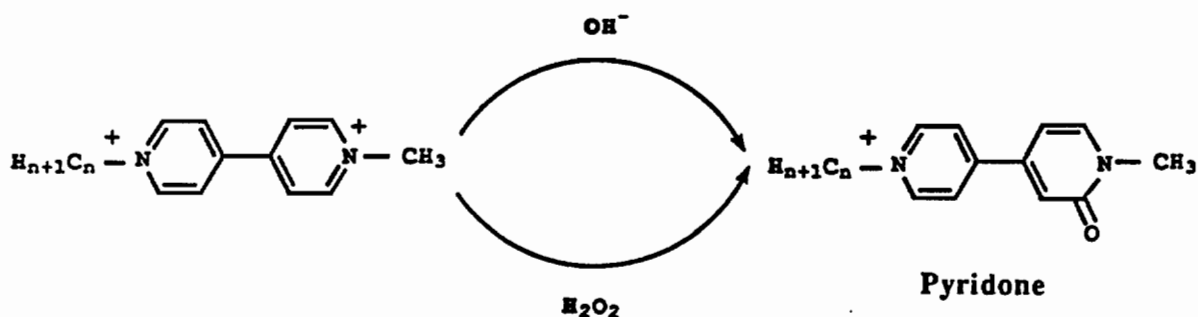
Table 4-5
Buffer Dependence of Viologen Decomposition Rate

Buffer	Initial Decomp. Rate
No DHP, C_6MV^{2+}	
20 mM Imidazole, pH 7.5	0.042% /min.
1 mM En, 20 mM Imid., pH 7.5	0.062% /min.
20 mM Tris, pH 8.0	0.069% /min.
20 mM Tricine, pH 8.2	0.32% /min.
20 mM Glycine, pH 9.5	1.65% /min.
2 mM DHP, $C_{16}MV^{2+}$	
20 mM Glycine, pH 9.5	< 0.01% /min.
20 mM Tricine, pH 8.1	0.15 /min.
20 mM TES, pH 7.5	0.29% /min.
20 mM Imidazole, pH 7.5	0.31% /min.
20 mM Tris, pH 7.5	0.51% /min.
20 mM Tris, pH 8.5	0.61% /min.

En - ethylenediamine

Tricine - N-tris[hydroxymethyl]-methylglycine

TES - N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid



Scheme I

The vesicles were least stable in the systems with the slowest degradation rates. It was therefore necessary to find the medium that simultaneously minimized the decomposition rate yet provided sufficient vesicle stability that the kinetic experiments could be made. Imidazole buffer was the best choice according to these criteria. In this buffer, degradation rates decreased with increasing ionic strength. Initial decomposition rates for $\text{C}_{16}\text{MV}^{2+}$ on DHP vesicles as measured directly in the stopped-flow apparatus were 0.85, 0.57, and 0.30 percent per minute for 20, 50, and 75 millimolar imidazole buffer respectively. This can be compared to 1.20% per minute for 20 mM Tris-HCl buffer under the same conditions. Above 75 mM imidazole, DHP vesicles were unstable. This medium was used to study transmembrane electron transfer kinetics of the long-chain viologens, $\text{C}_{12}\text{MV}^{2+}$ and $\text{C}_{16}\text{MV}^{2+}$, across DHP vesicle bilayers.

II.b) Dimer Formation: At high bulk or surface concentration, viologen radicals are known to form dimers or multimers^(11,34,35). Multimeric viologen is spectroscopically distinct from the monomeric form; the multimer spectrum exhibited isosbestic points with the monomer spectrum,

suggesting that it is dimeric as opposed to higher order aggregates. The forces involved in the dimer formation are believed to arise from π interactions between the bipyridine rings in each viologen^(36,37). This results in a cofacial pair where the bipyridine rings are coplanar. The interaction is also referred to as "pimerization".

Since the stopped-flow data are acquired at a single wavelength it was necessary to determine the viologen radical monomer-dimer isosbestic point(s) in order to eliminate changes in transmittance due to dimerization. By monitoring the reaction at an isosbestic point, formation of viologen radical with time could be accurately measured without complications arising from subsequent aggregation. For viologens loaded on the outer surface of DHP vesicles the degree of dimerization of reduced viologen increased with increasing alkyl chain length of the viologen at a given concentration.

Viologen dimerization was minimal in buffer solutions in the absence of DHP vesicles for the short chain viologens. For example, MV^{2+} solutions of up to 1 mM showed only moderate concentrations of dimer (<30%) upon reduction and the addition of 10% methanol eliminated almost all of the dimer. Long chain viologens in buffer reduced with an excess of reductant formed a yellow precipitate, presumably a doubly reduced viologen species. In the presence of DHP vesicles the reduction of MV^{2+} still led to predominantly monomeric product. A 50 μM MV^{2+} solution with 2 mM DHP gave only about 25% dimer. As opposed to MV^{2+} , a 50 mM solution of $C_{16}MV^{2+}$ reduced in the presence of 2 mM DHP vesicles showed significant quantities of dimer (~50%). As will be shown below, the amount of dimer present can be quantitated.

To determine the monomer-dimer isosbestic point(s) of reduced viologens loaded on the outer surface of DHP vesicles, multiple samples were prepared with varying concentrations of either MV^{2+} or $C_{16}MV^{2+}$ loaded onto the vesicles. The concentration of viologen was determined spectrophotometrically before reduction for each sample. The viologen radical spectra were then all scaled to a common concentration to determine whether or not any isosbestic points existed. The results for MV^{2+} are shown in Figure 4-2 and isosbestic points in the UV/visible region were found at 371, 552, and 760 nm. The same isosbestic points were found for the systems with $C_{16}MV^{2+}$ loaded on DHP vesicles. In both cases the molar absorptivity of the isosbestic point at 552 nm was calculated to be $9300 \text{ M}^{-1}\text{cm}^{-1}$. This is the most useful isosbestic point since it has a relatively high molar absorptivity and the slopes of the spectra are not excessively steep in this region, which would lead to ambiguity in its location.

To derive a formula for the percentages of monomer and dimer in a given spectrum, a spectrum for pure dimer must be obtained. It was not possible to obtain a viologen radical spectrum showing predominantly dimer when viologen was bound only at the external interface. However, if $C_{16}MV^{2+}$ was reduced in Tris buffer with a less than stoichiometric amount of SO_2^- from dithionite ion, an essentially pure dimer spectrum was obtained as evidenced by the almost complete disappearance of the characteristic monomer peak at 397 nm. This dimer spectrum had peaks at 361 and 544 nm. The pure monomer spectrum was obtained by reducing a small concentration of MV^{2+} in a 10 cm pathlength cell. Since the dimer had a significant absorbance at 1000 nm ($\epsilon \approx 4200 \text{ M}^{-1}\text{cm}^{-1}$) and the

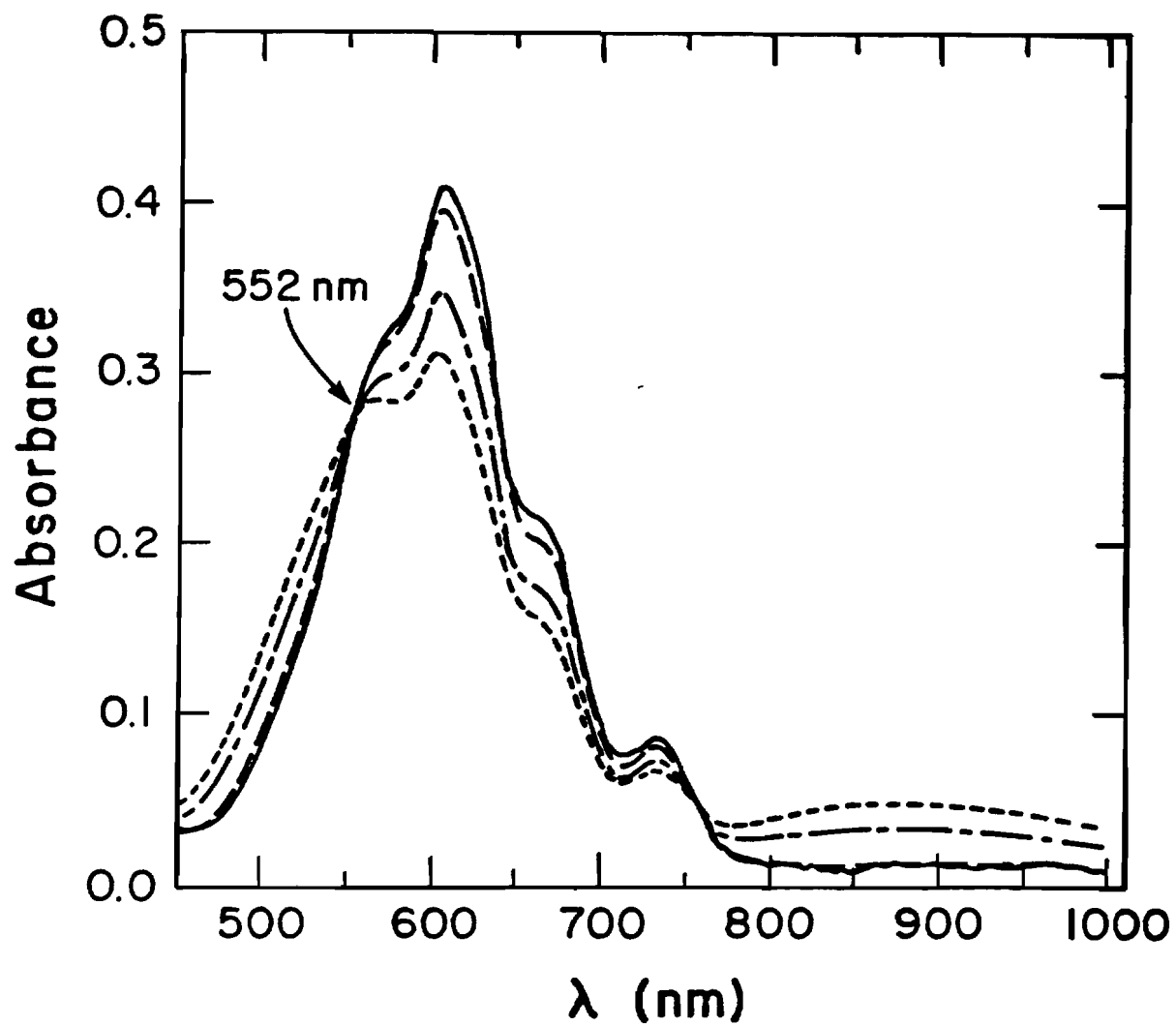


Figure 4-2: Spectra from dithionite-reduced MV^{2+} adsorbed onto 4 mM DHP vesicles. $[MV^{2+}]$ by decreasing absorbance at 605 nm - 9.0, 30.0, 111.1, 225.3 μM . All spectra scaled to 30.0 μM . 20 mM Tris-HCl, pH 8.0.

monomer appeared to have no absorbance in this region, a lower limit on the purity of the monomer formed could be calculated. This was determined to be at least 96% monomer and looked similar to spectrum a) in Figure 4-2. Using these two spectra an equation was derived for the percentage of viologen molecules present in the monomeric state for a given viologen radical spectrum, i.e.,

$$\% \text{ monomer} = \frac{\text{Abs}_{605}/\text{Abs}_{552} - 0.59}{0.98} \quad (4-7)$$

This formula is valid both for reduced systems with viologen in solution as well as externally adsorbed to DHP vesicles.

A significantly shifted dimer spectrum was found for reduced viologen systems where MV^{2+} was originally localized on both the inner and outer vesicle surfaces. A very high percentage of dimer was formed by systems containing initially high, equimolar distributions of MV^{2+} inside and outside of DHP vesicles. The spectral shift may be due to the dimer residing in a different environment. As will be discussed in section III.c, dimer formed from these types of systems appears to be localized inside the vesicles. The apparent pure dimer spectrum was obtained by digitally subtracting out a monomer spectrum equal to 10% of the total viologen radical present, thus eliminating the characteristic monomer peak at 397 nm. This dimer spectrum has peaks at 358 and 519 nm and is shown in Figure 4-3.

By varying the ratio of viologen concentration inside and outside of the vesicles the percentage of dimer formed could be controlled. In this manner an isosbestic point for this dimer with viologen monomer was

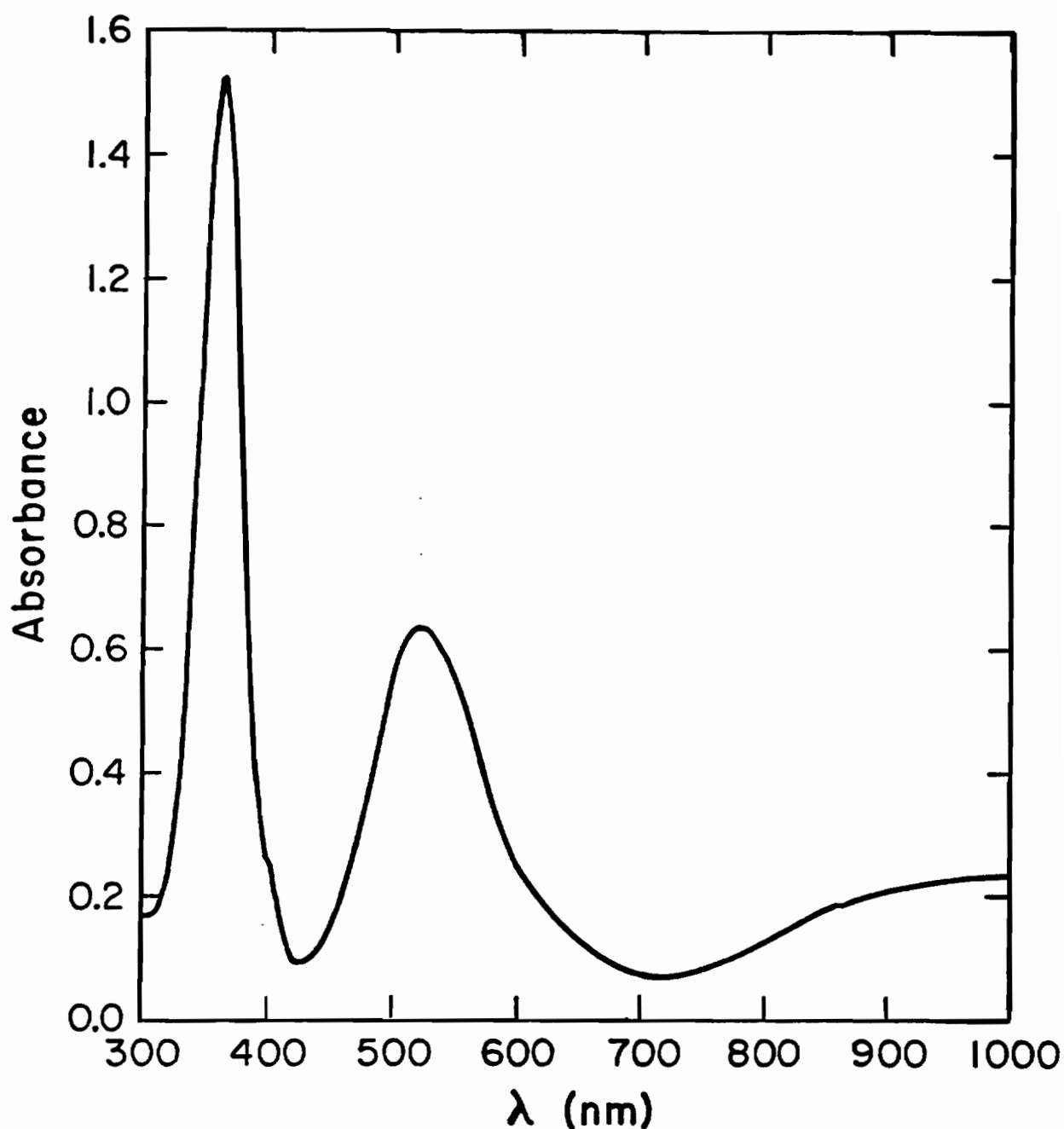


Figure 4-3: Absorption spectrum of dithionite-reduced system initially containing approximately 40 μM MV^{2+} inside and 40 μM MV^{2+} outside of 2 mM DHP vesicles in 20 mM Tris-HCl, pH 8.0. Spectrum of 8 μM MV^{+} monomer digitally subtracted to give theoretical 100% dimer spectrum.

determined and was calculated to be at 546 nm for DHP systems with MV^{2+} . The molar absorptivity at this isosbestic point was found to be 8200 $M^{-1}cm^{-1}$. Some sample spectra used for this determination are shown in Figure 4-4. From the dimer spectrum given in Figure 4-3 and the known monomer spectrum, a formula for percent monomer for this type of system could be derived and is given in Equation 4-8.

$$\% \text{ monomer} = \frac{\text{Abs}_{605}/\text{Abs}_{546} - 0.41}{1.20} \quad (4-8)$$

This formula is valid for reduced systems when the concentration of viologen entrapped inside the vesicle is greater than or equal to the concentration of viologen outside the vesicle. This restriction can be relaxed somewhat for MV^{2+} since it does not dimerize as readily on the outer surface. However, for the longer alkyl chain viologens, when excess viologen is present on the outer surface, two spectroscopically different forms of dimer will be formed in addition to viologen monomer and the isosbestic point will no longer exist.

These results show that what was previously thought to be pure viologen dimer⁽¹¹⁾ is actually a mixture of monomer and dimer. Only when the reduced viologen molecules are constrained to reside close together are high percentages of dimer observed. In this example the viologen molecules are constrained to the vesicle interior. Another example is the formation of MV^+ in its solid state which results in a spectrum characteristic of viologen dimer⁽³⁸⁾ with peaks at 369 nm and 556 nm making it slightly different from either dimer spectrum discussed here.

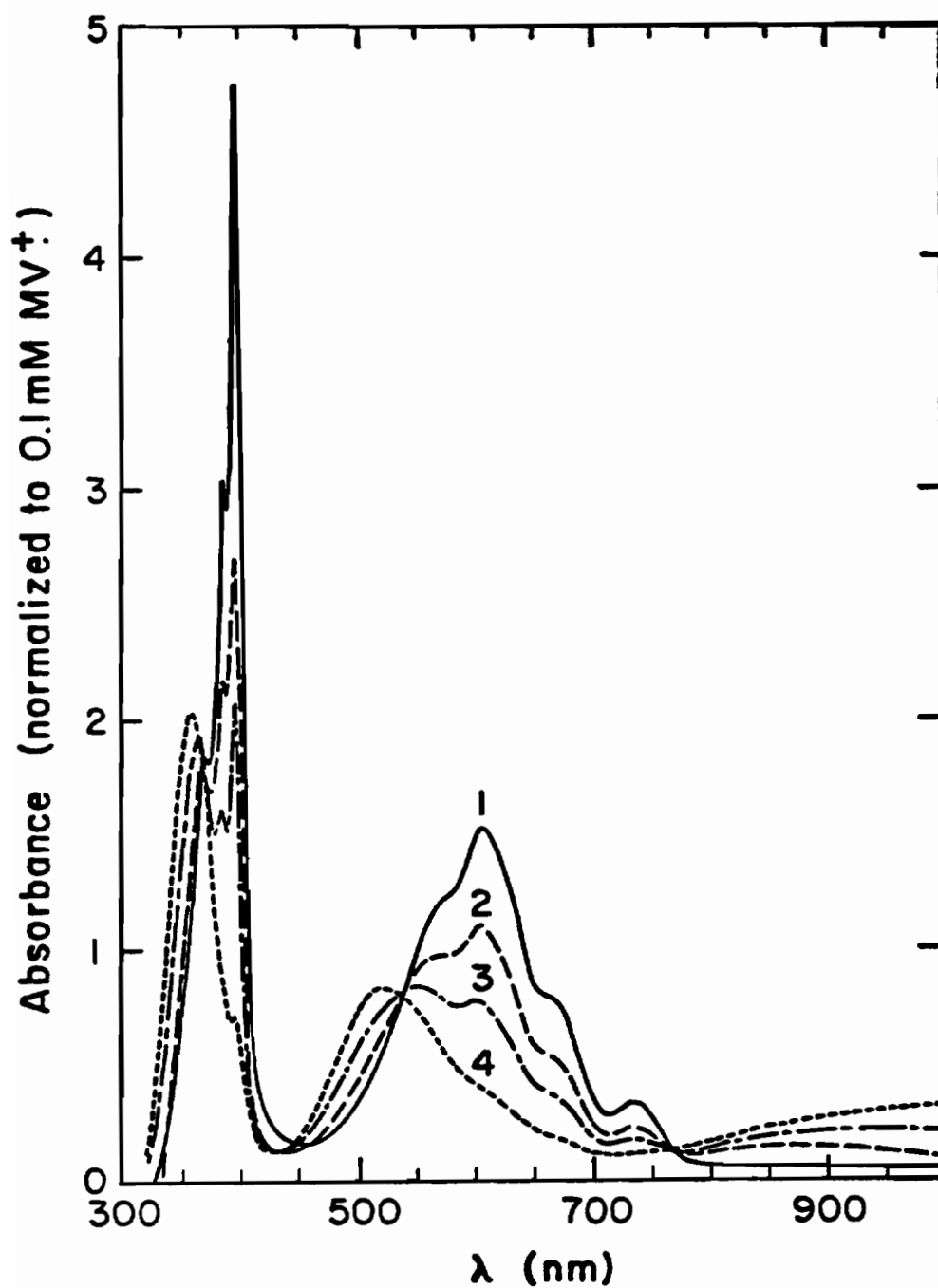


Figure 4-4: Absorption spectra of dithionite-reduced MV^{2+} bound to DHP vesicles - Initial conditions:

1) $[MV^{2+,out}] = 10 \mu M$. 2) $[MV^{2+,out}] = 160 \mu M$.

3) $[MV^{2+,out}] > [MV^{2+,in}]$. 4) $[MV^{2+,out}] < [MV^{2+,in}]$.

All in 20 mM Tris-HCl, pH 8.0. Monomer/dimer isosbestic points exist at 546 and 760 nm.

II.c) Reduction Kinetics: The kinetics of dithionite reduction of alkyl viologens adsorbed to the surface of DHP vesicles in Tris buffer at pH 8 have been extensively studied in this lab⁽¹⁴⁾. These data, given in Figure 1-2, are useful when studying the reduction of vesicle systems with viologen localized on both the inner and outer vesicle surfaces. One would expect the rate for reduction of the outer viologen in these systems to be comparable to the systems containing viologen adsorbed exclusively to the outer vesicle surface.

Since these data were not taken in pH 7.5 imidazole buffer, new experiments had to be run. These data were needed for the transmembrane redox studies done with $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$. It was found that, similar to the experiments in Tris buffer, these reactions showed biphasic behavior consisting of two simultaneous pseudo-first-order curves for MV^+ formation. The reduction also showed the expected half-order dependence on the dithionite ion concentration. The waveforms obtained were fitted to Equation 4-9.

$$y = A_0 + A_1 e^{-k_1 t} + A_2 e^{-k_2 t} \quad (4-9)$$

In all cases the experimentally determined A_0 was approximately zero. This implies a rate equation of

$$\frac{d[C_b MV^{2+}]}{dt} = A_1 k_1 [C_b MV^{2+}][SO_2^-] + A_2 k_2 [C_b MV^{2+}][SO_2^-] \quad (4-10)$$

The results for $C_{12}MV^{2+}$ are given in Table 4-6. The reduction rates might be expected to be slightly higher for vesicles with lower DHP to

Table 4-6
Rate Constants for Dithionite Reduction of $C_{12}MV^{2+}$
Adsorbed on DHP Vesicles ¹

$[C_{12}MV^{2+}]$	DHP: $C_{12}MV^{2+}$	A_1	$10^{-4} \times k_1$ ($M^{-1}s^{-1}$)	A_2	$10^{-4} \times k_2$ ($M^{-1}s^{-1}$)
126 μM	32	0.37	7.0	0.63	2.2
118 μM	34	0.21	8.2	0.79	3.3
91 μM	44	0.46	6.2	0.54	2.5
69 μM	58	0.43	6.5	0.57	3.0

¹ Conditions: 75 mM imidazole-HCl, pH 7.5, 2 mM DHP, 10 to 30-fold excess of $S_2O_4^{2-}$.

$C_{12}MV^{2+}$ ratios since the charge repulsion between SO_4^{2-} ion and the vesicle surface would be decreased due to charge neutralization. The values for k_1 possibly exhibit this trend, although the k_2 values seem fairly random. These rate constants can be compared with the corresponding values of $k_1 = 4.5 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $k_2 = 7 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ taken from Figure 1-2. The slightly higher rates in imidazole buffer may simply be due to the higher ionic strength which would serve to diminish the charge repulsion between reactants.

The results for the reduction of $C_{16}MV^{2+}$ adsorbed on DHP vesicles are given in Table 4-7. These data show a strong dependence on the DHP: $C_{16}MV^{2+}$ ratio. Again, as expected, the k_1 values increase with the decreasing DHP: $C_{16}MV^{2+}$ ratio, probably due to decreases in charge repulsion between reactants. Similar to the $C_{12}MV^{2+}$ experiments, the values of k_2 are more randomly distributed but might indicate a slight trend in the opposite direction. The $C_{16}MV^{2+}$ results also indicate that increasing the DHP: $C_{16}MV^{2+}$ ratio causes a higher percentage of the reduction to take place via the faster reduction step. The rate constants listed in Table 4-7 can be compared with the values of $k_1 = 1 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ and $k_2 = 1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ taken from Figure 1-2 for experiments done in Tris buffer. Again, the rates in 75 mM imidazole are slightly faster possibly due to the increased ionic strength.

Table 4-7
Rate Constants for Dithionite Reduction of $C_{16}MV^{2+}$
Adsorbed on DHP Vesicles^a

$[C_{16}MV^{2+}]$	DHP: $C_{16}MV^{2+}$	A_1	$10^{-5} \times k_1$ ($M^{-1}s^{-1}$)	A_2	$10^{-5} \times k_2$ ($M^{-1}s^{-1}$)
72 μM ^b	28	0.06	17.5	0.95	1.9
71 μM ^b	28	0.05	15.6	0.95	2.0
59 μM ^b	34	0.06	13.8	0.95	2.0
41 μM ^b	49	0.15	10.2	0.85	2.4
74 μM ^c	54	0.14	9.8	0.86	2.3
62 μM ^c	65	0.17	8.8	0.82	2.3

^a Conditions: 75 mM imidazole-HCl, pH 7.5, 10 to 30-fold excess of $S_2O_4^{2-}$.

^b Approximately 1 mM DHP.

^c Approximately 2 mM DHP.

III) Reduction of Alkyl Viologens Adsorbed to the Inner and Outer Surfaces of Dihexadecylphosphate Vesicles.

III.a) General: When alkyl viologens were entrapped inside DHP vesicles, with no viologen on the outer surface, virtually no reduced viologen was formed upon the addition of dithionite ion to the deoxygenated solution. In 20 mM Tris buffer at pH 8.0 less than 10% reduction of the inner viologen occurred within 30 minutes. When viologen was present on the outer vesicle surface before reduction, addition of dithionite would cause not only its reduction but also some or all of the inner viologen. The extent of inner viologen reduction depended upon the amount and alkyl chain length of the externally added viologen. This behavior indicates that external viologen molecules were able to mediate transmembrane oxidation-reduction across vesicle bilayers.

Another interesting feature of these systems with viologen initially bound to both inner and outer DHP vesicle interfaces is that the reduced viologen product spectra showed a higher percentage of dimer than would be expected if all of the viologen was on the outer surface. This effect is most pronounced in systems containing MV^{2+} at high concentrations. As will be discussed in section III.c this is probably due to the fact that the outer viologen is migrating into the vesicles which has a concentrating effect, thereby promoting dimerization. These effects were discussed in section II.b.

III.b) Stoichiometry of Inner Viologen Reduction: The addition of viologen to the outer vesicle surface mediated the reduction of inner

viologen, but only to the extent that no more than an equivalent number of ions inside were reduced than were initially present outside the vesicle. The exact stoichiometry of this transmembrane redox process varied with the nature of the viologen and the total viologen concentration. In most cases systems containing MV^{2+} were uncomplicated and there was a one-to-one correspondence between the number of inner viologen molecules reduced and the number of viologen molecules initially present on the outer surface. The results of an experiment which shows this stoichiometry are given in Table 4-8. In all of these cases where the concentration of MV^{2+} inside was greater than the concentration outside the radical product spectra contained a high percentage of dimeric species.

If other viologens were used, the stoichiometry fell below the one-to-one correspondence ratio (i.e. less than one viologen molecule inside was reduced for each viologen molecule outside). For example, assuming that $C_{16}MV^{2+}$ has an equilibrium distribution of one-third inside and two-thirds outside, certain conditions gave very little or no transmembrane electron transfer. For total $C_{16}MV^{2+}$ concentrations of 11, 15, 26, and 71 μM , using 4 mM DHP, the amounts of viologen radical observed after reduction were 53, 57, 71, and 78 percent respectively. This implies that at the lower concentrations possibly not even all of the outer viologen is being reduced, although the viologen distribution may vary at the lower loadings. In either event, $C_{16}MV^{2+}$ does not mediate transmembrane electron transfer as efficiently as MV^{2+} in these systems. Under no circumstances was viologen able to mediate the transfer of more than one electron into the vesicle per molecule. The reason for this

Table 4-8
Ratio of Externally Added MV^{2+} to MV^+ Formed
After Dithionite Reduction^a

$[MV^{2+}]$ added	Additional $[MV^+]$ ^b	Ratio
9.8 μM	21.8 μM	1:2.2
14.4 μM	29.8 μM	1:2.1
18.5 μM	39.8 μM	1:2.2
24.9 μM	52.2 μM	1:2.1
34.5 μM	66.3 μM	1:1.9

^a Approximately 4 mM DHP, 20 mM Tris-HCl, pH 8.0, 46 μM MV^{2+} initially inside (95% unreducible by dithionite), 5-fold excess of dithionite used for reduction.

^b $[MV^+]$ formed minus the $[MV^+]$ obtained when no MV^{2+} is added outside.

limitation will be discussed in the next section.

III.c) Viologen Migration: As was briefly mentioned in some of the previous sections, it was found that the reduction of DHP vesicle systems with viologen bound at the inner and outer interfaces resulted in the translocation of some of the viologen on the outer surface into the vesicles. Systems containing MV^{2+} followed precise stoichiometries and showed the highest percentage of viologen migration.

This phenomenon was first noted by oxidizing a reduced sample which originally had MV^{2+} on inner and outer vesicle surfaces by purging with air. Upon deoxygenating and adding more dithionite most of the viologen was now unreducible. To confirm that the viologen itself had not been chemically modified, the detergent sodium dodecylsulfate (SDS) was added anaerobically to the system. SDS solubilizes the DHP molecules, breaking up the vesicles and exposing their contents to bulk solution. When this was done all of the viologen in the system was immediately reduced. In this case the reduced viologen was in the monomeric state since it was no longer confined within the DHP vesicles. The spectra following each step described above are given in Figure 4-5.

The degree to which the viologen migration took place depended on factors such as the viologen used (both inside and outside), the amount of dithionite added, and the viologen to DHP ratio. This was quantitated by calculating a value for percent migration which is defined as the percentage of viologen originally on the outer vesicle surface which appears to have migrated into the vesicle. First we define a value α for systems with excess viologen inside, which is

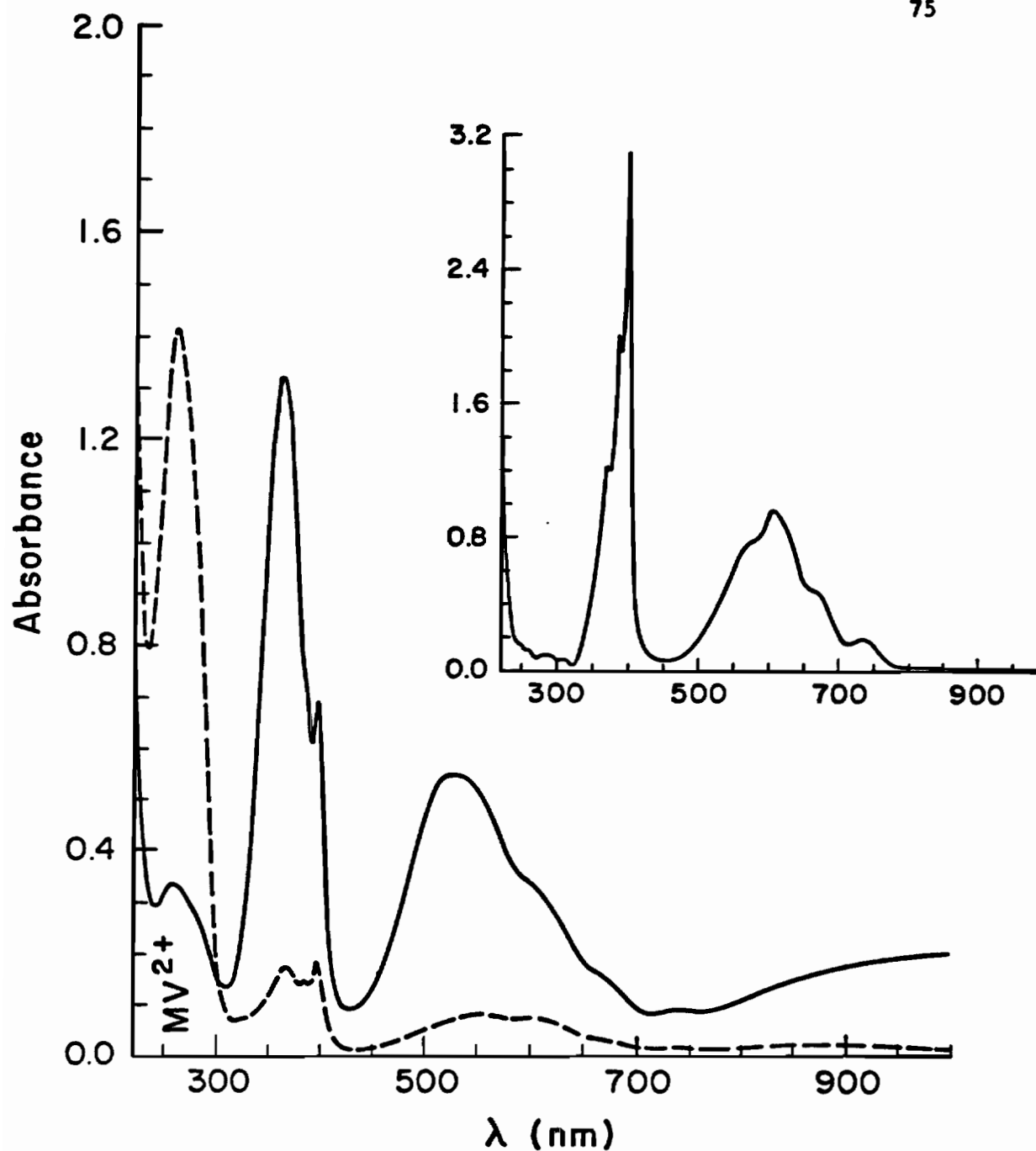


Figure 4-5: Product spectra from system initially containing 30 μM MV^{2+} inside and 30 μM MV^{2+} outside of 2 mM DHP vesicles in 20 mM Tris-HCl, pH 8.0. Solid line - Reduction by 5-fold excess of dithionite. Dashed line - After subsequent oxygenation, deoxygenation by argon purge, and re-reduction by dithionite. Inset - Subsequent anaerobic addition of sodium dodecylsulfate.

given in Equation 4-11.

$$\alpha \equiv \frac{[C_1MV^{\cdot}] \text{ formed after reduction}}{[C_1MV^{2+}] \text{ originally outside}} - 1 \quad (4-11)$$

This value is the average number of electrons translocated per viologen ion originally on the outer vesicle surface. As long as there is initially more viologen dication inside the vesicle than outside, the amount of viologen on the outer surface can be calculated by dividing the concentration of radical formed after reduction by the average number of radical ions formed per viologen on the outer surface, which is given by $(\alpha+1)$. This calculation is necessary for determining percent migration. Values for α can be determined from systems with known inside and outside viologen concentrations. In general for a given viologen, α values, as well as percent dimer and percent migration, increased with increasing viologen to DHP ratios. As already discussed, MV^{2+} inside and outside systems have an α value of approximately 1.0. Values of α and percent migration of various symmetric and asymmetric viologen distributions are given in Table 4-9.

From these results it can be concluded that all of these alkyl viologens mediate transmembrane electron transfer to some degree since all α values are greater than 0.0. Apparently MV^{2+} is the most efficient mediator regardless of the identity of the inner viologen. When an anaerobic solution of MV^{2+} was added to a reduced system with $C_{16}MV^{2+}$ distributed on both vesicle surfaces, the additional amount of viologen radical formed coincided with the amount of added MV^{2+} . No additional $C_{16}MV^{2+}$ was reduced initially even though an excess of $C_{16}MV^{2+}$

Table 4-9
Percent Migration and α Values For Various Alkyl
Viologens Inside and Outside of DHP Vesicles^a

<u>Viologen Inside</u>		<u>Viologen Outside</u>		α^b	% migration ^b
Identity	Concentration	Identity	Concentration		
MV ²⁺	21 μ M	MV ²⁺	16 μ M	1.0	87%
C ₆ MV ²⁺	27 μ M	C ₆ MV ²⁺	20 μ M	0.58	66%
C ₈ MV ²⁺	20 μ M	C ₈ MV ²⁺	17 μ M	0.51	38%
C ₁₀ MV ²⁺	20 μ M	C ₁₀ MV ²⁺	18 μ M	0.45	37%
C ₆ MV ²⁺	27 μ M	MV ²⁺	12 μ M	0.94	61%
C ₈ MV ²⁺	20 μ M	"	17 μ M	0.88	49%
C ₁₀ MV ²⁺	20 μ M	"	15 μ M	0.69	56%
MV ²⁺	23 μ M	C ₆ MV ²⁺	17 μ M	0.62	62%
"	"	C ₈ MV ²⁺	19 μ M	0.67	56%
"	"	C ₁₀ MV ²⁺	17 μ M	0.64	65%
"	"	C ₁₂ MV ²⁺	17 μ M	0.68	74%
"	"	C ₁₄ MV ²⁺	17 μ M	0.72	75%
"	"	C ₁₆ MV ²⁺	15 μ M	0.39	49%

^a Conditions: 20 mM Tris-HCl, pH 8.0, 1.5 mM DHP, 5-fold molar excess of S₂O₄²⁻.

^b See text for definition (Eq. 4-11).

still existed in the system. Over about 20 minutes further reduction of $C_{16}MV^{2+}$ did occur but this rate was much slower than expected for electron transfer mediated by MV^+ . This reduced rate might be due to differences in reduction potential between the two viologens. Reduction potentials of alkyl viologens are known to change in the presence of lipid vesicles⁽³⁹⁾.

In the case of DHP vesicles, MV^{2+} entrapped inside of vesicles appears to have a slightly lower reduction potential than external MV^{2+} . This was concluded from an experiment where a system with equimolar MV^{2+} inside and outside was titrated with dithionite ion. Initially only the viologen on the outer surface was reduced as evidenced by the fact that no dimer was formed and no migration took place. Not until the concentration of electrons available from $S_2O_4^{2-}$ exceeded the concentration of MV^{2+} originally outside did dimer formation occur, accompanied by viologen migration. This implies that MV^+ outside of DHP vesicles cannot reduce MV^{2+} inside unless there is an excess of dithionite driving the reaction. Therefore the reduction potential of MV^{2+} externally bound is greater than that for MV^{2+} internally bound. It is possible that internal $C_{16}MV^{2+}$ has a significantly lower reduction potential than both internal and external MV^{2+} and thus its reduction cannot be effected by MV^+ .

Ultimately it is the dithionite concentration which sets the Nernst potential by adjusting the MV^+ to MV^{2+} ratio. Thus, thermodynamically the MV^{2+}/MV^+ redox couple should be able to reduce $C_{16}MV^{2+}$ located inside the vesicle. As will be discussed, introduction of ionophores facilitates this reaction. However, the difference in reduction

potentials can affect the electron exchange rate. Since MV^+ seems to allow reduction of $C_{16}MV^{2+}$ inside at about the same rate as MMB^+ , which is not a reductant, it appears that the effect of MV^+ as an ionophore is much more significant than its role as an electron mediator.

At this point it is instructive to develop an hypothetical model which would explain the driving force for viologen migration and the fact that α never exceeds one. Subsequent experimental results can then be examined in light of this model and it can be determined whether or not they are consistent with it. The model is displayed pictorially for MV^{2+} in Figure 4-6.

When dithionite ion is added to a solution containing DHP vesicles with MV^{2+} bound at both membrane interfaces, the initial, most rapid reaction will be reduction of externally localized MV^{2+} . This step is represented by k_1 in Figure 4-6. Since internal MV^{2+} is reduced, there must be some kind of electron exchange between the reduced viologen on the outer surface and the internal viologen as SO_2^- alone will not directly reduce MV^{2+} that is separated by the bilayer membrane. This step is the second-order reaction designated as k_2 . The newly oxidized MV^{2+} is then re-reduced in the k_3 step which should occur with approximately the same rate constant as the k_1 step. At this point the net reaction has been reduction of both inner and outer viologen with transfer of an electron into the vesicle. As discussed in Chapter 1, transmembrane charge transfer leads to the polarization of the membrane and the development of a transmembrane potential. Since the system contains no lipophilic ions other than MV^+ one would expect this ion to move across the membrane in response to the electric field gradient.

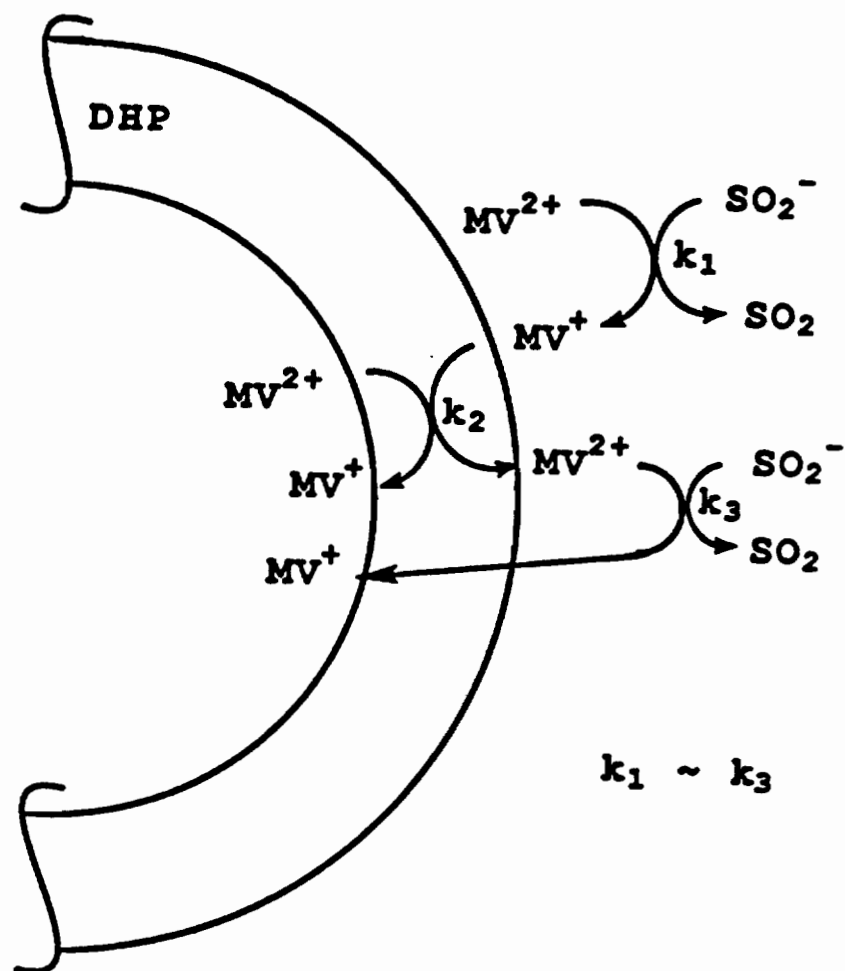


Figure 4-6: Proposed mechanism for transmembrane electron transfer when MV^{2+} is distributed on both the inner and outer surfaces of DHP vesicles.

This means that neutralization of the charge gradient requires one $MV^{\cdot+}$ to be translocated for each electron transferred. Therefore, after a $MV^{\cdot+}$ molecule mediates the transfer of an electron, it migrates into the vesicle and no longer participates in mediation of transmembrane redox. This stoichiometry involving 1:1 comigration of $MV^{\cdot+}$ with the electron would explain why α has a limiting value of one. Conditions leading to α values less than unity will be discussed in the next section.

The buildup of the electrical gradient and its dissipation by ion movement are illustrated in Figure 4-7 in which the net charge on both sides of the membrane is indicated during the stepwise reaction of one MV^{2+} transmembrane pair. The system is initially at equilibrium with no transmembrane potential. After electron transfer the net charge inside is minus one. A $MV^{\cdot+}$ molecule can then migrate inside to neutralize the negative charge and a viologen dimer is formed.

This scheme explains why MV^{2+} is found inside the vesicles after oxygenation. The amount of viologen found inside after oxygenation was dependent on the concentration of dithionite in solution before the oxygenation. This relationship, illustrated in Table 4-10, can be rationalized by the proposed mechanism. Even though molecular oxygen easily traverses the bilayer membrane, some viologen will react with O_2 near the outer surface (Scheme II). In this manner some of the viologen is drawn back out of the vesicle. But the amount of viologen radical which reacts with O_2 in this manner only accounts for a percentage of the overall oxidation. Large amounts of radical are oxidized within the vesicles. In fact if the system is slowly titrated with oxygen the viologen radical is slowly oxidized even though there is

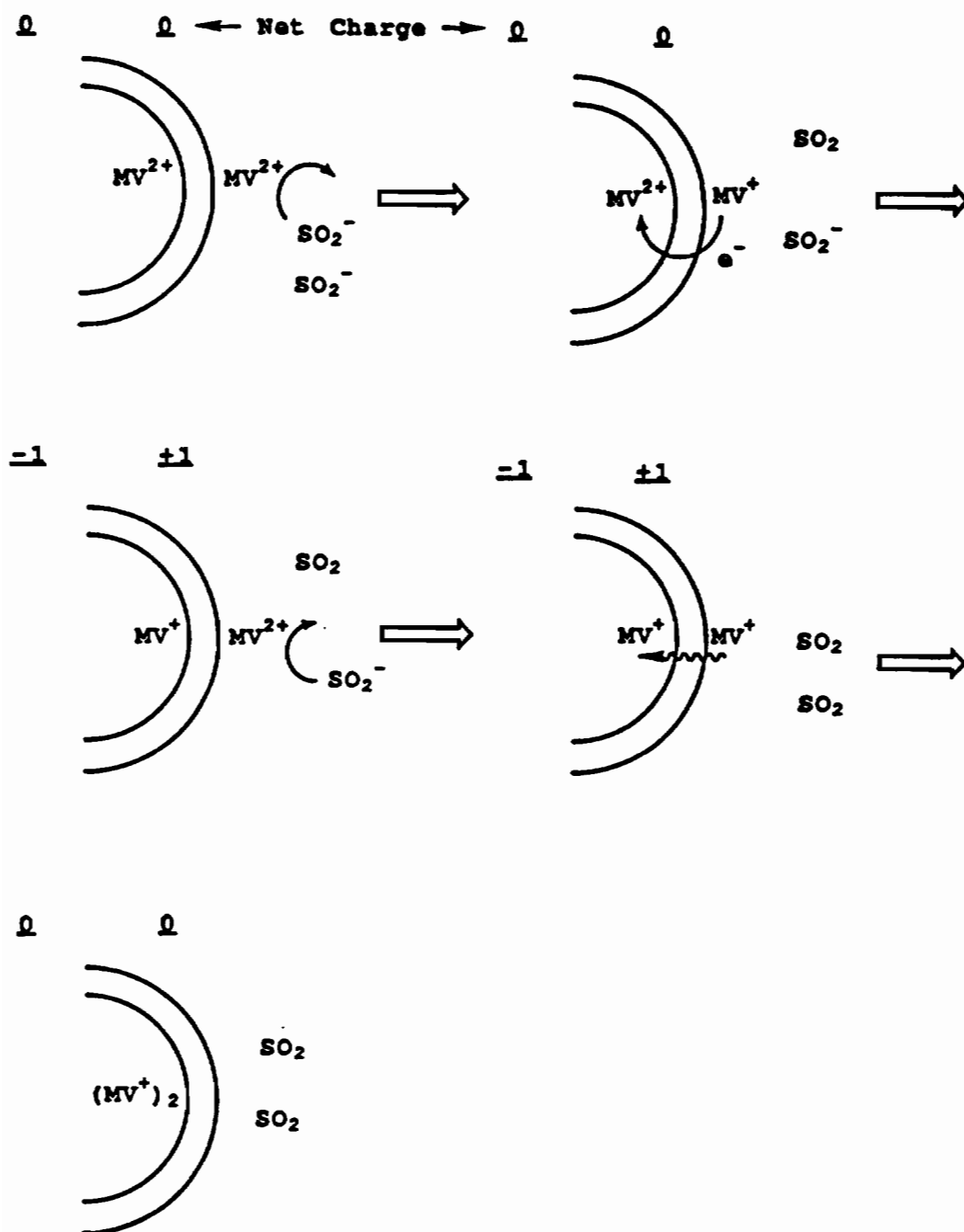


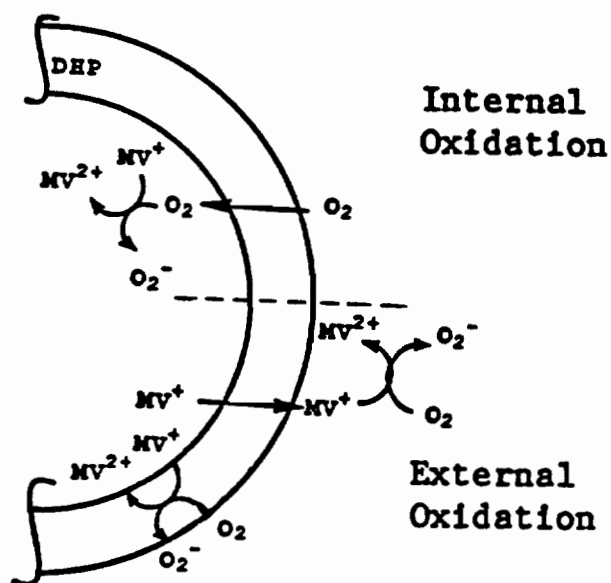
Figure 4-7: Schematic demonstration of MV^+ migration which is proposed to follow transmembrane electron transfer. Electroneutrality is restored at completion of entire process.

Table 4-10
Effect of $[S_2O_4^{2-}]$ on MV^{2+} Migration^a

Approximate $[S_2O_4^{2-}]$	$MV^{2+}:S_2O_4^{2-}$ molar ratio	% Migration ^b
50 μM	1:1	43%
100 μM	1:2	70%
250 μM	1:5	86%
500 μM	1:10	95%

^a 28 μM MV^{2+} inside, 24 μM MV^{2+} outside, 2 mM DHP, 20 mM Tris-HCl, pH 8.0.

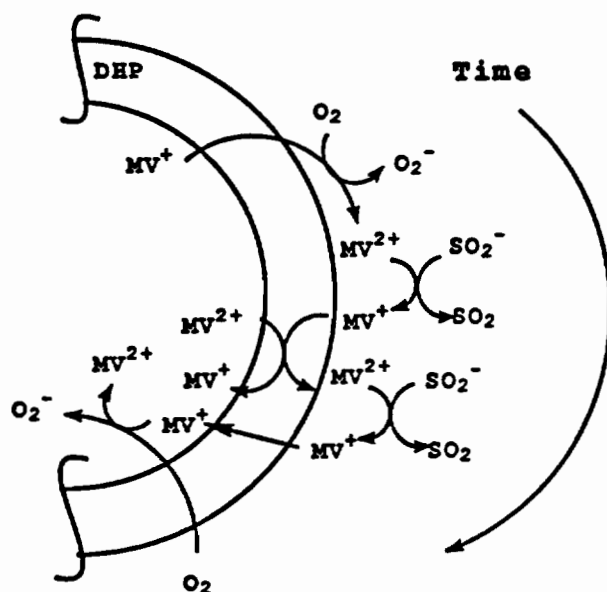
^b Percent migration defined in this section.



Scheme II

still an excess of dithionite ion in solution. Obviously some of the viologen being oxidized is inaccessible to the dithionite in bulk solution.

When a large excess of dithionite ion is in solution the outer MV^{2+} will be cycled back into the vesicle by the mechanism depicted in Figure



Scheme III

4-7. This sets up a cycle in and out of the vesicles (Scheme III). If this cycle was continued indefinitely all viologen radical molecules would eventually react with an O_2 molecule inside the vesicle and 100% of the MV^{2+} would be found inside. But since the supply of dithionite ion under these conditions is soon depleted this cycle never attains completion, but larger percentages of viologen are found inside after complete oxygenation as the initial dithionite concentration is increased.

To ensure that the phenomena of stoichiometric electron transfer and dimerization were independent of the reductant identity, $Cr^{II}EDTA$ was used as an alternate reductant. Exactly the same results were obtained as with $S_2O_4^{2-}$ ion. The reduction stoichiometries, the dimerization of MV^+ , and the migration into the DHP vesicles were all observed. Being anionic and therefore repelled from the vesicle surface, $Cr^{II}EDTA$ also was not capable of reducing MV^{2+} entrapped inside DHP vesicles when viologen was absent from the outer surface.

Another interesting result came from the addition of anaerobic $Fe(CN)_6^{3-}$ solutions to a reduced system which originally contained equimolar MV^{2+} inside and outside of DHP vesicles. Upon reduction most of the reduced viologen is inside the vesicles in the form of viologen dimers. Upon addition of excess ferricyanide the viologen in the system was oxidized with a reaction half-life of approximately 5 seconds. This rate is much slower than the oxidation of MV^+ or $C_{16}MV^+$ located outside DHP vesicles by $Fe(CN)_6^{3-}$ which occurred in less than 100 ms under similar conditions. Since earlier experiments had shown that $Fe(CN)_6^{3-}$ does not traverse the bilayer, it must be presumed that MV^+ oxidation is

confined to the outer vesicle surface. This reaction represents outward vectorial electron transfer. After complete oxidation, half of the viologen radical located inside the vesicle translocated to the outer surface. In effect, the original MV^{2+} distribution had been restored. If we assume that this mechanism is analogous to the reduction step shown in Figure 4-7, but in the opposite direction, we can then account for the viologen distribution. This mechanism is illustrated in Figure 4-8 for a single viologen dimer.

If a reduced system with MV^+ dimer inside of DHP vesicles was slowly titrated with $Fe(CN)_6^{3-}$ there was a progressive increase in the percentage of viologen radical monomer accompanying the loss in total viologen radical concentration. Apparently ferricyanide was able to react with some of the internal MV^+ , thereby pulling some MV^+ out of the vesicles by the mechanism shown in Figure 4-8. Since under these conditions there was no excess of ferricyanide ion in the bulk solution, this viologen would remain reduced on the external surface. This outer viologen radical then accounted for the increase in percent monomer which was being observed spectroscopically.

The exact mechanisms of the electron exchange reaction across the bilayer for both dithionite reduction and ferricyanide oxidation are still unknown. We cannot differentiate between transverse diffusion of reactants and electron tunneling as the reaction mechanism. This topic will be discussed further in Chapter 5. The most important conclusion of these experiments is that redistribution of viologen ions in these systems occurs in response to the transmembrane electrical potential generated by net transmembrane oxidation-reduction. Since the viologen

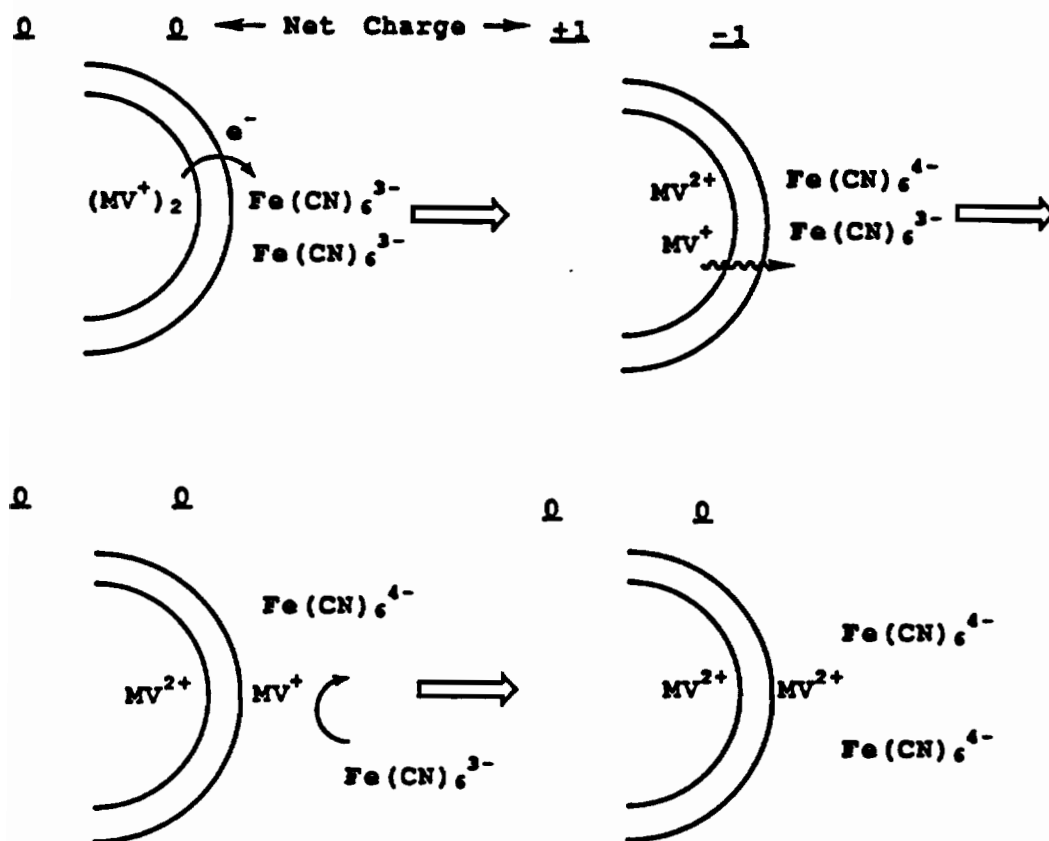


Figure 4-8: Schematic demonstration of outward MV^+ migration proposed to accompany oxidation by external ferricyanide. Electroneutrality is restored upon completion of the mechanism.

radicals are the only ions in the system which can readily permeate the bilayer, they are the only ions which can dissipate the electrical gradient. Additional proof of this concept is provided from studies on the behavior of systems which also contain additional lipophilic ions, as will be discussed in the next section.

Additional data which can be explained in terms of these concepts includes the reduction, oxygenation, and re-reduction of DHP systems with MV^{2+} localized on the outer vesicle surfaces. In this type of system there was no electron acceptor inside of the vesicles; therefore no electrical polarization of the membrane could occur and no viologen migration was observed. Similarly, as previously described, a system containing equimolar MV^{2+} inside and outside of DHP vesicles could be titrated with dithionite ion. If only sufficient dithionite was added to reduce the outer MV^{2+} , and then the system was oxygenated and re-reduced, no viologen migration was observed, again because no transmembrane electron transfer had occurred. If a similar system was completely reduced and then partially oxidized with $Fe(CN)_6^{3-}$ the addition of an excess of dithionite would regenerate the original MV^+ dimer spectrum. In this case, electrons withdrawn by oxidation of internal MV^+ by external ferricyanide were subsequently returned by the external dithionite, so the overall effect was no net electron transfer and the final distribution of MV^+ did not change.

All of the systems discussed so far had an excess or equal concentration of viologen inside the vesicles. This allows translocation to take place to completion. If external viologen was in excess, only an amount of electrons equivalent to the moles of internal

MV^{2+} could be transferred into the vesicles. Any stoichiometric excess of viologen on the outer surface had no available internal electron acceptor and therefore could not be transported inwardly. For example, a system with 20 μM MV^{2+} inside and 40 μM MV^{2+} outside could at best achieve 50% migration; i.e. 20 μM of the outer viologen could translocate. Some sample data for one of these types of systems as well as with $MV^{2+}_{in} > MV^{2+}_{out}$ are listed in Table 4-11.

The first thing to note from these results is that a larger-than-expected percent migration can be achieved with high dithionite concentrations. This effect can be explained by realizing that during oxygenation the internal viologen radicals are being oxidized while dithionite ion still exists in bulk solution. This provides new electron acceptors for the external viologen radicals which leads to their subsequent inward migration. Notice that the total yield of MV^{2+} after the oxygenation step also decreased somewhat with increasing dithionite concentration. This is due to viologen degradation as discussed in section II.a. Since the viologen redistribution occurring during oxygenation is dithionite dependent, it is difficult to draw conclusions about the viologen distribution following reduction from the final distribution of the oxidized system. A better gauge of the MV^+ distribution following reduction is the percentage of dimer in the system. The viologen radical inside the vesicles is expected to be about 80% dimer as determined from a similar system with approximately equimolar concentrations of internal and external MV^{2+} and the external viologen should approach 100% monomer at these concentration levels. Therefore from the overall percentage of dimer in the system we should

Table 4-11
Physical Properties Describing MV²⁺ Distribution on DHP
Vesicles After Dithionite Reduction^a

<u>Starting Distribution</u>		Molar excess of S ₂ O ₄ ²⁻	<u>Final Distribution^b</u>		% Dimer after red. [MV ⁺]	Total [MV ⁺]
In	Out		In	Out		
31 uM	59 uM	1-fold	55 uM	35 uM	48%	90 uM
"	"	5-fold	72 uM	10 uM	48%	90 uM
"	"	10-fold	73 uM	6 uM	48%	90 uM
31 uM	16 uM	5-fold	43 uM	4 uM	59%	31 uM
31 uM	22 uM	5-fold	46 uM	3 uM	65%	43 uM
28 uM	25 uM	5-fold	49 uM	3 uM	82%	46 uM

^a Conditions: Approximately 3 mM DHP, 20 mM Tris-HCl, pH 8.0.

^b Calculated distribution after reduction, 5 minute incubation, and subsequent oxygenation.

be able to estimate the viologen distribution from Equation 4-12.

$$\% MV^+ \text{ Inside} = \% \text{ Dimer} / 0.80 \quad (4-12)$$

If Equation 4-12 is applied to the first entry in Table 4-11 the calculated MV^+ distribution indicates 60% of the total viologen radical inside of the vesicles, compared to the predicted value of 69% if a concentration of outer viologen equal to the inner viologen concentration translocated quantitatively according to the proposed model.

We have found that MV^{2+} is more efficient than the longer alkyl chain viologens in mediating transmembrane electron transfer and in translocating into DHP vesicles. The reason for this may not be due to the actual electron transfer event but rather the required ion movement which must follow; i.e. MV^+ is a more efficient ionophore than the longer chain analogs. Some evidence for this will be presented in the next section. Either way, the end result is that much lower percentages of migration are observed in systems with longer alkyl chain viologens. For example, an equilibrium distribution of $C_{16}MV^{2+}$ on DHP vesicles would have approximately one-third of the total viologen localized on the inner surface. This distribution is similar to that for the first MV^{2+} vesicle system listed in Table 4-11. However, four reduction-oxygenation cycles with a 10-fold molar excess of dithionite were required to achieve the same percentage of translocation in the $C_{16}MV^{2+}$ system as was observed after one such cycle in the MV^{2+} system.

III.d) Effect of Ionophores on Viologen Migration: It was hypothesized in the previous section that viologen migration is dictated by transmembrane electrical potential gradients. It follows that if this gradient is partially or completely dissipated by some other mechanism before the viologens can respond, the extent of redistribution of viologen molecules across the membrane would be altered. This perturbation can be accomplished by adding ionophores, i.e., lipophilic ions capable of traversing the bilayer membrane, to the system. The results from these types of experiments support the hypotheses that have been advanced.

Potential ionophores were first checked for efficiency in DHP systems. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) are well-known uncouplers of oxidative phosphorylation in biological systems and function as protonophores⁽⁹⁾. The functioning of these protonophores was checked by preparing vesicles containing a high internal buffer concentration in a low buffer concentration medium. Vesicles were prepared in 50 mM Tris buffer, then gel chromatographed using 2 mM Tris buffer eluent. The external pH was jumped from about 8 to 6 with 0.1M HCl and the relaxation curves were monitored with a combination pH electrode. The relaxation had a Δ pH of about 0.2 pH units and took about 5 minutes to equilibrate. Neither CCCP nor DNP showed appreciable enhancement of these relaxation rates.

The ability of valinomycin to transport potassium ion across DHP bilayers was also tested. Vesicles were prepared in 20 mM Tris buffer containing 20 mM KCl. These were chromatographed on a Sephadex G-50 Fine column using 20 mM Tris buffer as the eluent. A potassium ion

sensing electrode (Orion model 93-19) was then used to monitor potassium leakage out of the vesicles. It was found that the addition of small aliquots of a methanol solution of valinomycin had no more effect on the potassium ion leakage rate than the addition of methanol alone.

Apparently the valinomycin was not incorporated properly into the bilayer, since if the valinomycin was added to the vesicles in the latter stages of the sonication procedure complete leakage of K^+ was observed during the residence time on the column. Thus valinomycin appeared to function properly if incorporated with sonication.

However, a functional ionophore was needed that could simply be added to the vesicle system at any time after the vesicles had been formed. Another lipophilic ion which has been used in biological studies is tetraphenylphosphonium ion (TPP^+)⁽⁹⁾. Based on its effect upon redox-initiated viologen redistribution this ion appeared to function as an ionophore in DHP vesicles. Also, since methyl viologen radical appeared to rapidly dissipate the electrical gradient in DHP vesicles, it seemed likely that the structurally similar 1-methyl-4,4'-bipyridinium ion (MMB^+) would also function as an ionophore in these systems.

Experiments were done with TPP^+ to determine its effect on systems with MV^{2+} localized in an equimolar distribution on both vesicle surfaces. All experiments were made in 20 mM Tris-HCl buffer at pH 8.0. As is typical of these systems, dithionite addition resulted in reduction of 90% of the viologen in the system and transfer of 86% of the outer viologen into the vesicles after oxygenation and re-reduction. When TPP^+ was added externally in a concentration equal to the total

viologen concentration, dithionite reduction yielded 100% viologen radical and only about 50% migration. The radical spectrum in this case also showed a considerably higher percentage of viologen monomer than the analogous system without TPP^+ . These results can be explained by hypothesizing that the TPP^+ on the outer surface of the vesicles competes with MV^+ to dissipate the electrical gradient developed by the transmembrane electron transfer event. Thus TPP^+ can migrate into the vesicles in place of MV^+ . Therefore, some of the MV^+ remained on the outer vesicle surface, accounting for its lower percent translocation and increased percentage in the monomeric form. The incomplete reduction in this system in the absence of TPP^+ might be due to the developing MV^+ concentration gradient which opposes the charge gradient that develops from the electron transfer step. Thus electron transfer ceased without reaching completion. In the presence of the ionophore, TPP^+ can migrate inwardly to dissipate the electrical gradient allowing the MV^+ concentration gradient to also dissipate. The intrinsic permeability of TPP^+ appeared to be lower than MV^+ . The effects of TPP^+ usually were not apparent within the first minute of the reaction. It was only after 5 or 10 minutes that increased reduction yields were observable. This permeability difference is reasonable since TPP^+ is larger and bulkier than MV^+ .

The difference in diffusion rates is most evident in experiments involving oxidation of internally bound MV^+ by $\text{Fe}(\text{CN})_6^{3-}$ in the external solution. Unlike systems involved with reduction of equimolar MV^{2+} inside and outside of vesicles, once this reaction is completed no further redistribution of MV^{2+} is expected because of the inability of

MV^{2+} to cross the DHP bilayer. In these oxidative reactions the effects of TPP^+ are very minimal. This is because these reactions are usually completed within one to two minutes and on that timescale MV^+ is better suited to react to the electrical gradient formed (see Figure 4-8). If the ionophore was able to diffuse across the membrane at a rate which was faster than that for MV^+ we would expect to see more MV^{2+} remaining inside the vesicles at the end of the reaction when the ionophore was present. Alternatively, since TPP^+ is added to the external vesicle surface, it may not be able to effectively neutralize an electrical gradient which is negative outside. The membrane is polarized in the opposite sense during the reduction reactions, so TPP^+ is expected to be more useful in these cases. TPP^+ was completely removed by cation exchange chromatography and therefore could not be entrapped inside.

More extensive experiments were done using the ionophore MMB^+ . It had a profound effect on viologen distribution in many different systems and appeared to react more quickly to dissipate electron gradients than did TPP^+ . One property that MMB^+ affected was viologen dimerization. As hypothesized earlier, the formation of viologen dimer is caused by the localization of viologen radicals in the confines of the vesicle interior. At the end of the reduction reaction there exists a large viologen radical concentration gradient directed outwardly. Since there are no charge-compensating ions in the system, the radical cannot leak out of the vesicles down this gradient. In the time-course spectra shown in Figure 4-9 we see the formation of MV^+ monomer and its subsequent conversion to viologen radical dimer accompanying transmembrane reduction, which is then invariant with time; i.e. there

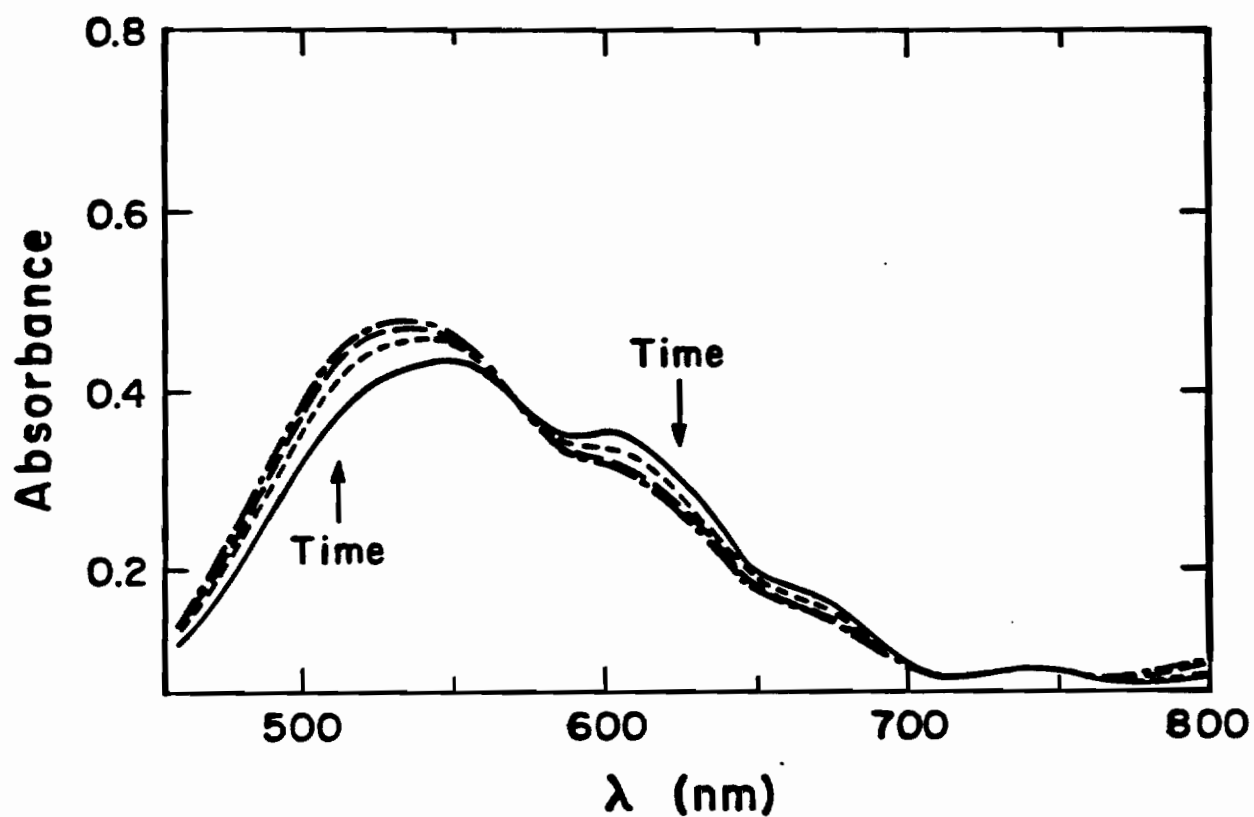


Figure 4-9: Dithionite-reduction spectra for a DHP vesicle system containing 42 μM MV^{2+} inside and 26 μM MV^{2+} outside. Spectra taken at 2, 5, 10, and 20 minutes after reduction. 20 μM Tris-HCl, pH 8.0.

was no conversion of dimer to monomer over this time period. This implies no leakage of MV^+ out of the vesicle since MV^+ on the outer vesicle surface would be predominantly monomeric. If the vesicles were prepared in imidazole buffer the results were different. Imidazole is known to act as a lipophilic ion⁽⁴⁰⁾. The time-course spectra in Figure 4-10 show that after the initial formation of viologen dimer there is a slow shift toward monomer over time. This indicates that there was a gradual leakage of MV^+ down its concentration gradient until an equilibrium distribution of MV^+ inside and outside of the vesicles was reached. After approximately 20 minutes no further changes in the monomer-dimer equilibrium took place. Using Equation 4-12 we can calculate that equilibrium occurred after just over half of the viologen radical had leaked out of the vesicles.

It was expected that the addition of MMB^+ would speed up the rate of the MV^+ leakage out of the vesicles and thus the attainment of an equilibrium percentage of viologen dimer. In 75 mM imidazole-HCl buffer the time for equilibration went from about 20 minutes in the absence of MMB^+ to less than 5 minutes in the presence of 100 μM MMB^+ . In 20 mM Tris-HCl buffer where this equilibration time was normally on the order of tens of hours it took about 10 minutes in the presence of 200 μM MMB^+ . The results in imidazole buffer imply an initial MV^+ leakage rate of about 15 μM per minute when starting with approximately 80 μM MV^+ inside. That MV^+ was actually leaking out was confirmed by showing that the percent migration in these systems dramatically declined as the percentage of monomer increased; e.g. a MV^{2+} system which showed >90% translocation of outer MV^+ into DHP vesicles in the absence of MMB^+

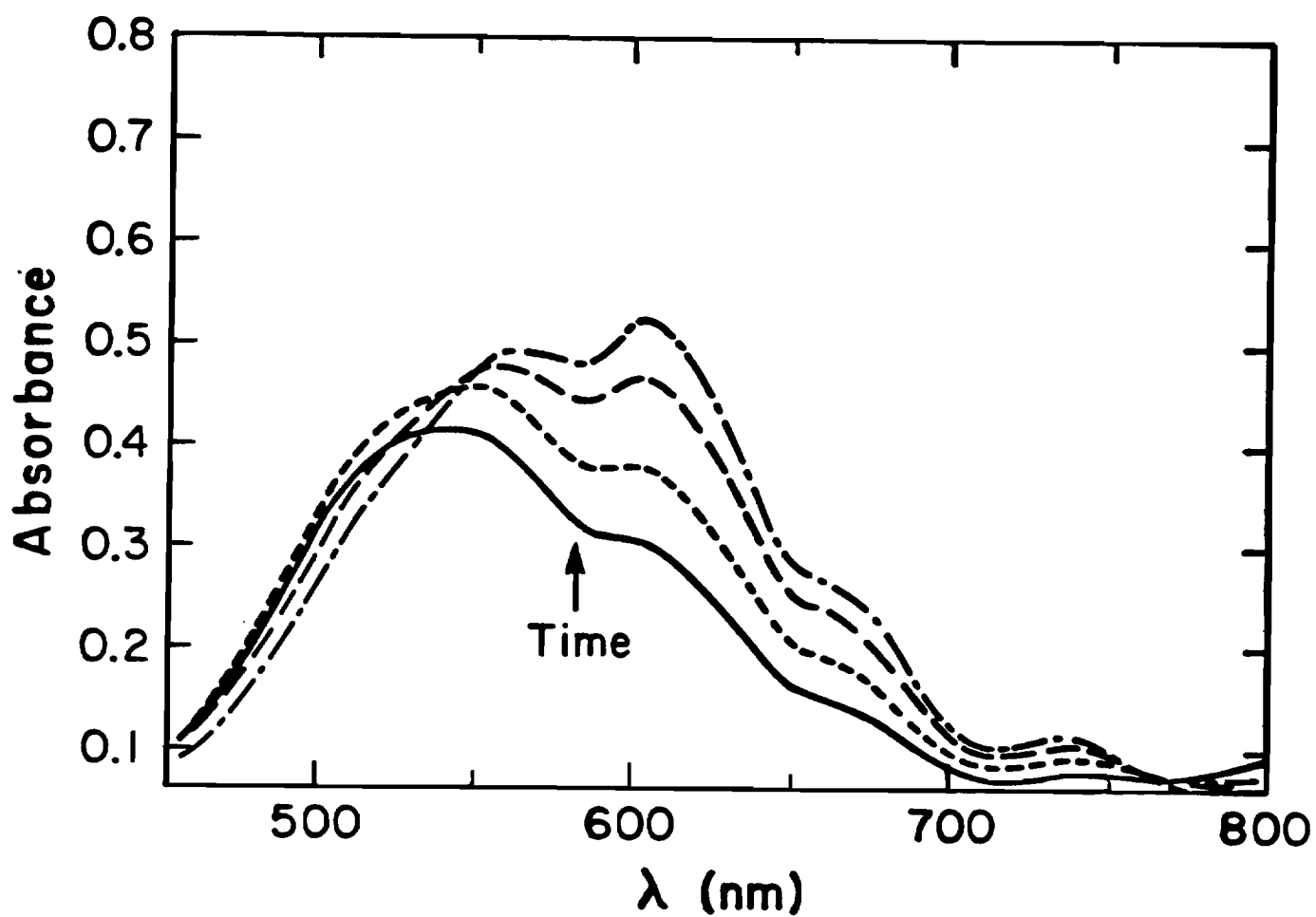


Figure 4-10: Dithionite-reduction spectra for a DHP vesicle system containing 34 μM MV^{2+} inside and 25 μM MV^{2+} outside. Spectra taken at 2, 5, 10, and 20 minutes after reduction. 75 mM imidazole-HCl, pH 7.5.

would have less than 50% migration in the presence of a two-fold molar excess of MMB^+ after monomer/dimer equilibrium was attained.

The fact that MMB^+ promoted leakage of MV^+ out of the vesicles implied that it would be possible to achieve values for α which were greater than one in certain systems. A system was prepared in 20 mM Tris-HCl buffer with 32 μM MV^{2+} inside and 22 μM MV^{2+} outside of DHP vesicles. In the absence of ionophores, dithionite reduction of this system resulted in the formation of 42 μM MV^+ which gives an α value of 0.91. This is very close to the expected α value of 1.0. If 50 μM MMB^+ was added to this system, complete reduction of all of the viologen was observed after the addition of excess dithionite, giving an α value of 1.45. This result is explained by proposing that MV^+ is continually leaking out of the vesicles and mediating further electron transfer. Its migration into the vesicle does not preclude its ability to participate further in the electron transfer process since it is now able to come back out of the vesicle. This implies that only catalytic amounts of MV^{2+} would be necessary on the outer vesicle surface to mediate the reduction of large quantities of internal MV^{2+} provided that enough ionophore was present to compensate for the number of electrons transferred inward. Thus very large values of α could be observed.

This effect was seen when MMB^+ was added to DHP vesicles containing MV^{2+} only on the inner vesicle surface. The presence of the ionophore did not cause any leakage of MV^{2+} out over periods as long as 30 minutes. This was, of course, also the case in the absence of ionophores. The difference occurred after the addition of excess dithionite ion. In the absence of MMB^+ less than one percent reduction

of MV^{2+} took place over a 10 minute period. If a two-fold molar excess of MMB^+ was added before the addition of dithionite ion, reduction of MV^{2+} occurred almost completely over a 10 minute period (Figure 4-11). The shape of the reduction curve was that of an autocatalytic reaction⁽⁴¹⁾. This can be explained by proposing a cycling of MV^+ across the membrane which builds on itself. After one molecule is reduced on the outer vesicle surface the electron transfer event proposed in Figure 4-6 can occur resulting in two MV^+ inside the vesicle. But these can now freely leak out because of the presence of the ionophore. This sets up a 2^n progression as the number of MV^+ molecules available on the outer surface for electron mediation essentially doubles with each cycle. Thus the small amount of MV^{2+} which initially leaks out or that was not removed by chromatography catalyzes the reduction of all of the viologen in the system.

The data presented so far have not given any information on the nature of the rate-limiting step in the electron transfer reaction. Referring to Figure 4-6 we can assume it is either the actual electron exchange step (k_2) or the migration of the compensating charged ion (k_3). In the absence of ionophores this ion is a viologen radical. Kinetic arguments tell us that if the k_2 step is rate-limiting the electron transfer step should be second-order. If it is the diffusion of ions we would expect the overall process to be first-order in the absence of ionophores. These types of arguments will be discussed in the next section and tend to support the following data.

The rate of transmembrane electron transfer was studied as a function of MMB^+ concentration. If counterion flow was the rate-

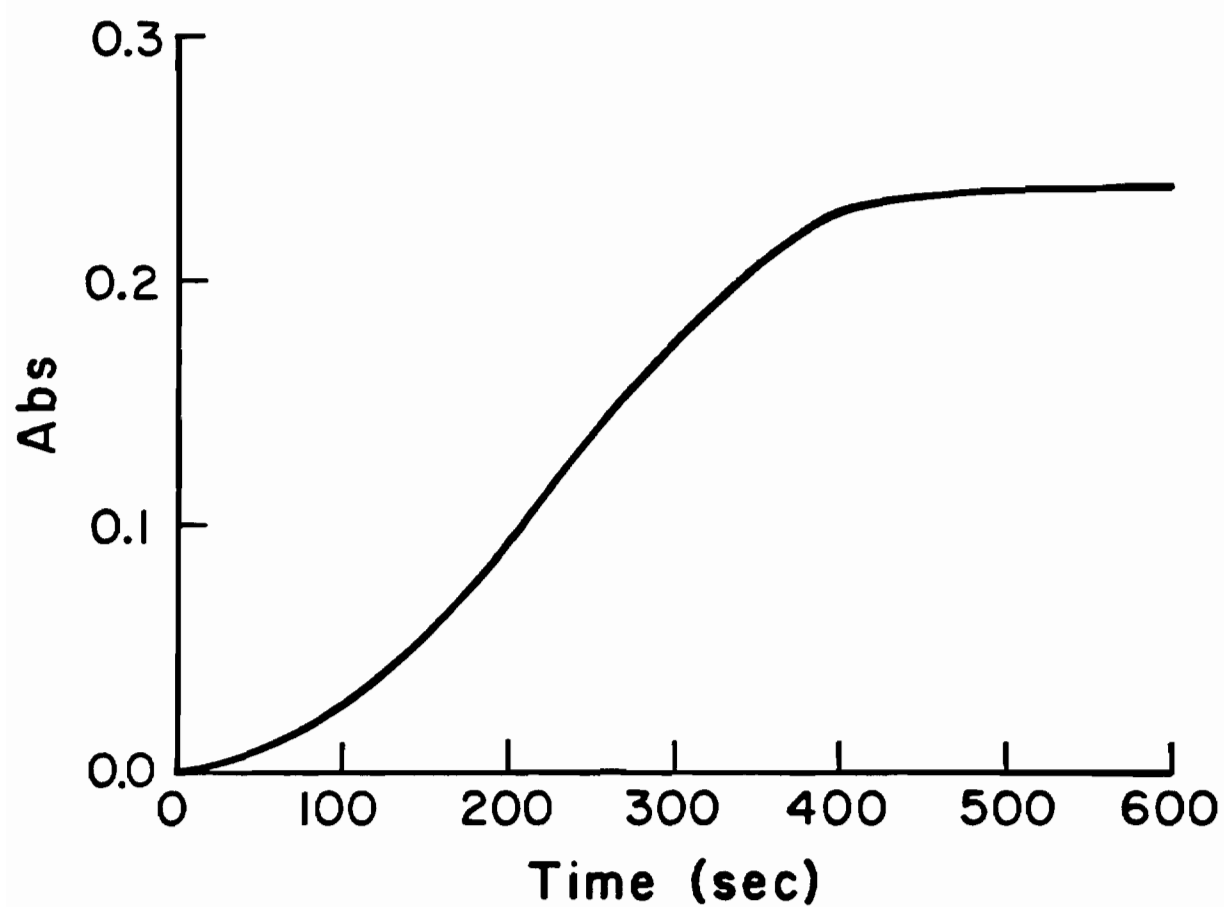


Figure 4-11: Dithionite reduction of 2 mM DHP vesicle system containing 25 μM internal MV^{2+} and 50 μM external MMB^{+} . 20 mM Tris-HCl, pH 8.0. Monitored at 546 nm.

limiting factor in electron transfer the rates would increase significantly with increasing MMB^+ concentration. DHP vesicles were prepared in both 20 mM Tris and 75 mM imidazole buffers with approximately 100 μM MV^{2+} equally distributed inside and outside. In the case of imidazole buffer the time for completion of the reaction shortened significantly with MMB^+ concentrations up to 100 μM . In the absence of MMB^+ the reaction was complete after about 10 minutes; with 100 μM MMB^+ added the reaction was complete after only 1.5 minutes, but the initial reduction rates of internal MV^{2+} did not change. This was probably due to the catalytic mechanism just discussed. Because MMB^+ was present, large quantities of MV^+ were leaking out during the reaction and thus rapidly mediating electron transfer to the remaining MV^{2+} inside. This conclusion is supported by the observation that the product spectrum started to shift toward higher percentages of monomer immediately after reduction when MMB^+ was present.

The results in Tris buffer were essentially the same except for one complicating factor. It appeared that in this system the MMB^+ was affecting the spectral characteristics of the viologen radical dimer. The isosbestic point at 546 nm no longer gave the correct viologen radical concentration but was overestimating it. Possibly the MMB^+ itself was dimerizing with the viologen radical. MMB^+ had no effect whatsoever on viologen radical monomer spectra in buffer.

Vesicle systems were also prepared in 20 mM Tris and 75 mM imidazole buffers with equilibrium distributions of $\text{C}_{16}\text{MV}^{2+}$ inside and outside. As already discussed in section III.b the addition of dithionite ion to these systems resulted in less than 100% reduction

yields in Tris buffer. However, it was observed that 100% reduction was obtained in the presence of MMB^+ or if the vesicles were prepared in imidazole buffer. This implies that transmembrane electron transfer is being restricted by the buildup of a potential gradient in the absence of lipophilic ions. Apparently $\text{C}_{16}\text{MV}^{2+}$ is not very effective at dissipating this gradient.

Experiments were done to see how the reduction rates would be affected by the MMB^+ concentration in systems with $\text{C}_{16}\text{MV}^{2+}$ localized at both vesicle surfaces. In Tris buffer, similar to the MV^{2+} system, the initial rate of reduction was not significantly affected by increasing concentrations of MMB^+ . The major difference was that the magnitude of the reduction curve was greater since all of the viologen was now being reduced. In imidazole buffer the rate was not significantly affected by the addition of MMB^+ . This is not surprising since, as mentioned earlier, imidazole can function as an ionophore. Apparently it is sufficiently rapid to dissipate any potential generated by the slow intrinsic electron transfer rate of these systems.

These results imply that, at least for the initial part of the transmembrane step, the measured electron transfer rates were limited by the rate of actual electron transfer and not by the rate of counterion diffusion. Other factors besides the concentration of lipophilic ions in the system would have to be changed in order to significantly speed up electron transfer.

The transmembrane electron transfer properties of other viologens were also studied. One of these was 1-1''-propanediylbis(1'-methyl-4,4'-bipyridinium) (PMB^{4+}). This diviologen could not be removed from

the vesicle surface by chromatography so only equilibrium distributions could be studied. When dithionite ion was added to such a system only about 65% of the total PMB^{4+} was reduced. This is about the amount of viologen expected to be on the outer surface of vesicles with an equilibrium distribution and implies that no transmembrane electron transfer has occurred. This conclusion is also supported by the fact that after oxygenation and re-reduction the same amount of radical is formed, implying that no viologen migration had taken place. If MV^{2+} was added to this system before reduction it was able to mediate electron transfer to internal PMB^{4+} with the total number of electrons transferred equal to the initial concentration of added MV^{2+} . After oxygenation and re-reduction it was found that most of the MV^{2+} was inside the vesicles and only PMB^{4+} which was outside in the original system was reduced. If MV^{2+} was entrapped inside of DHP vesicles and PMB^{4+} was added to the outer surface, no transmembrane redox or viologen translocation was observed. If the ionophore MMB^+ was added to this system in a 4-fold molar excess, complete reduction was observed via a zero-order reduction process. Apparently reduced PMB^{4+} is not capable of traversing the bilayer membrane. Since PMB^{2+} is almost structurally identical to MV^+ dimer⁽⁴²⁾, we can conclude that MV^+ dimer probably does not readily diffuse across the bilayer membrane either. This same conclusion was reached in a study of the formation of MV^+ by CdS colloids in DHP vesicles⁽⁴³⁾.

Another viologen studied was 1-methyl-1'-sulfonato-n-propyl-4,4'-bipyridinium (MSPB^+). Equilibrium dialysis showed almost no binding of MSPB^+ to DHP vesicles; consequently entrapment within DHP vesicles was

negligible. To check for possible MSPB^+ -mediated transmembrane oxidation-reduction a system was set up in 20 mM Tris-HCl at pH 8.0 with equal concentrations of MV^{2+} inside and MSPB^+ outside. When this system was reduced with dithionite, only about 20% of the inner MV^{2+} was reduced. However, if the ionophore MMB^+ was added at a concentration equal to the total viologen concentration, all of the viologen in the system was reduced.

This result can again be explained by the electrogenic nature of transmembrane electron transfer. Although the MSPB^0 molecule carries no net charge and therefore creates no transmembrane electrical potential by traversing the membrane, the reduction of inner MV^{2+} represents transfer of an electron into the vesicle. This causes polarization of the vesicle with excess negative charge inside. This polarization impedes further inward transfer of electrons. Since the MSPB^0 radical is neutral it cannot dissipate this potential gradient, so net electron transfer stops. But, if an ionophore is present which can alleviate the buildup of charge, then transmembrane electron transfer can continue unrestricted. This was the case when MMB^+ was added to the system. Therefore MSPB^+ is capable of mediating transmembrane electron transfer but, unlike MV^{2+} , is not capable of dissipating the electrical gradient. Since it is neutral when reduced, MSPB^0 is not affected by the membrane polarization and therefore is not drawn into the vesicles. The fact that the reduction curve did not show the autocatalytic shape characteristic of systems with MV^{2+} inside and MMB^+ outside proves that the MSPB^+ radical participated in the transmembrane electron transfer step. The curve shape was now similar to that for MV^{2+} -mediated

transmembrane oxidation-reduction (Figure 4-12). Complete reduction was observed within two minutes which is also significantly faster than the autocatalytic system. This system is unique because transmembrane electron transfer can be modulated by regulating the ion flow and is discussed more completely in section IV.d in this chapter.

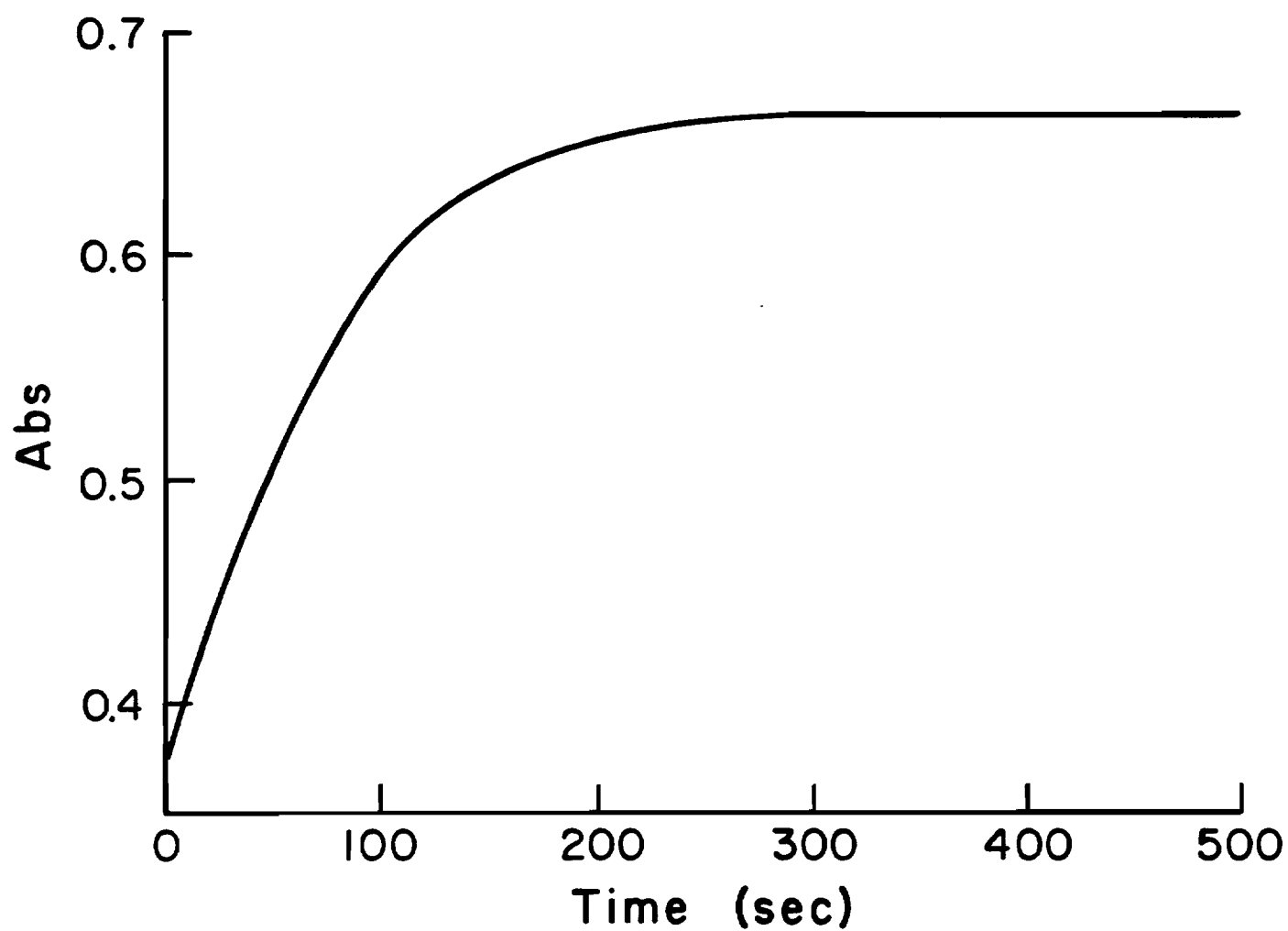


Figure 4-12: Dithionite reduction of 2 mM DHP vesicle system containing 36 μM internal MV^{2+} and 31 μM external MSPB^+ in the presence of approximately 100 μM external MMB^+ . 20 mM Tris-HCl, pH 8.0. Monitored at 546 nm.

III.e) Kinetics: The kinetics of viologen reduction by dithionite ion were studied with alkyl viologens distributed on both the inner and outer surfaces of DHP vesicles. Systems utilizing MV^{2+} and C_6MV^{2+} were prepared with equal quantities of the viologen inside and outside of the vesicles. This turned out to simplify the kinetic analyses. Systems prepared with $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ had to be used with their equilibrium distributions since chromatography would not remove these viologens from the outer vesicle surface. The equilibrium distribution for these viologens is expected to be approximately one-third inside and two-thirds outside. Viologen reduction in all of these systems was expected to consist of two distinct steps. The first would be the reduction of the outer viologen at approximately the same pseudo-first-order rate as was found in the systems containing only externally bound viologen (Figure 1-2). The second, possibly much slower, step would then be the reduction of the inner viologen. If this was kinetically distinct it could then be studied independently of the outer reduction step and its reaction order could be determined. For the most part it proved possible to temporally separate these steps.

The first system studied was MV^{2+} equally distributed between the inner and outer surfaces of DHP vesicles prepared in 20 mM Tris, pH 8.0. Two distinct kinetic steps were observed upon reduction by a large stoichiometric excess of dithionite ion. The first step was very rapid and was essentially complete after a few hundred milliseconds. The calculated second-order rate constant, k_1 , was consistent with that for reduction of outer bound MV^{2+} . The average calculated value for k_1 was $1.6 \pm 0.4 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$.

The slower reduction step could not be fit by one or two single exponential functions implying that it was not a first-order or pseudo-first-order process. However the slow step fit well to a simple second-order function as described in Chapter 3. The kinetic results are given in Table 4-12. The rate was independent of dithionite concentration as would be expected for entrapped viologen which is inaccessible to dithionite ion in solution. This implies that the externally localized reduced viologen transferred the electron to the inner viologen dication.

One thing to note about the transmembrane rate constants (k_2) for these systems is that the units are not really appropriate for the reactions taking place. The rates are controlled not by the bulk concentration of viologen but by the surface concentration on the vesicles. Therefore, for a given viologen concentration we would expect the rate constants to decrease as the DHP concentration increases. The rate constant for the interfacial outer reduction (k_1) is aided by lower charge repulsion between reactants at higher surface concentrations of viologen and is therefore also expected to increase with decreasing DHP/ C_pMV^{2+} ratios. These effects, particularly for the transmembrane step, can be observed in the data in Table 4-12 where the average value for k_2 was $1.4 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ when the DHP concentration was approximately 1 mM but was only $1.1 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ when the DHP concentration was about 2 mM.

The set of reactions thought to take place in this system have already been illustrated schematically in Figure 4-6. There are several pieces of evidence in the kinetic data that support this hypothesis.

Table 4-12

Kinetics of Dithionite Reduction of MV^{2+}
Inside/Outside of DHP Vesicles^a.

Total $[MV^{2+}]$ (μM)	Approx. [DHP] (mM)	$[S_2O_4^{2-}]$ (mM)	$10^{-7} \times k_1$ ($M^{-1}s^{-1}$)	$10^{-4} \times k_2$ ($M^{-1}s^{-1}$)
25	1	0.28	1.3	1.3
39	1	0.41	0.7	1.9
61	1	0.60	2.1	1.2
70	1	0.70	1.6	1.1
81	1	0.80	2.0	1.6
53	2	0.50	1.4	1.6
"	"	1.50	b	1.7
58	2	0.56	1.8	0.7
70	2	0.57	2.0	0.7
"	"	1.17	b	0.7
111	2	1.06	1.7	c
"	"	2.17	b	c
Average:			1.6+/-0.4	1.3+/-0.4

^a Approximately equal $[MV^{2+}]$ inside and outside, 20 mM Tris, pH 8.0.

^b Reaction too fast to accurately measure by this method.

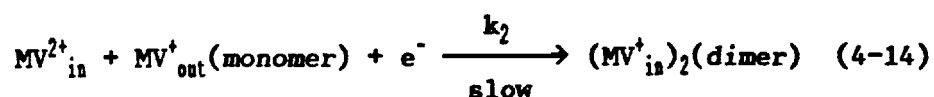
^c Slow step did not fit homogeneous second-order plot though still dithionite independent.

First, the rate constant for the fast reduction step was approximately the same as that for MV^{2+} bound only to the external interface of DHP vesicles and this process accounted for about 50% of the total reaction. This supports the idea that the initial step in the reaction is the rapid reduction of all of the MV^{2+} outside of the vesicles. The reduction of the rest of the MV^{2+} in the system, presumably the inner viologen, then occurs via a single second-order process which is independent of dithionite concentration. This implies that the transmembrane reaction is between two viologen molecules. We have hypothesized that it is net transmembrane redox between an inner MV^{2+} and an outer MV^+ . Rapid re-reduction of the newly formed MV^{2+} outside would lead to the apparent second-order kinetics.

The question also arises as to the rate of the charge compensating flow of MV^+ into the vesicles. There are two pieces of evidence that suggest that this is not rate-limiting. First, such a transmembrane diffusional process would be expected to show a pseudo-zero-order or first-order rate, which is not consistent with the observed second-order kinetics. Secondly, some information can be gained by monitoring the reaction at different wavelengths. We know that MV^{2+} reduced outside of DHP vesicles results in a uv-visible spectrum characteristic of MV^+ monomer, but the product spectrum for the complete reduction of systems with equimolar MV^{2+} inside and outside of DHP vesicles is characteristic of viologen dimer. This spectral property has been taken as an indication of the MV^+ being localized within the vesicles (section III.c).

An experiment was performed where the reduction of a single system was monitored at three different wavelengths. The voltage curve for the first wavelength is shown in Figure 4-13. This was monitored at 546 nm, which is the isosbestic point for MV^+ radicals in either the monomeric or dimeric forms (section II.b). This condition implies that the molar absorptivity of the dimer unit at this wavelength is twice that of the monomer. At this wavelength we are watching the reduction of MV^{2+} independently of the relative amounts of monomer or dimer being formed. The sharp breakpoint at about 200 msec in the curve indicates the completion of the outer reduction step and was calculated to represent about half of the total absorbance change.

The voltage curve in Figure 4-14 was taken at 575 nm where the molar absorptivities for MV^+ monomer and dimer are equivalent. In this case we can see that very little change in the percent transmittance takes place after 200 ms. This implies that after the initial reduction of the outer MV^{2+} to form MV^+ monomer the subsequent reduction of the inner MV^{2+} involves the direct formation of MV^+ dimer after the transfer of an electron. If dimer formation was slow relative to reduction of inner MV^{2+} the percent transmittance would increase with time as monomer converted to dimer instead of being invariant with time as observed. Thus the proposed reactions would be as follows.



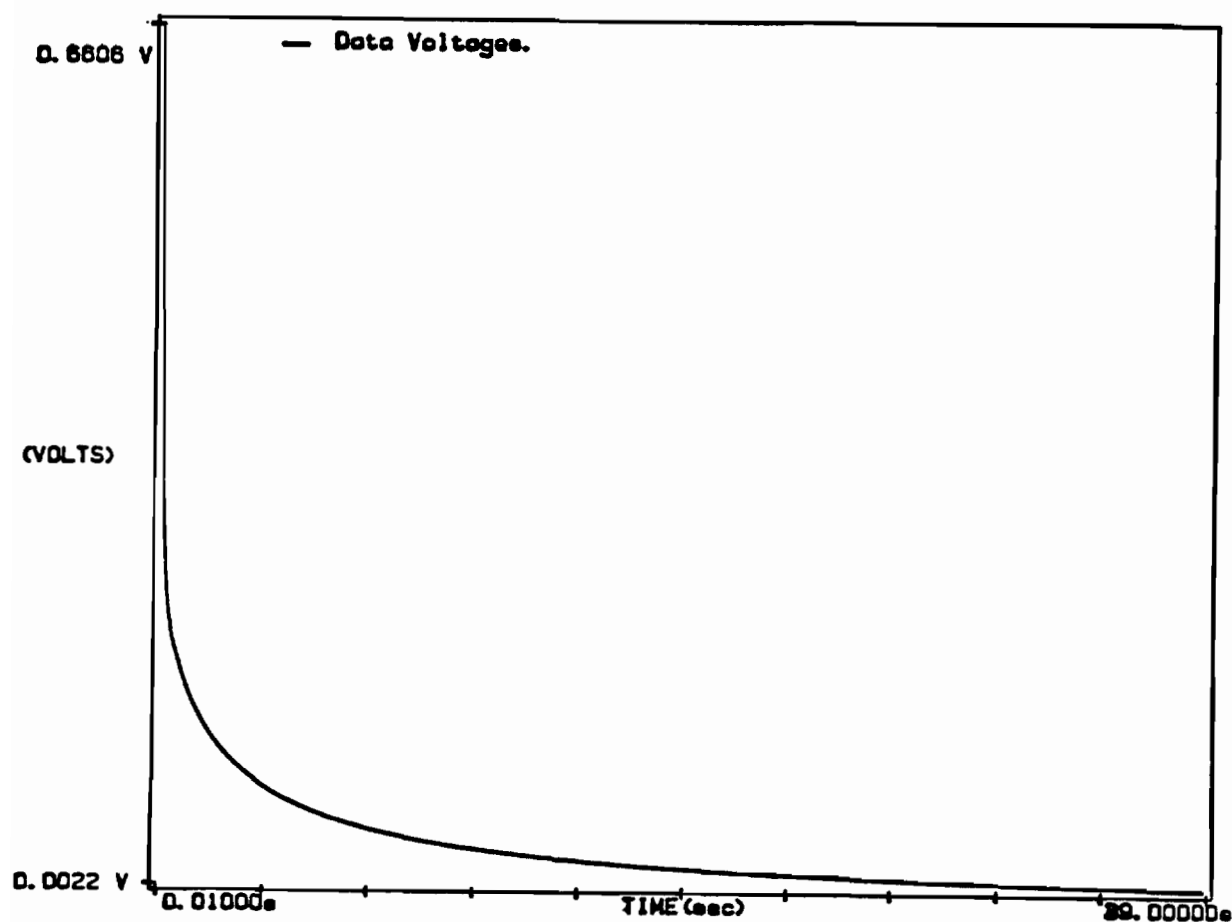


Figure 4-13: Voltage curve representing the change in percent transmittance at 546 nm for a DHP vesicle system with approximately equal concentrations of MV^{2+} inside and outside of the vesicles. Viologen radical chromophores in either the monomeric or dimeric form absorb equally at this wavelength.

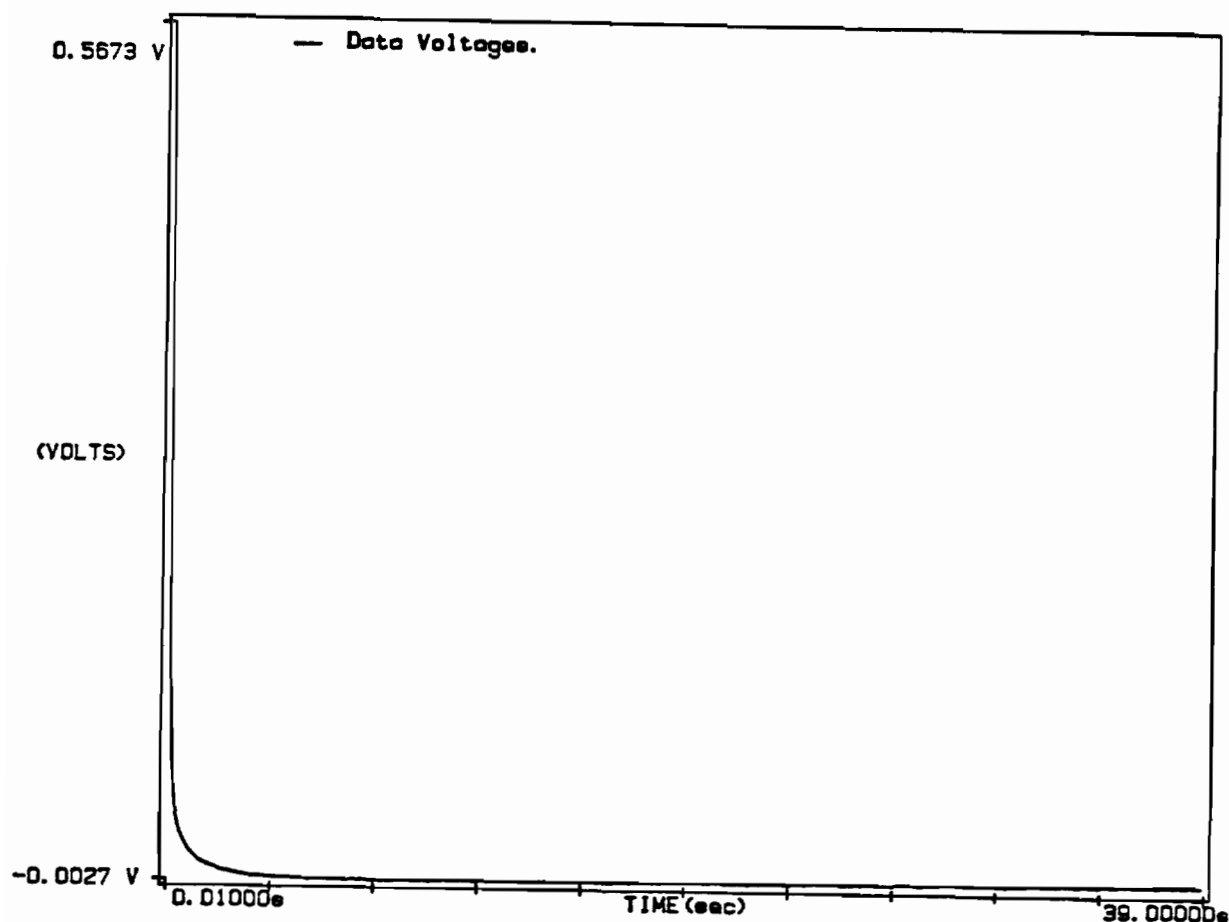


Figure 4-14: Voltage curve representing the change in percent transmittance at 575 nm for a DHP vesicle system with approximately equal concentrations of MV^{2+} inside and outside. Viologen radical chromophores in the monomeric state absorb twice as much as those in the dimeric state at this wavelength.

Dimerization occurs simultaneously with, or rapidly follows, the transmembrane redox step and is believed to proceed through the mechanism given in Figure 4-6. This observation implies that MV^+ migration into the vesicles is not rate-limiting; otherwise a transition of the reduced inner viologen from monomer to dimer would be observed as the outer monomeric MV^+ diffused inward.

Figure 4-15 shows the same reaction being monitored at 520 nm where the molar absorptivity of the dimer is four times that of the monomer. Here the breakpoint at 200 msec represents about 25% of the total absorbance change in the system. And the calculated rate constant for the reduction of inner MV^{2+} agreed with the k_2 calculated at the isosbestic point. This would not be the case if the conversion of monomer to dimer were rate-limiting. Again this implies that the reaction consists of the rapid reduction of half of the viologen in the system to form MV^+ monomer followed by the slower reduction of the remaining viologen with the immediately detectable product being MV^+ dimer.

All of the evidence presented so far supports the mechanism proposed in Figure 4-6. The nature of the electron transfer step is still unknown and may require more sophisticated techniques to uncover. But we can now write a rate law for the reduction of MV^{2+} by dithionite in equimolar inside and outside systems⁽⁴⁴⁾.

$$\frac{d[MV^+_{total}]}{dt} = k_1[MV^{2+}_{out}][SO_2^{2-}] + k_2[MV^{2+}_{in}]^2 \quad (4-15)$$

This simplified rate law is valid where $[MV^{2+}_{in}] = [MV^{2+}_{out}]$ due to the

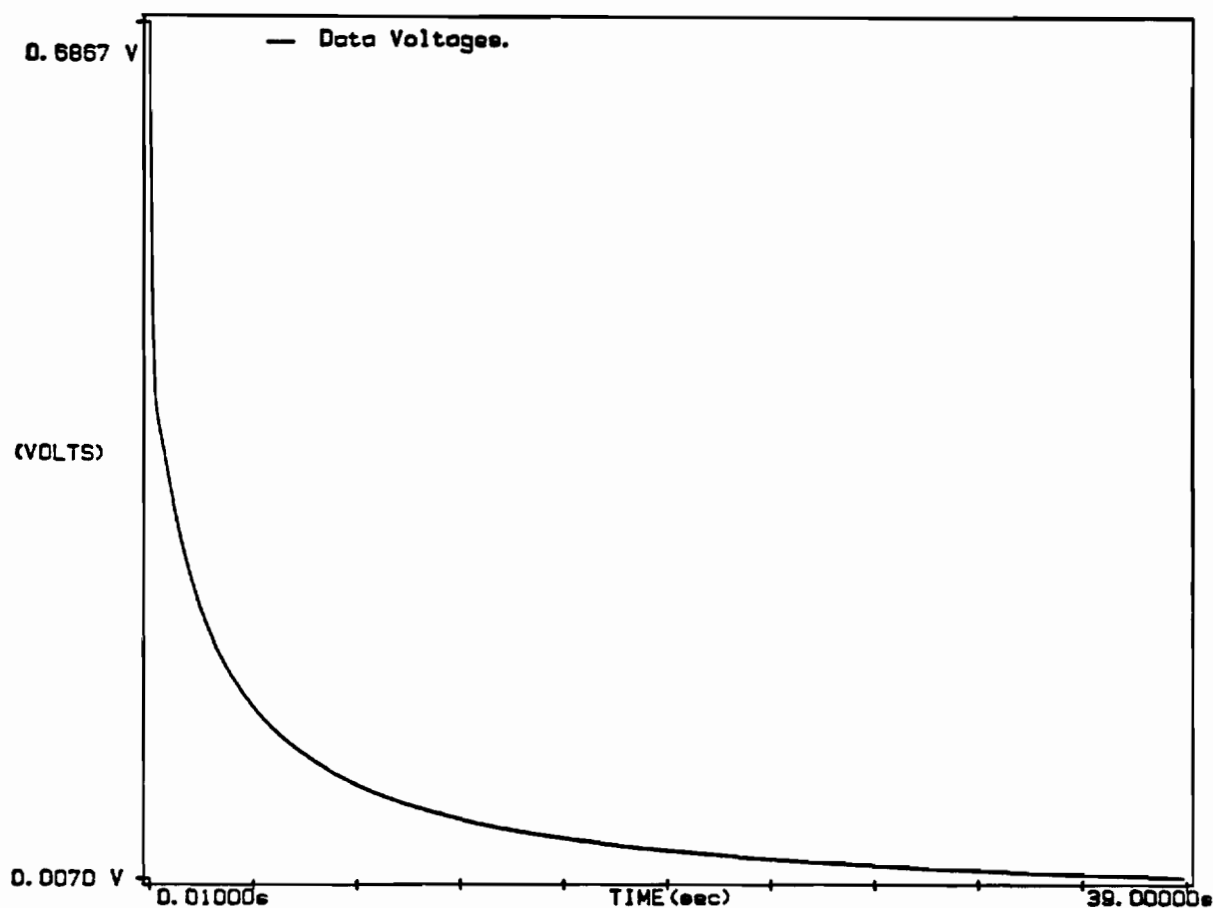


Figure 4-15: Voltage curve representing the change in percent transmittance at 520 nm for a DHP vesicle system with approximately equal concentrations of MV^{2+} inside and outside. Viologen radical chromophores in the dimeric state absorb twice as much as those in the monomeric state at this wavelength.

equimolar initial distribution.

A separate set of experiments was performed using C_6MV^{2+} as the electron mediator. DHP vesicles were prepared with equal quantities of C_6MV^{2+} on either side of the vesicle bilayer. Again the systems were reduced by a large molar excess of dithionite ion in the stopped-flow apparatus. The results are summarized in Table 4-13.

The analysis of the C_6MV^{2+} data was identical to that for MV^{2+} except that, in general, the second-order fits to the slow step were not so good, especially at longer times. This may be an indication that the charge-compensating viologen radical migration is becoming rate-limiting near the end of the reaction. The slow step could not be fitted adequately to one or the sum of two exponential curves.

The results of these experiments were similar to those for the MV^{2+} systems. The transmembrane reduction step was independent of dithionite concentration and the magnitude of k_2 was found to be inversely dependent on the DHP/ C_6MV^{2+} ratio. Again this is probably due to the increased surface concentration of viologen as the total vesicle surface area decreases. The magnitude of k_1 was independent of DHP concentration within experimental uncertainty. The value of k_1 decreased about 40% in going from MV^{2+} to C_6MV^{2+} while the value of k_2 decreased almost an order of magnitude. The value of $1.0 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for k_1 with 4 mM DHP agreed well with that for the reduction of C_6MV^{2+} loaded only outside of 4 mM DHP vesicles, where k_1 was $1.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Figure 1-2).

The monitoring wavelength was also varied and in this case also it appeared that dimer formation coincided with transmembrane oxidation-

Table 4-13

Kinetics of Dithionite Reduction of C_6MV^{2+}
Inside/Outside of DHP Vesicles ^a.

Total $[C_6MV^{2+}]$ (μM)	Approx. [DHP] (mM)	$[S_2O_4^{2-}]$ (mM)	$10^{-7} \times k_1$ ($M^{-1}s^{-1}$)	$10^{-3} \times k_2$ ($M^{-1}s^{-1}$)
24	1	0.52	0.81	3.3
26	1	0.57	0.93	3.7
31	1	0.66	0.99	3.0
32	1	0.62	0.97	b
32	2	0.33	0.97	b
42	2	0.83	0.70	1.8
"	"	3.52	0.89	1.9
44	2	0.50	0.88	0.7
46	2	0.53	0.93	b
47	2	0.86	0.87	1.8
53	2	0.57	0.90	0.7
55	2	0.63	0.88	1.2
"	"	1.48	1.04	1.3
56	2	0.65	0.99	1.7
69	2	0.74	0.76	1.0
73	2	0.69	0.97	2.6
"	"	2.13	1.18	2.4

(cont.)

(Table 4-13 cont.)

45	4	0.82	0.84	0.90
62	4	1.18	1.17	1.0
67	4	1.29	0.96	1.5
69	4	1.17	1.00	0.95
Averages:	1	0.52-0.66	0.91+/-0.07	3.3+/-0.3
	2	0.50-3.52	0.93+/-0.12	1.6+/-0.6
	4	0.82-1.29	0.99+/-0.12	1.1+/-0.2

^a Approximately equal [$C_i MV^{2+}$] inside and outside of vesicles, 20 mM Tris, pH 8.0.

^b Slow step did not fit homogeneous second-order plot.

reduction. The value of k_2 was found to be independent of the monitoring wavelength again implying the direct formation of the final product. This system also showed the magnitude of the fast step accounting for one-half of the total viologen in the system, implying that the fast step is due to the reduction of the outer viologen.

Kinetic studies were also done with systems containing an equilibrium distribution of $C_{12}MV^{2+}$ or $C_{16}MV^{2+}$ across DHP vesicle bilayers. These systems presented similar problems and had similar properties so they will be discussed together. These systems turn out to be quite different from the two just discussed.

The first problem encountered with these systems was the relatively rapid decomposition of the reduced viologens in 20 mM Tris-HCl buffer at pH 8.0 (section II.a). After much study, it was found that 75 mM imidazole-HCl, pH 7.5 was a suitable buffer for these experiments. The major difference between these two buffers, as far as electron transfer is concerned, is that the imidazole ion is an ionophore⁽⁴⁰⁾ and allows for complete reduction of the inner viologen. These viologens in Tris buffer were not completely reduced by dithionite ion (sections III.b,d). The imidazole ion provides an alternate mechanism for the dissipation of the transmembrane potential generated by electron transfer. The kinetic results for $C_{12}MV^{2+}$ are given in Table 4-14 and those for $C_{16}MV^{2+}$ are given in Table 4-15.

The rate constants determined for the outer reduction steps, k_1 and k_2 , were very similar to those determined for $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ bound only externally to DHP (section II.c). These rates in 75 mM imidazole buffer were only slightly faster than in 20 mM Tris buffer. The

Table 4-14

Kinetics of Dithionite Reduction of $C_{12}MV^{2+}$
Inside/Outside of DHP Vesicles ^a.

$[C_{12}MV^{2+}]$ (μM)	$[S_2O_4^{2-}]$ (mM)	$10^{-6} \times k_1$ ($M^{-1}s^{-1}$)	$10^{-6} \times k_2$ ($M^{-1}s^{-1}$)	$k_3(s^{-1})$ 1st order approx.	$A_1/A_2/A_3$ ^b
40	0.42	4.4	1.0	0.034	0.46/0.27/0.23
"	1.02	5.1	1.1	0.042	0.44/0.23/0.25
56	0.68	5.5	1.3	0.041	0.35/0.37/0.26
"	1.47	5.7	1.0	0.050	0.39/0.31/0.24
58	0.62	5.2	1.1	0.052	0.42/0.29/0.28
81	0.90	6.2	1.3	0.046	0.33/0.40/0.26
"	2.23	6.6	1.3	0.051	0.35/0.34/0.26
Averages:		5.5+/-0.7	1.2+/-0.1		

^a Equilibrium distribution of $C_{12}MV^{2+}$ inside and outside, 75 mM imidazole-HCl, pH 7.5; approximately 2 mM DHP.

^b Relative fraction of: fast outer reduction/slow outer reduction/transmembrane reduction.

Table 4-15

Kinetics of Dithionite Reduction of $C_{16}MV^{2+}$
Inside/Outside of DHP Vesicles ^a.

$[C_{16}MV^{2+}]$ (μM)	$[S_2O_4^{2-}]$ (mM)	$10^{-6} \times k_1$ ($M^{-1}s^{-1}$)	$10^{-5} \times k_2$ ($M^{-1}s^{-1}$)	$k_3(s^{-1})$ 1st order approx.	$A_1/A_2/A_3$ ^b
47 ^c	0.66	0.90	1.5	0.0067	0.09/0.60/0.31
"	1.62	0.98	1.7	0.0070	0.11/0.57/0.33
48 ^c	0.65	1.2	2.1	0.0070	0.08/0.59/0.33
"	1.45	1.2	2.1	0.0073	0.12/0.57/0.31
33 ^d	0.53	1.2	2.6	0.0062	0.08/0.49/0.43
33 ^d	0.52	0.92	2.2	0.0043	0.11/0.46/0.43
"	1.36	0.78	2.0	0.0059	0.17/0.41/0.42
34 ^d	0.54	1.3	2.6	0.0042	0.07/0.50/0.43
75 ^d	1.15	0.95	1.8	0.0061	0.10/0.55/0.35
"	2.66	0.87	1.7	0.0062	0.15/0.51/0.34
Averages:		1.0+/-0.2	2.0+/-0.4		

^a Equilibrium distribution of $C_{16}MV^{2+}$ inside and outside, 75 mM imidazole-HCl, pH 7.5.

^b Relative fraction of: fast outer reduction/slow outer reduction/transmembrane reduction.

^c Approximately 1 mM DHP.

^d Approximately 2 mM DHP.

relative contributions of the two outer reduction pathways are somewhat different in this case from the outside-only case but qualitatively, the reduction of outer viologen for $C_{12}MV^{2+}$ is shared about equally between the two pathways while for $C_{16}MV^{2+}$ the slower pathway dominates.

Because there was not an equimolar distribution of viologen between the inner and outer vesicle surfaces the simplified version of the rate law given in Equation 4-15 did not apply and the slow step could not be analyzed as it was for the MV^{2+} and C_6MV^{2+} systems. Consequently, the rate constant for the slow step is given as a first-order approximation for these systems. This approximation was adopted for the purpose of determining if any significant changes in the initial slope of the slow step occurred upon increasing the dithionite concentration. In most instances, the observed dithionite dependence was small. However, it was clear from the analysis that the only way to quantitatively evaluate whether or not the transmembrane step was independent of dithionite concentration was to calculate the actual second-order rate constant. This analysis was also essential to compare the relative rates of the k_2 steps with those for the $MV^{2+}/+$ and $C_6MV^{2+}/+$ transmembrane reactions. Qualitatively, it was obvious that transmembrane electron transfer was much slower in the systems with the longer alkyl chain viologens since the total time for complete reduction was much longer. One way to obtain approximate second-order rate constants for the transmembrane step was to fit the data curves to a simulated mechanism. The simulation program used is given in Appendix C.

The computer simulation monitors changes in the concentration of each species in the system over very small increments of time according to the postulated mechanism. In order to accommodate the biphasic outer reduction process found with the longer chain alkyl viologens the more complicated mechanism depicted in Figure 4-16 was used for these simulations. This mechanism accounts for the fact that there can be two outer reduction processes occurring simultaneously which leads to the possibility of two transmembrane reduction pathways that can proceed at different rates.

Again this mechanism assumes that, at least initially, the diffusion of reduced viologen down the charge gradient is not rate-limiting. This implies that $k_3, k_5 \ll k_4$ and is supported by the fact that the transmembrane step cannot be fitted to the sum of two exponential functions. The first test of the simulation program was to fit some of the MV^{2+} and C_6MV^{2+} data which were known to be well-behaved. In most cases good fits could be obtained using rate constants very near those determined by analyzing the fast and slow steps separately. Then one experiment was done with a nonequimolar distribution of MV^{2+} across the vesicle bilayer membrane. Again the simulation program was able to adequately fit the data using rate constants similar to those obtained from equimolar systems. One such fit is shown in Figure 4-17. It should be noted that the simulation program does not incorporate any kind of fitting algorithm, but requires that the user to input all of the kinetic parameters. Thus the fits obtained are are usually the result of several hours of trial and error and are not necessarily the best possible although some of the values, such as rate constants for

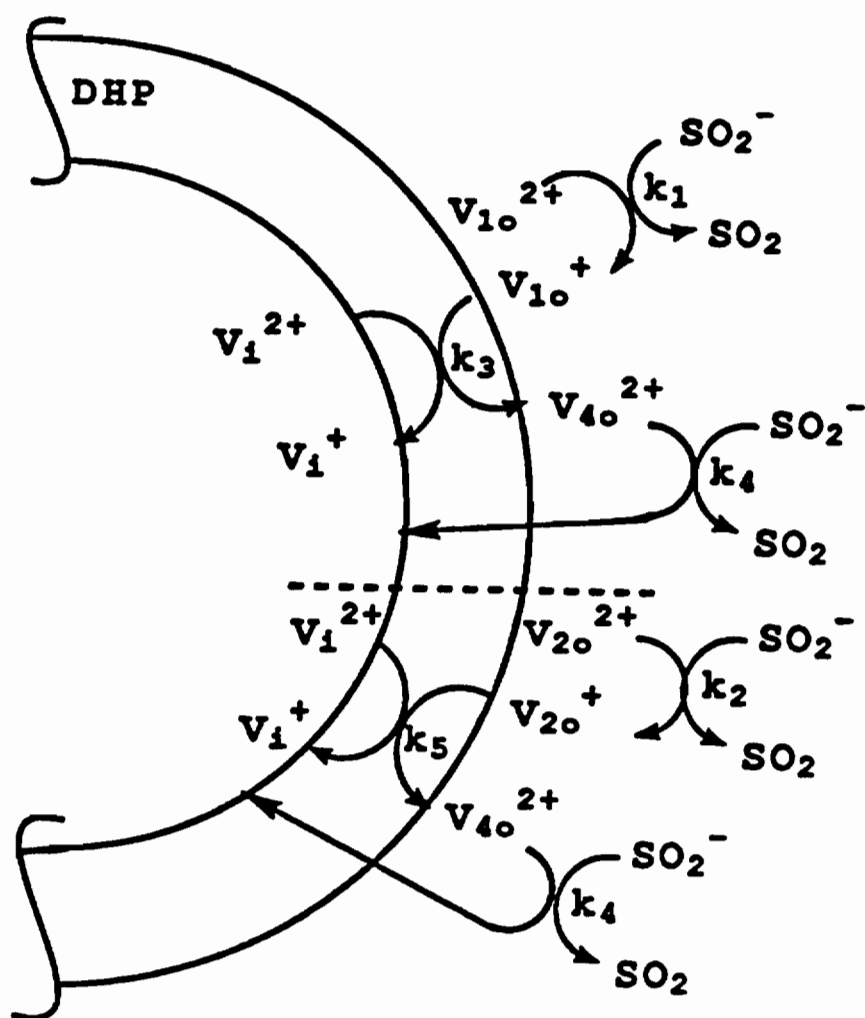


Figure 4-16: Mechanism for viologen-mediated transmembrane oxidation-reduction used for computer simulations.

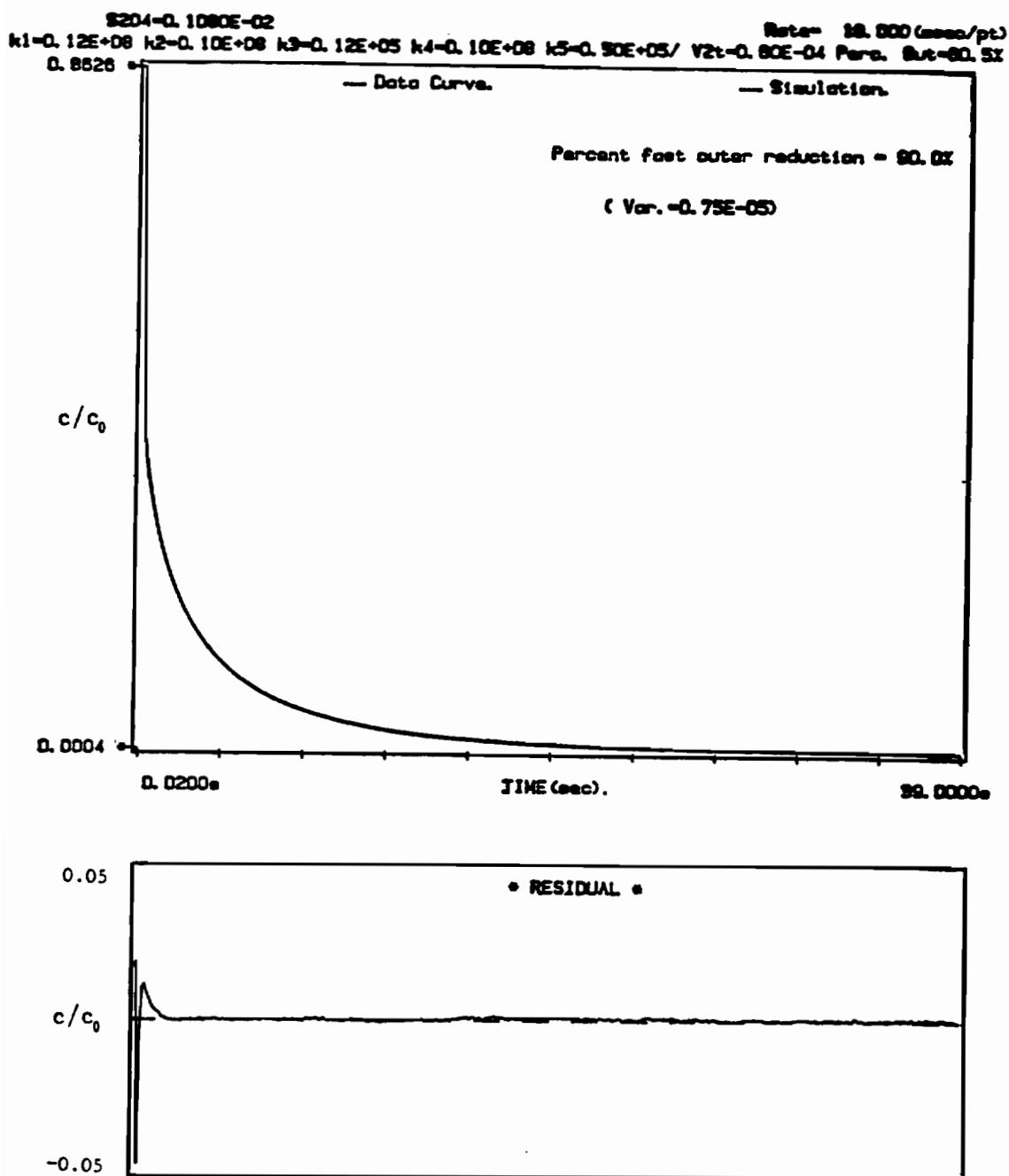


Figure 4-17: Comparison of dithionite reduction curve with simulated curve created from mechanism shown in Figure 4-14. Percent fast outer reduction is fraction of k_1 step compared to k_2 step. Experimental conditions: 18 μM MV^{2+} inside, 24 μM MV^{2+} outside, 2 mM DHP, 20 mM Tris-HCl, pH 8.0.

the outer reduction steps, are already known.

After the simulation program was determined to be satisfactory for the simpler systems, it was used to analyze some of the $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ systems. In all cases the values used for the outer reduction rate constants, k_1 and k_2 , were nearly identical with those from the previous analyses listed in Tables 4-14 and 4-15. In general the fits for these systems were not as good as for the shorter alkyl chain length viologens, but they could provide some estimated second-order rate constants for the transmembrane reduction step. These could then be compared to those from the MV^{2+} and C_6MV^{2+} systems.

Analyses from a $C_{12}MV^{2+}$ system are shown in Figures 4-18 and 4-19. The only difference between these two experiments is the change in dithionite concentration. One can see that the rate constants for the transmembrane reduction step, k_3 and k_5 , are little changed upon the increase in dithionite concentration and in both cases the viologen reduction is equally distributed between the k_1/k_3 and k_2/k_5 pathways as shown in Figure 4-16. Note also that the simulation implies that about two-thirds of the viologen is localized on the outer surface as expected from the relative inner and outer surface areas (section I.b).

The results for $C_{16}MV^{2+}$ were similar and are shown in Figures 4-20 and 4-21. Again very little adjustment of the kinetic parameters was necessary to fit the curve at the higher dithionite concentration. In both cases the majority (approx. 70%) of the reduction reactions are carried out through the faster pathway (designated k_2/k_5 in Figure 4-16). And as with $C_{12}MV^{2+}$ about two-thirds of the viologen was determined to be outside of the vesicles. A compilation which summarizes the rate

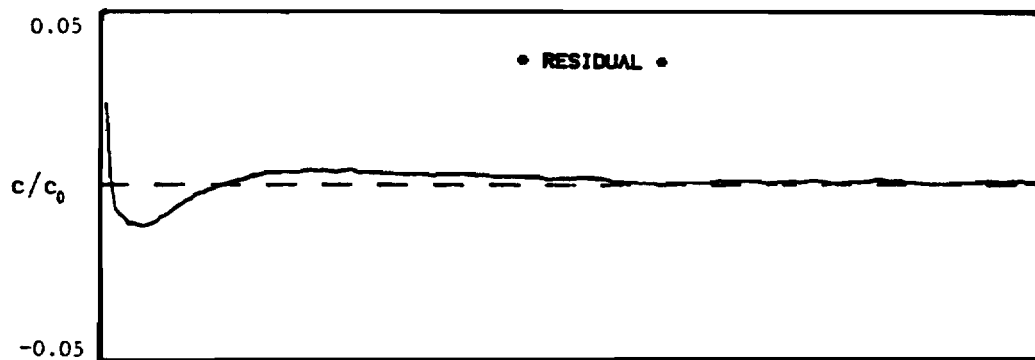
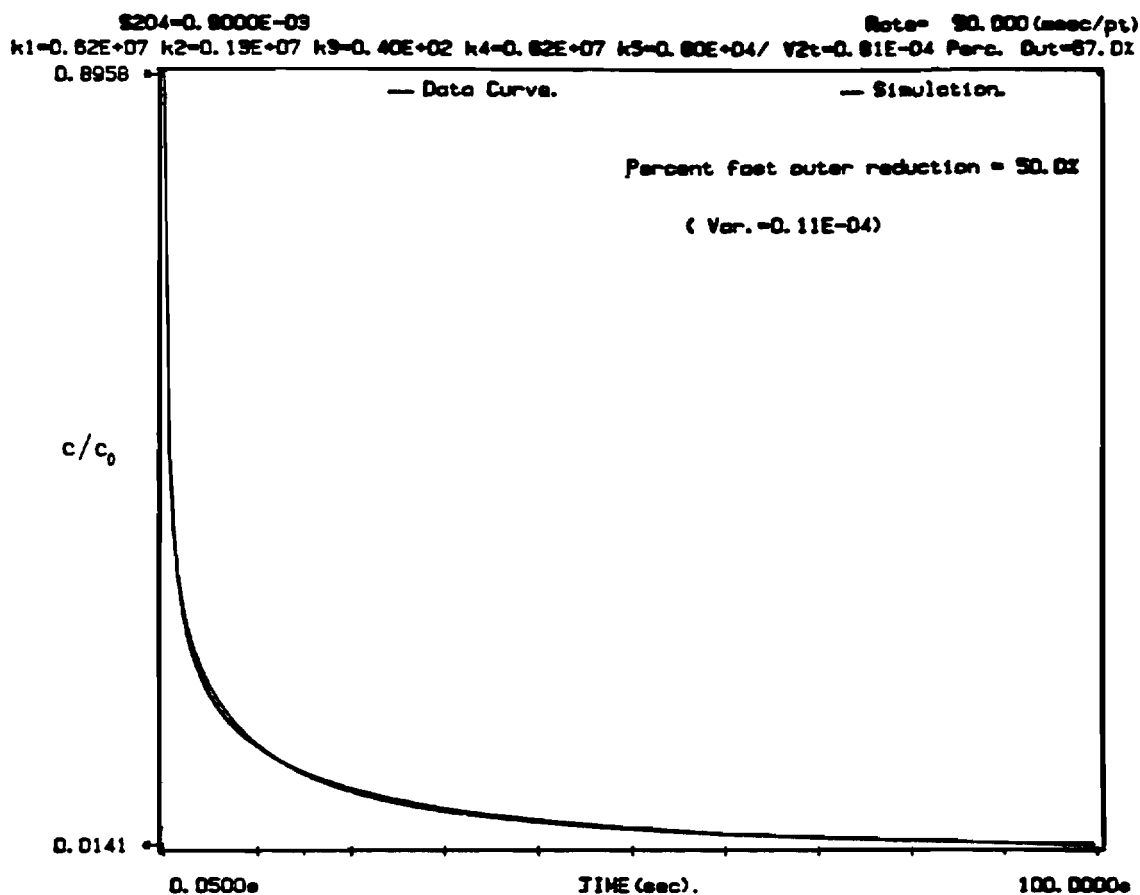


Figure 4-18: Comparison of 11-fold excess dithionite reduction curve with simulated curve generated from mechanism shown in Figure 4-14. Percent fast outer reduction is fraction of k_1 step compared to k_2 . Experimental conditions: 81 μM $\text{C}_{12}\text{MV}^{2+}$ with equilibrium inside/outside distribution, 2 mM DHP, 75 mM imidazole-HCl, pH 7.5.

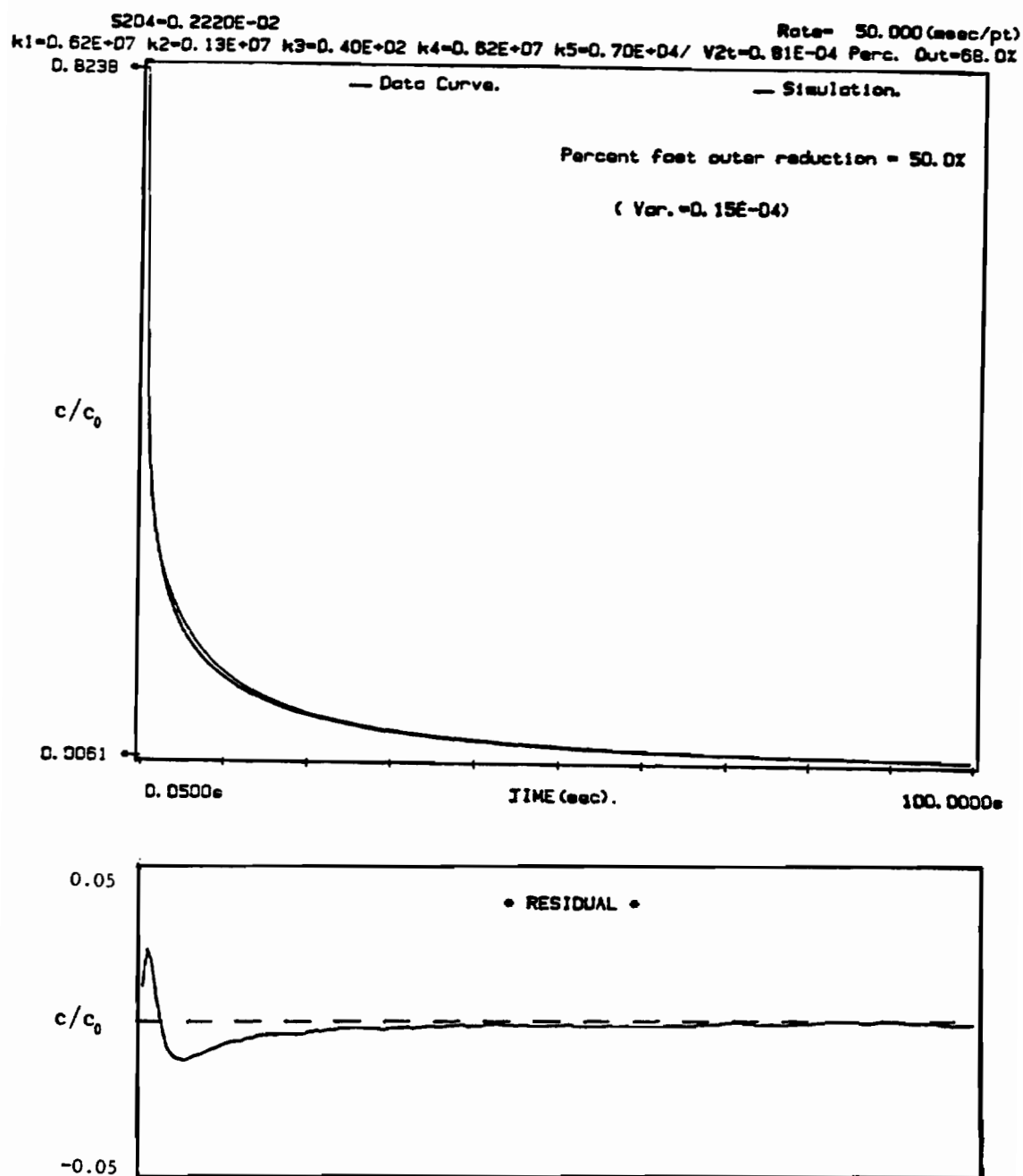


Figure 4-19: Comparison of 27-fold excess dithionite reduction curve with simulated curve generated from mechanism shown in Figure 4-14. Experimental conditions: Same as in experiment shown in Figure 4-16 except for increase in dithionite concentration.

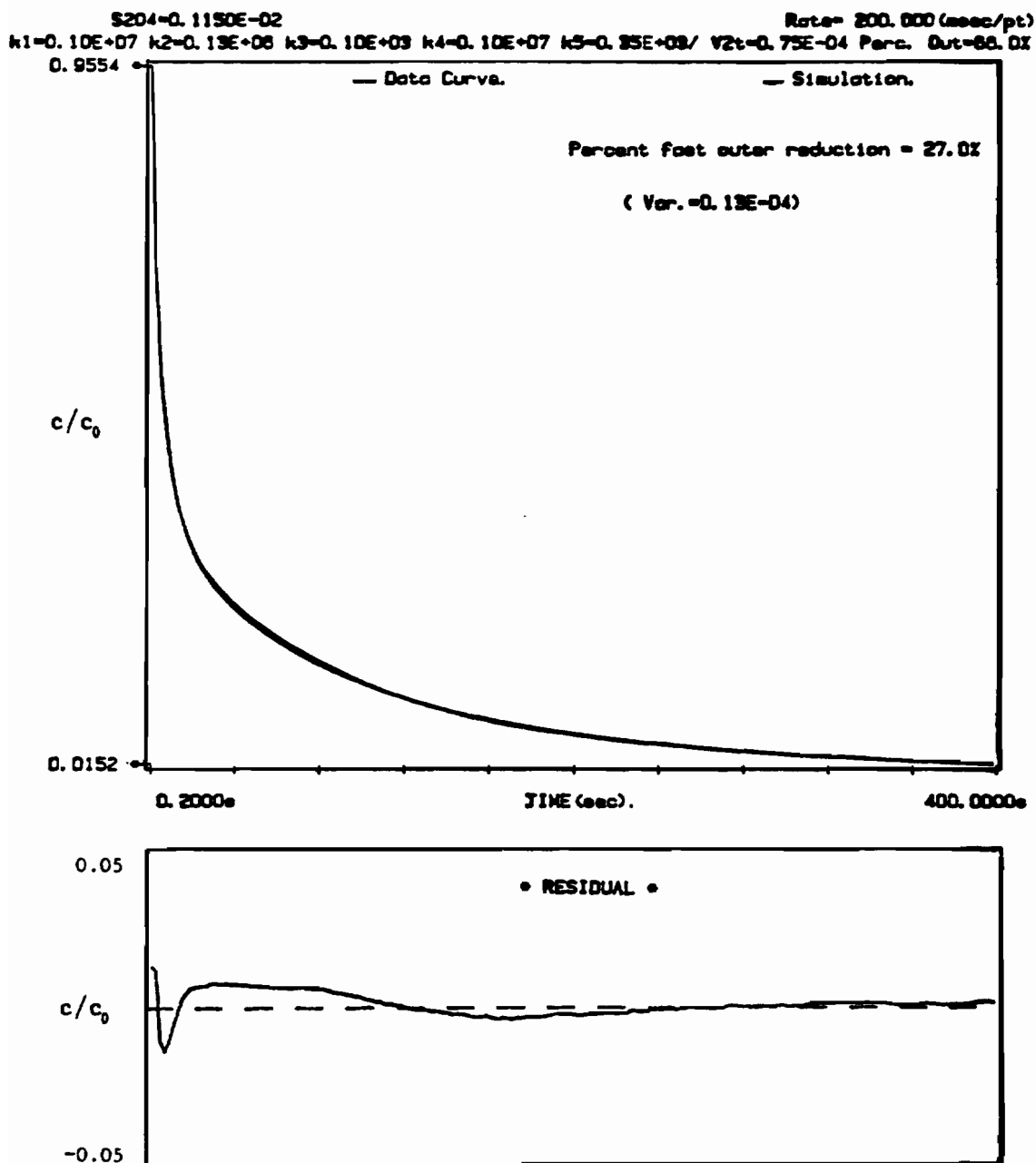


Figure 4-20: Comparison of 15-fold excess dithionite reduction curve with simulated curve generated from the mechanism shown in Figure 4-14. Experimental conditions: 75 μM $\text{C}_{16}\text{MV}^{4+}$ with equilibrium inside/outside distribution, 2 mM DHP, 75 mM imidazole-HCl, pH 7.5.

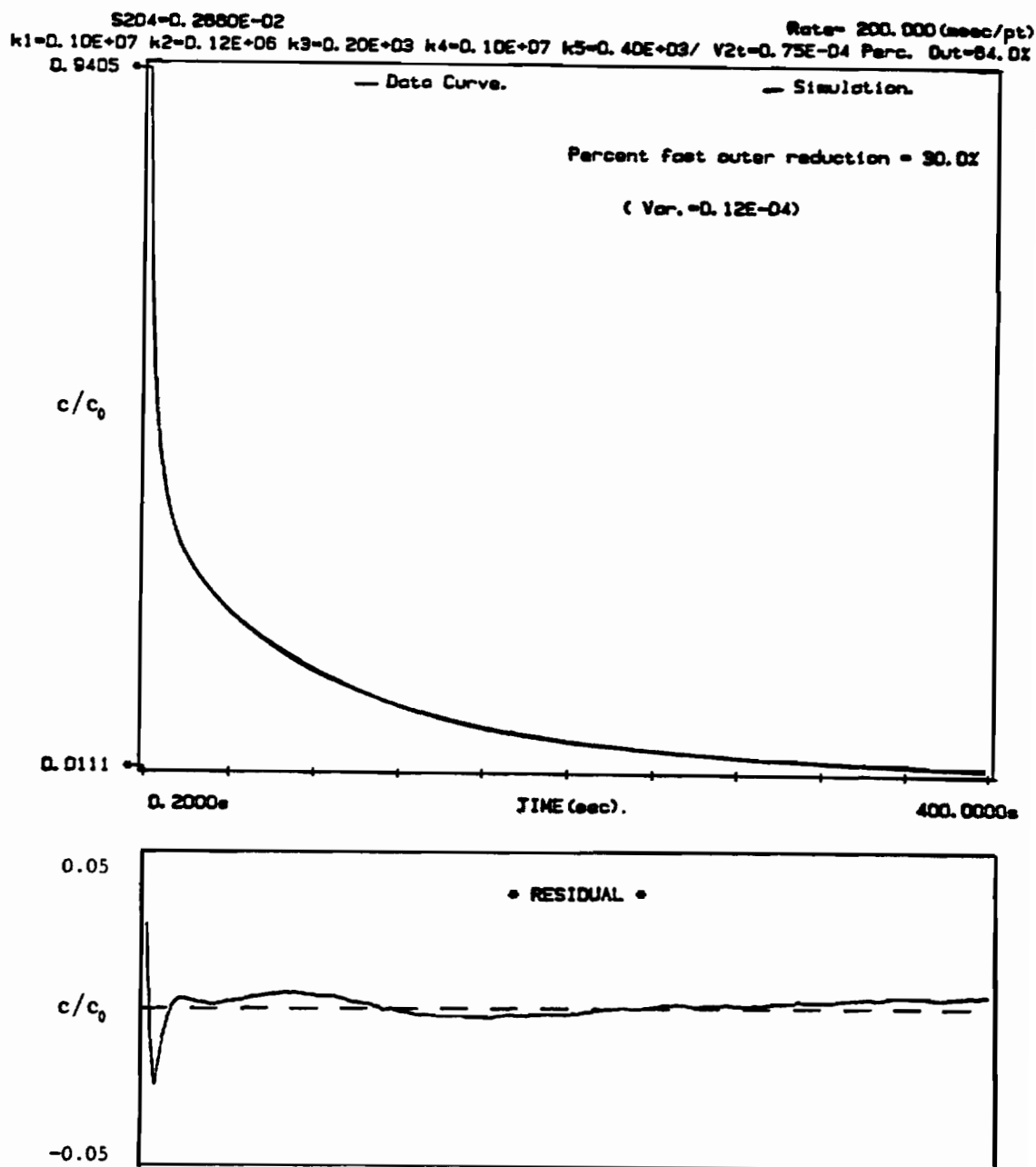


Figure 4-21: Comparison of 35-fold excess dithionite reduction curve with simulated curve generated from the mechanism shown in Figure 4-14. Experimental conditions: Same as in experiment shown in Figure 4-18 except for the increase in dithionite concentration.

data for transmembrane oxidation-reduction is given in Table 4-16.

The $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ systems appear to have a pathway available for electron transfer which is more rapid than that for C_6MV^{2+} as well as a pathway which is slower. If one follows the slower pathway the trend is for slower rates of transmembrane electron transfer with increasing alkyl chain length. In general this is apparent in the longer times needed for complete reduction of the system as the alkyl chain length increases.

Table 4-16
Summary of Transmembrane Redox Rate Constants.

Viologen	Conditions	Rate Constant ($\text{M}^{-1}\text{s}^{-1}$)	Pathway Percentage
MV^{2+}	a	$1.3(+/-0.4) \times 10^4$	Approximately 100%
C_8MV^{2+}	b	$1.6(+/-0.6) \times 10^3$	Approximately 100%
$\text{C}_{12}\text{MV}^{2+}$	c	$6.5(+/-0.5) \times 10^3$	Approximately 50%
		$4.0(+/-0.0) \times 10^2$	Approximately 50%
$\text{C}_{16}\text{MV}^{2+}$	d	$6.9(+/-3.1) \times 10^3$	Approximately 65%
		$1.1(+/-0.5) \times 10^2$	Approximately 35%

a 1-2 mM DHP, 20 mM Tris-HCl, pH 8.0.

b 2 mM DHP, 20 mM Tris-HCl, pH 8.0.

c 2 mM DHP, 75 mM imidazole-HCl, pH 7.5.

d 1-2 mM DHP, 75 mM imidazole-HCl, pH 7.5.

IV) Miscellaneous Transmembrane Redox Systems.

IV.a) Viologen Outside / Ferricyanide Ion Inside of Dehexadecyl-phosphate Vesicles: Systems were prepared with $\text{Fe}(\text{CN})_6^{3-}$ inside and either MV^{2+} or PMB^{4+} outside of DHP vesicles in 20 mM Tris, pH 8.0. These were prepared by cosonicated the DHP with 20 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and then running the vesicles down a gel column to remove external $\text{Fe}(\text{CN})_6^{3-}$ (Caution - HCN is released during sonication procedure!). Typically 15-25 μM $\text{Fe}(\text{CN})_6^{3-}$ could be entrapped as determined spectrophotometrically. A molar absorptivity of $1.04 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ for $\text{K}_3\text{Fe}(\text{CN})_6$ was determined experimentally at 420 nm. In all cases either MV^{2+} or PMB^{4+} was then added to the exterior vesicle surface at a concentration roughly equal to the entrapped ferricyanide concentration.

In the absence of viologen, addition of excess $\text{S}_2\text{O}_4^{2-}$ to the anaerobic solution resulted in reduction of entrapped $\text{Fe}(\text{CN})_6^{3-}$ which was complete after a period of about 20 minutes. This was slow enough not to interfere significantly with the viologen-mediated reduction of ferricyanide.

In systems with MV^{2+} outside of the vesicles, addition of a 5-fold molar excess of $\text{S}_2\text{O}_4^{2-}$ resulted in complete reduction of the entrapped ferricyanide as well as all of the MV^{2+} within two minutes. In this case, most of the MV^+ formed was present in the monomeric state and it was calculated that after oxygenation greater than 80% migration of viologen into the vesicles had occurred. The amount of MV^{2+} on the outer vesicle surface was calculated by dividing the concentration of MV^+ formed upon re-reduction with dithionite by $(\alpha+1)$ (see section

III.c, this chapter). These reaction dynamics can be easily explained in terms of the reaction mechanisms proposed in Figure 4-6. The only difference is that ferricyanide ion is the electron acceptor in this case instead of MV^{2+} . Since the standard reduction potential for $Fe(CN)_6^{3-}$ is 0.46 volts⁽⁴⁵⁾, this reaction is energetically downhill by about 0.9 volts.

The data are interpreted as follows: After external MV^{2+} was reduced by dithionite ion, it reduced an internal ferricyanide ion in a transmembrane redox step. The newly formed MV^{2+} was rapidly re-reduced by external dithionite ion and migrated into the vesicle to compensate for the transmembrane potential generated by the transmembrane redox event, accounting for the high percentage of viologen uptake in the system. One major difference in this system as compared to the equimolar MV^{2+} system is that the radical formed was monomeric instead of dimeric even though it appears to be localized inside of the vesicles. Possibly the $Fe(CN)_6^{4-}$ ion strongly pairs with $MV^{\bullet}$ ions and prevents dimer formation.

If the concentration of dithionite added was less than half that of the entrapped ferricyanide, the blue color initially produced would slowly disappear over a period of about 5 minutes. It was found that variable amounts of translocation of methyl viologen occurred in this case, with the amount of methyl viologen migrating inward always being slightly less than the amount initially reduced on the outer surface. This reaction then appears to be just the first part of the above mechanism. The MV^{2+} was reduced to the extent of two ions per molecule of $S_2O_4^{2-}$. The methyl viologen radicals formed then reacted with the

entrapped ferricyanide resulting in entrapped ferrocyanide and MV^{2+} which are both colorless.

The other system of this type which was investigated used PMB^{4+} instead of MV^{2+} as the external dopant ion. As was discussed in section III.c, PMB^{4+} does not appear to mediate transmembrane electron transfer. This conclusion was confirmed in this system since reduction of entrapped ferricyanide and viologen migration were not observed regardless of the dithionite concentration. Therefore, PMB^{4+} and its reduced forms do not appear to be capable of traversing this membrane.

In general, it would seem that any electron acceptor with the correct reduction potential when entrapped inside of DHP vesicles would allow methyl viologen-mediated transmembrane oxidation-reduction from dithionite ion. If no other lipophilic ions are present in the system viologen translocation will also occur.

IV.b) Reduction of Ferricyanide Ion Inside of Egg Phosphatidylcholine Vesicles Mediated by $C_{16}MV^{2+}$: Approximately 4 mM egg phosphatidylcholine (PC) vesicles were prepared from a chloroform lipid stock solution in 1.0 M KCl and 0.3 M $K_3Fe(CN)_6$ at pH 9.0. The solution was cooled with an ice bath during sonication (Caution - HCN is released during the sonication procedure!). In some cases 500 μM $C_{16}MV^{2+}$ was also present during sonication. After annealing the vesicles for 18 hours at room temperature the solution was chromatographed on a gel column and eluted with 1.0 M KCl. The chromatographic process removed both external $Fe(CN)_6^{3-}$ and $C_{16}MV^{2+}$. About 200 μM $Fe(CN)_6^{3-}$ was entrapped within the vesicles; if 500 μM $C_{16}MV^{2+}$ was added about 50 μM of the viologen was

entrapped.

A large molar excess of dithionite ion was added to a system containing 200 μM ferricyanide and 50 μM $\text{C}_{16}\text{MV}^{2+}$ inside and 50 μM $\text{C}_{16}\text{MV}^{2+}$ outside the vesicles. The anaerobic solution immediately turned blue and then went virtually colorless within one second. Excess dithionite was still present as confirmed spectroscopically. All of the ferricyanide in the system was reduced. It was also determined after oxygenation that most of the external $\text{C}_{16}\text{MV}^{2+}$ had migrated into the vesicles. This result is identical to that reported for a similar system⁽⁴⁶⁾ using N,N'-dihexadecyl-4,4'-bipyridinium ion. In that case the authors also found that more ferricyanide was reduced than $\text{C}_{16}\text{MV}^{2+}$ migrated inward. They explained the excess $\text{Fe}(\text{CN})_6^{3-}$ reduction by electron tunneling mechanisms, but this does not have to be the case since egg PC vesicles have been found to be quite leaky towards electrolytes compared to DHP vesicles⁽²²⁾. In the present experiment, dithionite reduction of entrapped ferricyanide was found to be fairly rapid in the absence of viologens. About one-half of the entrapped ferricyanide could be reduced within 2 minutes. Furthermore, if transmembrane redox occurred by electron tunneling it must be accompanied by diffusion of some other ion to neutralize the transmembrane potential. Thus, the PC membrane must be relatively permeable to either $\text{S}_2\text{O}_4^{2-}$ or electrolyte ions.

The most probable mechanism in this case is similar to that shown in Figure 4-6. Reduction of outer $\text{C}_{16}\text{MV}^{2+}$ leads to electron or diffusive exchange with an inner $\text{C}_{16}\text{MV}^{2+}$. Maintenance of electroneutrality then requires the inward migration of a $\text{C}_{16}\text{MV}^{\cdot+}$ radical ion. Both of these

viologen radicals are eventually oxidized by entrapped ferricyanide. This implies two ferricyanide molecules are reduced for every viologen molecule migrating inwardly. In the earlier work⁽⁴⁶⁾, it was assumed that only one ferricyanide molecule was reduced for each inner viologen reduced. This stoichiometric difference in the assumed mechanism could account for most of the discrepancy these workers reported since they found that the amount of ferricyanide reduced was just over twice the amount of viologen initially outside.

A few other experiments were also done with ferricyanide entrapped inside and various alkyl viologens distributed outside egg PC vesicles. These results were similar to the above case. If $C_{16}MV^{2+}$ was present only outside of the vesicles and entrapped ferricyanide was in two-fold molar excess the addition of an excess of dithionite led to only a slight blue/violet color indicating only a very small percentage net reduction of the viologen in the system. But, after oxygenation and re-reduction, it was found that greater than 80% of the viologen was on the inside of the vesicles. This suggests that in the first reduction the reduced $C_{16}MV^{2+}$ immediately transferred an electron to an inner ferricyanide. After being re-reduced on the outer surface it migrated inwardly to compensate for the transmembrane electrical potential and was subsequently oxidized by the entrapped ferricyanide. This mechanism is consistent with the other experiments that have been discussed and explains the observed results.

Almost identical results were observed if MV^{2+} was distributed only outside of the vesicles except that the addition of dithionite resulted in the appearance of a blue color which disappeared within about 10

seconds. This may be a consequence of the fact that MV^{2+} binds less strongly to PC vesicles than $C_{16}MV^{2+}$ and thus the reduced MV^{2+} requires more time to diffuse to the vesicle surface before it can react with the entrapped ferricyanide⁽²²⁾. Again greater than 90% inward translocation was observed.

In another experiment PMB^{4+} was bound externally to the vesicles. In this case about 80% of the total viologen was observed as radical ion upon dithionite addition. Therefore, it was not reacting very efficiently with the entrapped ferricyanide. After oxygenation and re-reduction it was found that less than 35% migration had occurred. But, compared with DHP vesicles, this observation was significant since almost no migration of PMB^{4+} was ever observed in these systems. This difference can be attributed to the increased permeability of the egg PC bilayer, which might be due in part to the fact that the experiments are performed well above its phase transition temperature ($0^{\circ}C^{(47)}$) and in part to the absence of strong electrostatic attraction to the surfactant headgroups.

IV.c) Reduction of MV^{2+} Inside and Outside of Dipalmitoylphosphatidylcholine Vesicles: Methyl viologen was distributed in an equimolar fashion inside and outside of dipalmitoylphosphatidylcholine (DPPC) vesicles. This was done to investigate the magnitude of transmembrane oxidation-reduction and viologen translocation in this system. These bilayers differ from DHP since the overall surface charge is neutral due to the zwitterionic lipid headgroups. They also differ from the egg PC bilayers since the experiments were conducted well below their phase

transition temperature of 42 °C⁽⁴⁸⁾.

Unfortunately, the neutral surface permitted very little binding of MV^{2+} , which in turn led to very low entrapment yields. In order to entrap just 5 μM viologen, the vesicles had to be prepared in the presence of 5 mM MV^{2+} . The experiments were done in 20 mM Tris-HCl, pH 8.0.

Vesicles were therefore prepared with 5 μM MV^{2+} inside and 5 μM MV^{2+} outside. Initial reduction of this system with a 10-fold molar excess of dithionite ion led to complete reduction of all of the viologen and the appearance of a visible spectrum characteristic of viologen dimer. If 10 μM MV^{2+} was reduced outside of DPPC vesicles the spectrum showed 100% monomeric MV^+ . If the system with equimolar MV^{2+} inside and outside was oxygenated and re-reduced it was determined that about 50% of the MV^{2+} originally on the outer surface had migrated inside the vesicles. It appeared that this system showed properties similar to those of the DHP systems.

IV.d) MV^{2+} Inside / $MSPB^+$ Outside of DHP Vesicles - MMB^+ Controlled

Electron Transfer: DHP vesicles were prepared in 20 mM Tris-HCl at pH 8.0 containing approximately equimolar MV^{2+} inside and 1-methyl-1'-sulfonato-n-propyl-4,4'-bipyridinium ($MSPB^+$) outside. The solution was made anaerobic by purging with argon and reduced by the addition of a large molar excess of dithionite ion.

This system showed an almost immediate reduction of about half of the viologen present. Presumably this was due to reduction of $MSPB^+$ which was external to the DHP vesicles. After several minutes about 60%

of the total viologen in the system was reduced and no further reduction was observed with time. If an anaerobic solution of 1-methyl-4,4'-bipyridinium (MMB^+) was then added in slight molar excess the reduction of the remainder of the viologen in the system was observed within a few minutes. MMB^+ has no redox chemistry under these conditions as observed spectroscopically and merely serves as a lipophilic cation.

In the present case, reduced MSPB^+ is neutral and thus cannot dissipate the electrical gradient generated by transmembrane electron transfer. Therefore, in the absence of MMB^+ , electron transfer cannot proceed to any significant extent due to the buildup of a transmembrane potential which impedes further electron flow. The addition of MMB^+ provides a mechanism whereby the electrical gradient can be dissipated by its migration into the vesicles.

One-to-one correspondence could be observed in quantitative studies between the number of electrons allowed to flow into the vesicles and the number of MMB^+ molecules added to the vesicle exterior. After addition of 10, 21, and 31 μM MMB^+ to different systems containing approximately 50 μM internal MV^{2+} and 50 μM external MSPB^+ it was observed that additional viologen radical was produced in concentrations of 12, 21, and 31 μM respectively. This supports the notion that, in the absence of MMB^+ , transmembrane redox is being held-up by the lack of a charge neutralization mechanism and, for every electron transferred after the addition of MMB^+ , one cation must comigrate with each electron.

In the previous work using MV^{2+} as the electron mediator the migration of MV^+ into the vesicle interior resulted in a dimeric

viologen radical spectrum. In the present case when MMB^+ is in excess the product spectra were characteristic of viologen radical monomers. This implies that viologen radicals are not concentrated in the vesicle interior. This is because MV^+ formed inside the vesicle can assume an equilibrium distribution inside and outside of the vesicles due to electroneutral exchange with the excess MMB^+ . Even with stoichiometric MMB^+ little dimerization occurs. The MMB^+ inside appears to restrict viologen dimerization possibly by forming a mixed dimer with MV^+ itself.

Further evidence that these types of migration phenomena are occurring comes from cation exchange chromatography performed on the system before and after reduction. A system was prepared with 36 μM MV^{2+} inside and 31 μM MSPB^+ outside of 4 mM DHP vesicles. Then 48 μM MMB^+ was added to the external medium. When this suspension was chromatographed over Chelex 100 cation exchange resin, 23 μM viologen was found to comigrate with the vesicles. Presumably this is just the internal MV^{2+} with the lower concentration reflecting dilution of the vesicles during chromatography. If this system was reduced with dithionite ion and then oxygenated after 10 minutes 9 μM viologen and 13 μM MMB^+ was found inside the vesicles after chromatography. This supports the notion that MMB^+ is "pumped" into the vesicles during transmembrane reduction of MV^{2+} and that MV^+ is able to redistribute by transmembrane exchange with MMB^+ until equilibrium across the bilayer is reached. MSPB^0 can also set-up an equilibrium concentration distribution since it does not generate an electrical potential upon transmembrane migration. These phenomena are shown schematically in Figure 4-22. This system is interesting because in this case electron

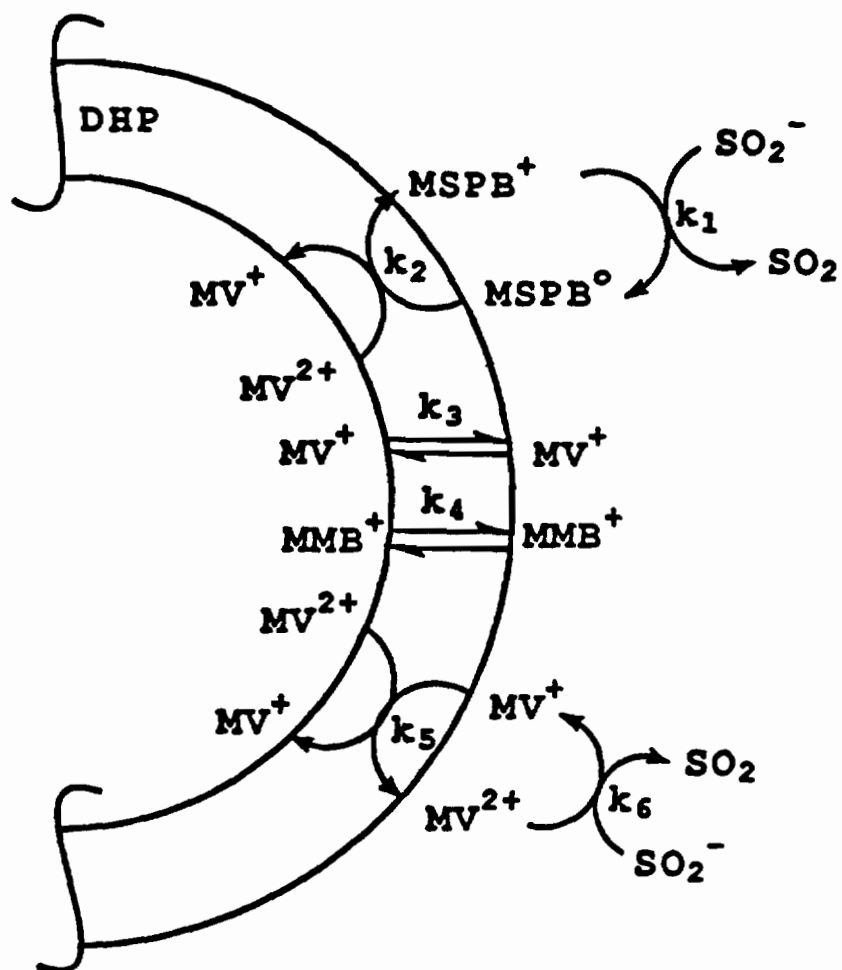


Figure 4-22: Proposed reaction processes in an equimolar internal MV^{2+} , external MSPB^+ system. The k_2 and k_5 steps represent transmembrane redox. The k_2 , k_3 , and k_5 steps are electrogenic and must be balanced such that overall electroneutrality is maintained. The k_6 step is a consequence of the k_5 step and is not initially present within the system.

flow is controlled by the addition of an external lipophilic cation and the number of electrons allowed to flow into the vesicle is approximately equal to the number of translocating MMB^+ molecules.

V) Charge Separation and Recombination in a Photochemical System Containing ZnTPPS^{4-} Ion and Alkyl Viologens Adsorbed on Dihexadecylphosphate Vesicles.

V.a) General: Interfacial electrostatics can be used to enhance photochemical charge separation processes. Early studies focused on continuous illumination of sensitized systems in the presence of a sacrificial electron donor in order to effect the reduction of an acceptor molecule.

One such system utilized a $\text{Ru}(\text{bpy})_3^{2+}$ derivative bound to sodium dodecylsulfate (SDS) micelles as a sensitizer⁽⁴⁹⁾. The electron acceptor was methyl viologen (MV^{2+}) and the sacrificial electron donor was cysteine. Constant irradiation of the system with visible light led to the slow accumulation of reduced MV^{2+} . In this case, the interfacial electrostatics are not optimal since both the sensitizer and electron acceptor are bound to the interface. This does very little to slow down the thermodynamically favorable back-reaction process.

Details of a system that utilized interfacial electrostatics more effectively was published a couple of years later⁽⁵⁰⁾. In this case the micelles were composed of cetyltrimethylammonium chloride (CTAC) molecules which results in an overall positive surface charge. The sensitizer was $\text{Ru}(\text{bpy})_3^{2+}$ and the electron acceptor was $\text{C}_{14}\text{MV}^{2+}$. Because of its positive charge, the sensitizer would be expected to be repelled from the micelle/water interface. The large hydrophobic force associated with the long alkyl "tail" on the acceptor caused it to stay associated with the micelle. This system was flashed in a laser flash photolysis system and the formation and decay of viologen radical was

monitored. This represents charge separation and recombination in the absence of sacrificial electron donors. It was found that the back reaction was slowed down over two orders of magnitude in the presence of CTAC compared to the absence of CTAC. The back reaction was determined to be second-order. In this case surface repulsion of the oxidized sensitizer after charge transfer results in greatly extended lifetimes of charge separated species.

Another similar system utilized positively charged vesicles composed of dioctadecyldimethylammonium chloride (DODAC)⁽⁵¹⁾. The sensitizer was zinc tetramethylpyridyl porphyrin (ZnTPyP^{4+}) and the electron acceptor was again $\text{C}_{14}\text{MV}^{2+}$. Similar results were obtained during laser flash photolysis experiments. The rate of back electron transfer was over 50 times slower in the presence of the vesicles than in their absence. Again the sensitizer is repelled from the interface containing the acceptor after the charge separation event thus slowing down the rate of recombination.

We wished to study the interfacial effects on photoinduced charge separation and recombination in systems containing alkyl viologens adsorbed on the surface of dihexadecylphosphate (DHP) vesicles. This should help to provide some insight into the nature of viologen binding to DHP vesicles. The sensitizer used in these experiments was ZnTPPS^{4-} . The kinetics of the charge separation and recombination steps were studied and some of the results have been published⁽¹²⁾.

The work presented in this chapter will concentrate on the charge recombination step since in the published work this step could not be well characterized due to limitations in the transient digitizer

originally used. It was not able to run slow enough to capture the entire recombination waveform. By assessing the factors which affect the recombination rate it is hoped that insight will be gained into the interfacial organization of the redox components.

V.b) Laser Intensity Dependence: One way to vary the concentration of reactants involved in charge recombination is to vary the intensity of the laser flash using neutral density filters. This was expected to reduce the number of reactant pairs as the filter absorbance increased. The recombination step was analyzed to determine its reaction order. Curiously, the reaction can be fitted by two simultaneous first-order processes. It is also possible that adequate fits could have been obtained from the sum of a first- and second-order process but a program for this was not available. Possible explanations for the reaction order will be discussed in section V.f. The data for these experiments are given in Table 4-17.

The data were fit to the equation $y = A_0 + A_1 e^{-k(1)t} + A_2 e^{-k(2)t}$. It can be seen that there is a lot of variability in the k_1 step for a given viologen whereas the k_2 values are more reproducible and apparently independent of laser intensity. This helps to confirm that it is indeed a first order process. The pathway percentages determined by comparing A_1 and A_2 are also quite variable. The sum of A_1 and A_2 does not equal unity since the maximum voltage in the waveforms associated with recombination could not be determined due to competing viologen radical formation and recombination reactions. Pathway percentages can be calculated by normalizing $(A_1 + A_2)$ to one.

Table 4-17
Dependence of $C_1MV^+/ZnTPPS^{3-}$ recombination
rates on laser intensity ^a

Viologen	$[C_1MV^{2+}]$ (μM)	Filter Abs.	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})	Voltage change ^b
C_1MV^{2+}	78	0.0	0.63	566	0.18	132	20 mV
"	"	0.3	0.61	567	0.28	146	11 "
"	"	0.6	0.64	485	0.24	117	9 "
"	"	1.0	0.58	525	0.34	125	3 "
C_2MV^{2+}	74	0.0	0.30	698	0.56	151	17 mV
"	"	0.3	0.18	882	0.65	177	10 "
"	"	0.6	0.24	407	0.66	125	7 "
"	"	1.0	0.46	375	0.59	63	3 "
$C_{1/2}MV^{2+}$	76	0.0	0.22	726	0.79	109	17 mV
"	"	0.3	0.33	498	0.65	92	27 "
"	"	0.6	0.26	540	0.73	97	11 "
"	"	1.0	0.36	525	0.65	91	6 "
$C_{1/4}MV^{2+}$	77	0.0	0.54	369	0.75	89	12 mV
"	"	0.3	0.43	454	0.85	77	8 "
"	"	0.6	0.34	295	0.85	83	6 "
"	"	1.0	0.80	875	0.95	84	3 "
$C_{1/8}MV^{2+}$	79	0.0	0.66	278	0.64	63	10 mV
"	"	0.3	1.07	620	0.94	67	6 "
"	"	0.6	0.85	473	0.91	77	4 "
"	"	1.0	c	c	c	c	2 "
$C_{1/16}MV^{2+}$	82	0.0	0.56	340	0.63	84	9 mV
"	"	0.3	0.41	446	0.89	88	6 "
"	"	0.6	0.44	366	0.84	72	4 "
"	"	1.0	c	c	c	c	2 "

^a Solutions prepared in 20 mM Tris-HCl, pH 8.0, 2 mM DHP. Samples purged with Ar or N₂ for 10 min. before flashing. Typically, 64 laser flashes were averaged for each sample.

^b Proportional to initial amount of viologen radical produced.

^c Waveforms too noisy to be accurately analyzed.

The fits were generally quite good. A typical example is given in Figure 4-23. The general trend seems to be for the recombination rates to slow down with increasing viologen alkyl chain length. This is shown graphically for averaged data in Figure 4-24. This is reasonable because viologen radicals with longer alkyl tails are expected to be more deeply embedded in the bilayer and thus are less accessible to reactants in bulk solution. This is consistent with the dithionite reduction data shown in Figure 1-2 where bound viologen dication is reacting with a bulk solution component. The amount of radical initially produced by the laser flash decreased with increasing absorbance of the neutral density filter as expected.

V.c) ZnTPPS⁴⁻ Concentration Dependence: In this set of experiments the concentration of reactants was varied by adjusting the sensitizer concentration. Fewer sensitizer molecules results in the formation of fewer charge-separated pairs after the laser flash. The results from this work are displayed in Table 4-18.

Again the data were adequately fit by the equation $y = A_0 + A_1 e^{-k(1)t} + A_2 e^{-k(2)t}$ indicating two simultaneous processes. In general, the rate constants for a given viologen were unaffected by the ZnTPPS⁴⁻ concentration. Thus the 4 data points for each viologen were averaged and Figure 4-25 was generated. Once more the general trend was for the rate constants to decrease as the viologen alkyl chain length increased.

V.d) Viologen Concentration Dependence: In order to gain more insight into the rate-limiting step of the recombination reaction the dependence of k_1 and k_2 on the viologen concentration was examined. Unfortunately,

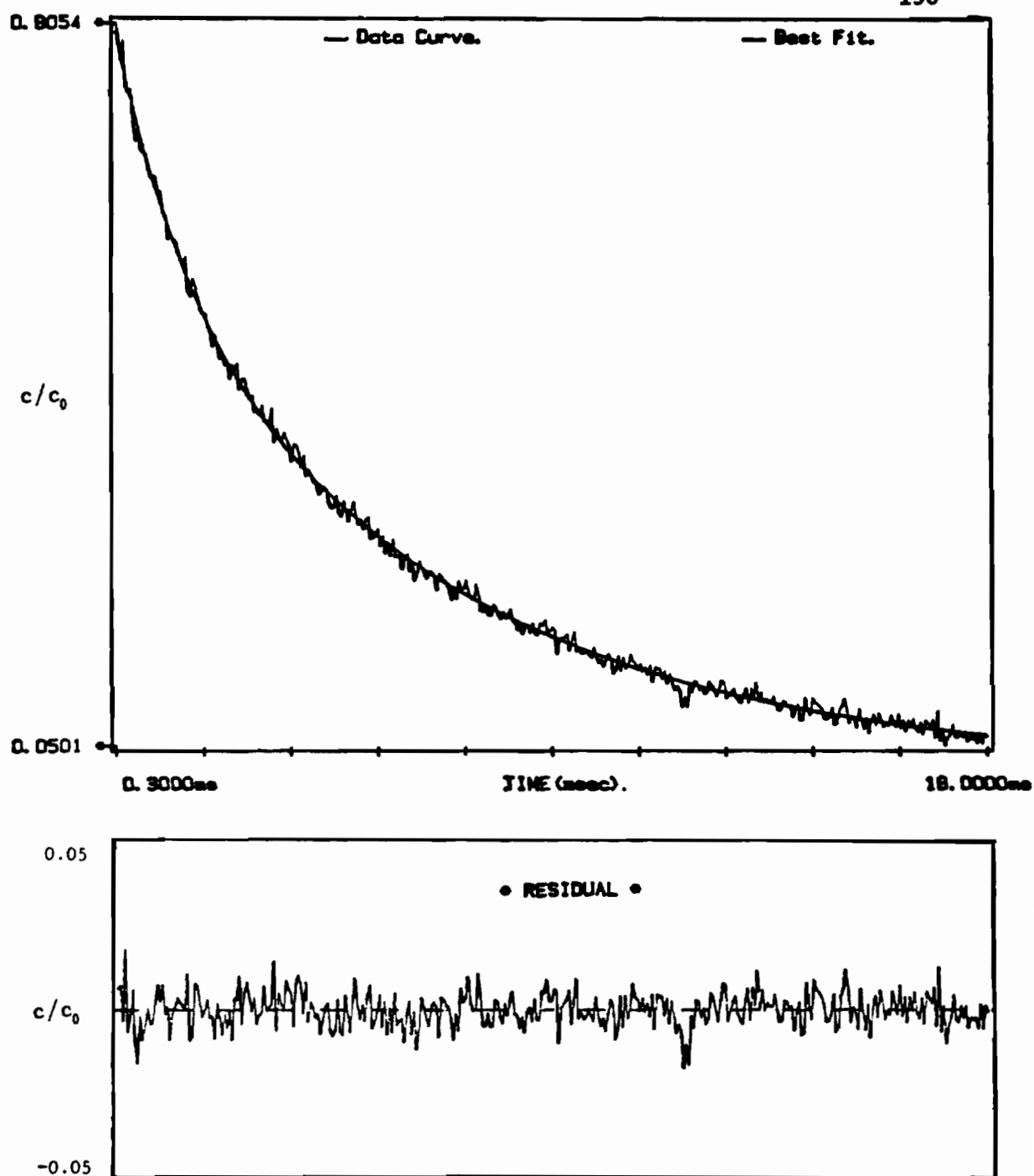


Figure 4-23: Typical laser flash data fit to the equation $A_0 + A_1 e^{-k(1)t} + A_2 e^{-k(2)t}$.

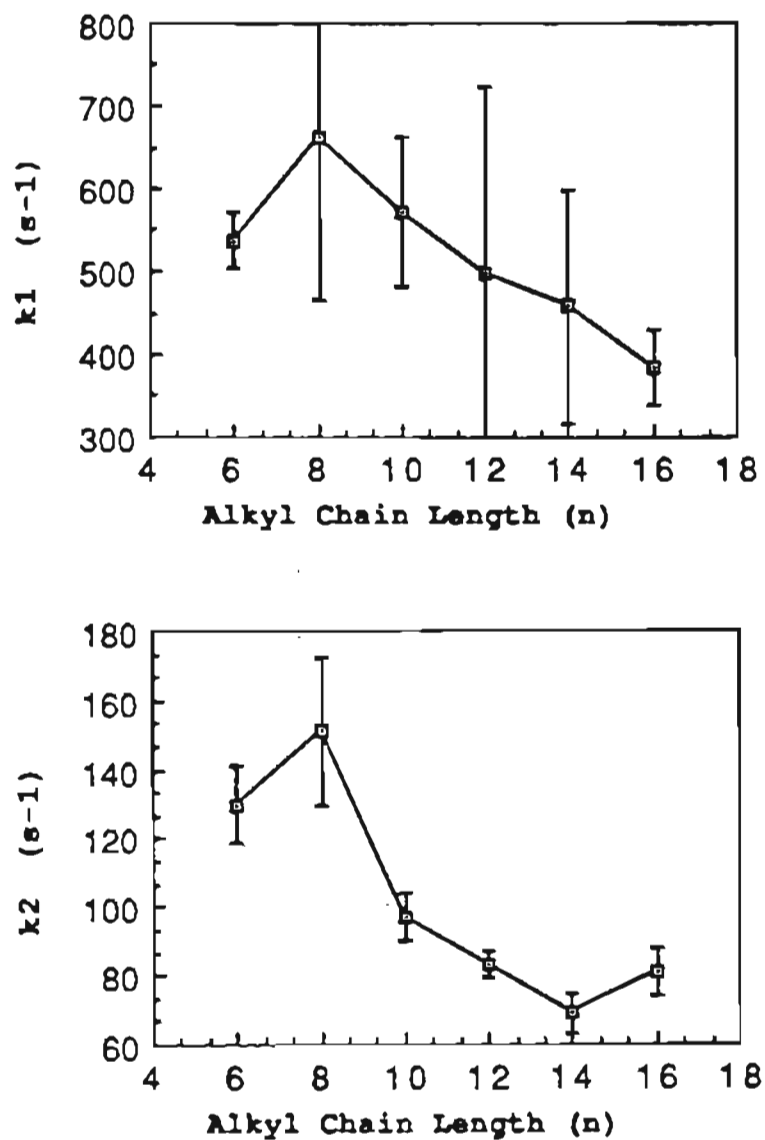


Figure 4-24: Dependence of rate constants for recombination on viologen alkyl chain length. Average of data from Table 4-17. Errors are standard deviations.

Table 4-18

Dependence of $C_0MV^+/ZnTPPS^{4-}$ recombination rates on initial $ZnTPPS^{4-}$ concentration ^a

Viologen	$[C_0MV^{2+}]$ (μM)	$[ZnTPPS^{4-}]$ (μM)	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})	Voltage change ^b
C_6MV^{2+}	131	18	0.55	642	0.33	158	17 mV
"	"	33	0.72	471	0.18	47	26 "
"	"	46	0.64	696	0.16	166	33 "
"	"	66	0.51	698	0.14	106	36 "
C_8MV^{2+}	116	23	0.56	471	0.28	104	17 mV
"	"	45	0.45	584	0.32	175	20 "
"	"	62	0.56	512	0.19	133	22 "
"	"	81	0.50	573	0.30	182	25 "
$C_{10}MV^{2+}$	75	23	0.47	396	0.57	85	23 mV
"	"	44	0.26	422	0.76	84	30 "
"	"	64	0.24	490	0.77	95	34 "
"	"	83	0.46	457	0.58	123	33 "
$C_{12}MV^{2+}$	67	23	0.77	557	0.79	109	11 mV
"	"	47	0.81	543	0.82	99	18 "
"	"	67	1.02	372	0.28	109	12 "
"	"	87	0.95	462	0.28	118	13 "
$C_{14}MV^{2+}$	75	24	0.78	562	0.81	126	7 mV
"	"	49	0.76	367	0.61	131	11 "
"	"	65	0.92	414	0.49	127	10 "
"	"	85	1.02	402	0.28	126	11 "
$C_{16}MV^{2+}$	82	19	0.72	486	0.82	104	6 mV
"	"	43	0.95	342	0.42	83	8 "
"	"	62	0.75	456	0.58	100	10 "
"	"	80	0.75	362	0.54	99	11 "

^a Solutions prepared in 20 mM Tris-HCl, pH 8.0, 2 mM DHP. Samples purged with Ar or N₂ for 10 min. before flashing. Typically, 64 laser flashes were averaged for each sample.

^b Proportional to initial amount of viologen radical produced.

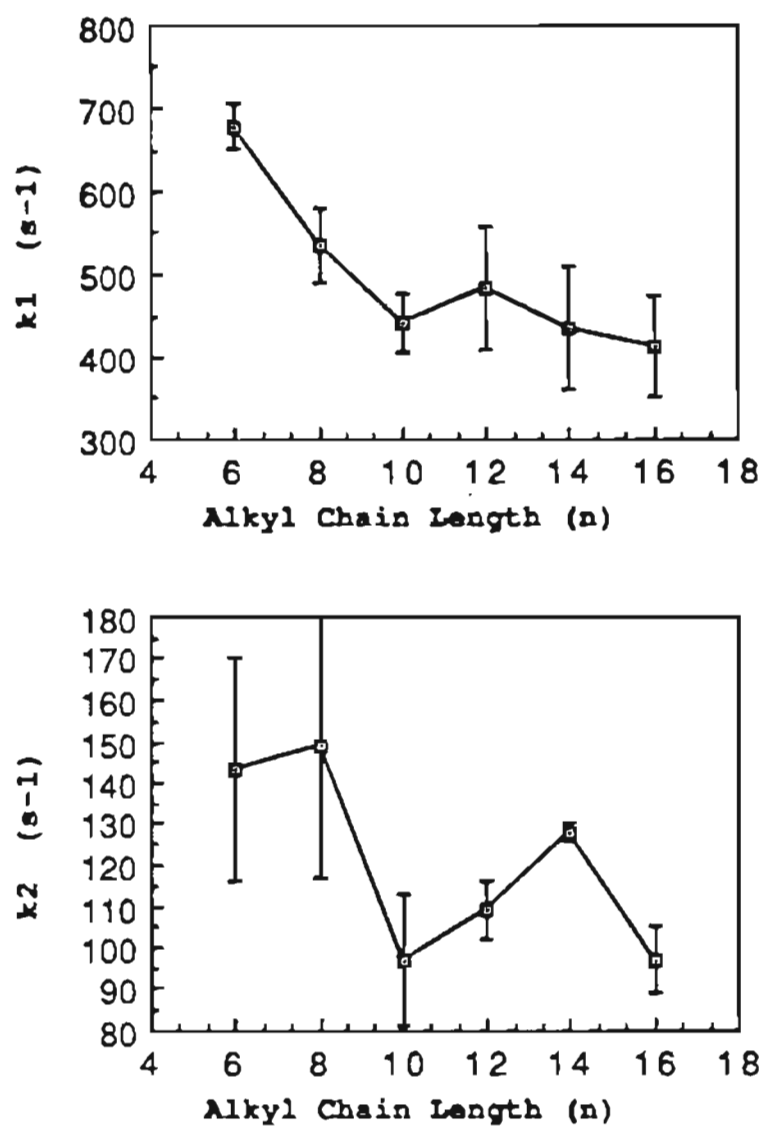


Figure 4-25: Dependence of rate constants for recombination on viologen alkyl chain length. Average of data in Table 4-18. Errors are standard deviations.

no discernable trends could be observed in these data. For the sake of completeness these data, along with some averaged data from Table 4-18, are presented in Table 4-19.

The amount of viologen radical initially formed is insensitive to the viologen concentration. This implies that within the viologen concentration range studied, the lifetime of the $^3\text{ZnTPPS}^{4-}$ species is long enough to react with a viologen molecule every time no matter what the viologen concentration. This in turn implies quantum yields near one since other deactivation pathways do not appear to compete on this timescale. This conclusion, though, is not consistent with the earlier experiments⁽¹²⁾.

V.e) Ionic Strength Dependence: For these experiments the recombination reaction was studied in buffer strengths of 5, 20, 50, 80, and 150 mM Tris-HCl at pH 8.0. The concentration of all other ions was kept constant. The purpose of these experiments was to determine whether or not the rate-determining step in the recombination reaction was between charged species. If the reaction was between like-charged species the rate would be expected to increase with increasing ionic strength; if it was between oppositely charged species the reaction rate should decrease with increasing ionic strength. The contribution of 2 mM DHP vesicles to the ionic strength at pH 8.0 has been previously determined to be 9 mM⁽¹²⁾.

The recombination reactions were again fitted to the sum of two exponential functions to obtain two first-order rate constants. A summary of the larger rate constant (k_1) is presented in Figure 4-26 and

Table 4-19

Dependence of C_1MV^+ /ZnTPPS³⁻ recombination rates
on viologen concentration ^a

Viologen	[C_1MV^{2+}] (μM)	[ZnTPPS ⁴⁻] (μM)	A_1	k_1 (s^{-1})	A_2	k_2 (s^{-1})	Voltage change ^b
C_6MV^{2+}	27	29	0.44	357	0.55	88	20 mV
"	60	27	0.69	321	0.36	77	18 mV
"	131	^c	0.61	627	0.20	119	^c
C_8MV^{2+}	23	30	0.71	273	0.42	72	16 mV
"	48	31	0.45	618	0.73	104	17 mV
"	116	^c	0.52	535	0.27	149	^c
$C_{10}MV^{2+}$	22	30	0.24	579	0.89	92	22 mV
"	51	32	0.38	437	0.69	88	25 mV
"	75	^c	0.36	441	0.67	97	^c
$C_{12}MV^{2+}$	34	28	0.64	87	0.51	38	8 mV
"	69	29	0.42	344	0.84	73	7 mV
$C_{14}MV^{2+}$	32	28	0.47	268	0.78	55	7 mV
"	75	^c	0.87	436	0.55	128	^c
$C_{16}MV^{2+}$	34	31	0.53	252	0.80	104	5 mV
"	62	34	0.62	548	0.90	79	6 mV
"	82	^c	0.79	412	0.59	97	^c

^a Solutions prepared in 20 mM Tris-HCl, pH 8.0, 2 mM DHP. Samples purged with Ar or N₂ for 10 min. before flashing. Typically, 64 laser flashes were averaged for each sample.

^b Proportional to initial amount of viologen radical produced.

^c Since no dependence on [ZnTPPS⁴⁻] was observed in Table 4-18, the data for this line represent the average of 4 samples from that table and the parameter in this column varied for each.

the smaller rate constant (k_2) in Figure 4-27. The two rate constants appear to be similarly affected by differing ionic strengths. A Debye-Hückel treatment of this data seems inappropriate since rate constants do not change progressively in the same direction with changing ionic strength.

Several qualitative trends can be seen in Figures 4-26 and 4-27. One trend is that, at the lower ionic strengths, the recombination rates for any given viologen increase with increasing ionic strength. The difference between 50 and 150 mM Tris-HCl, though, is negligible within experimental uncertainty. The reaction rates appear to have reached a maximum at 50 mM Tris-HCl. The other noticeable trend is that there is no rate dependence on the viologen alkyl chain length for the 5 and 20 mM Tris-HCl samples while the experiments run at 50, 80, and 150 mM Tris-HCl show a pronounced chain length dependence of the rate constants with the rate decreasing with increasing alkyl chain length.

The pre-exponential factors, A_1 and A_2 , varied considerably from sample to sample but showed no discernable trend. Qualitatively it appeared that the two recombination pathways each accounted for about one-half of the total reaction. At 5 mM Tris-HCl the slower pathway (k_2) disappeared altogether for C_6MV^{2+} and C_8MV^{2+} . The recombination curves for these cases were adequately fit by single exponential functions.

V.f) Mechanism: The data presented for this system provide much insight into the nature of the charge recombination process but are not sufficient for the proposal of an unambiguous mechanism. This section

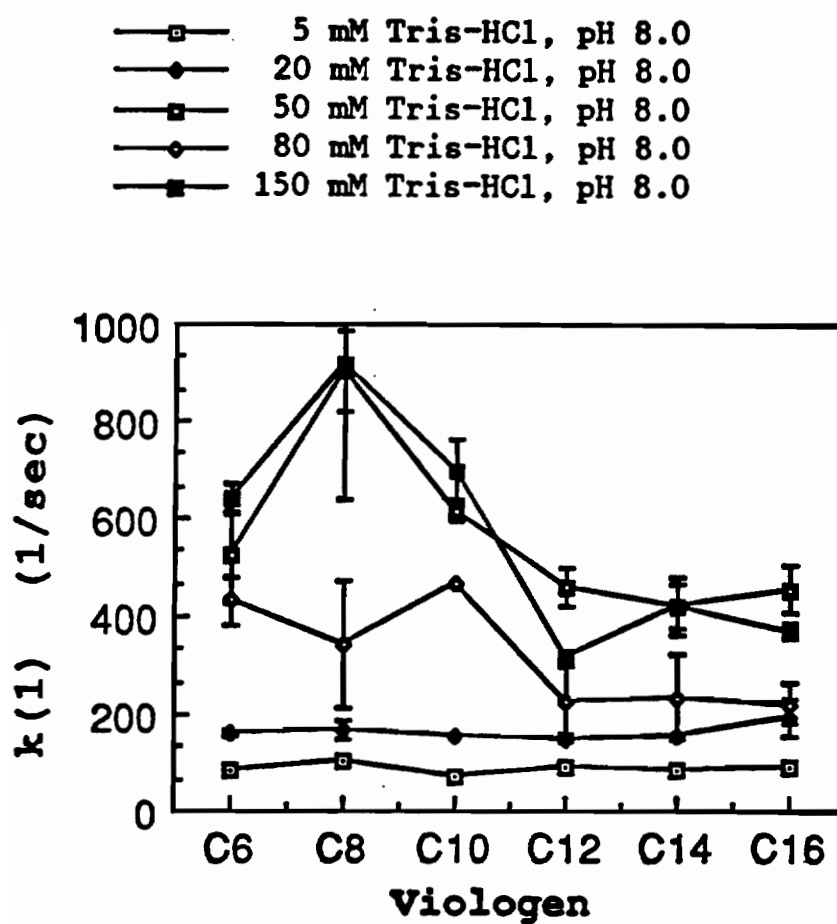


Figure 4-26: Ionic strength dependence of the fast (k_1) recombination rate constant for various alkyl chain length viologens. Errors are standard deviations.

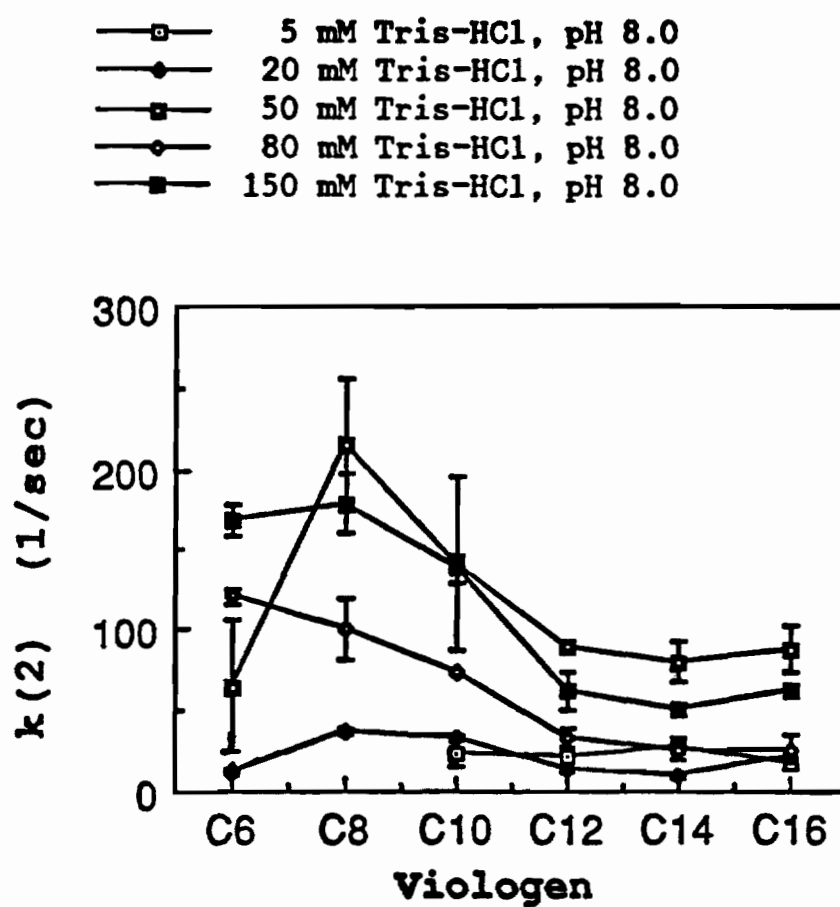


Figure 4-27: Ionic strength dependence of the slow (k_2) recombination rate constant for various alkyl chain length viologens. Errors are standard deviations.

will discuss possible reaction pathways which are supported by these data.

The fact that the data curves could be fitted to the sum of two exponential functions does not prove the existence of two first-order processes. Possibly, simultaneous first- and second-order functions would have also fit the data well (A computer program that would do this was not available at the time the data were analyzed). If the data curves are sums of exponential functions it needs to be determined if they are true first-order or pseudo-first-order processes.

A priori one might expect the recombination reaction to be a homogeneous second-order process with a first-order dependence on both C_1MV^+ and $ZnTPPS^{3-}$. This would be the case for these ions free in solution since the reactants are formed in pairs and the charge recombination is simply a result of a bimolecular collision. But neither of the two pathways observed for recombination appears to be of this nature. This is strongly supported by the fact that rate constants appear unchanged when the initial concentrations of reactants are varied (Tables 4-17,18). If these were truly second-order processes the first-order analysis would not give constant values for k_1 and k_2 under these conditions.

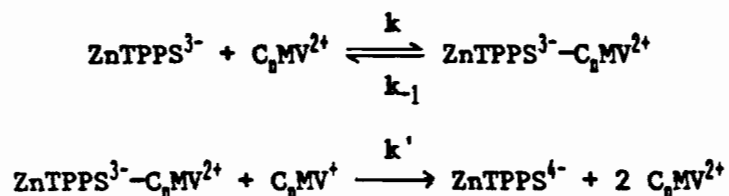
One mechanism which would show first-order kinetics involves diffusion of viologen radical from within the hydrophobic core of the bilayer to the vesicle surface as the rate-limiting step. One argument against this type of mechanism is the observed ionic strength dependence of the rate constants. Unless the membrane becomes progressively more fluid with increasing ionic strength, radical diffusion does not appear

to be a plausible rate-limiting process.

It is also known that bimolecular reactions occurring between reactants confined to small particles become first-order at low reactant concentrations (on the order of <10 reactant pairs per particle)^(52,53). In the present experiments the range of reactant pairs formed per vesicle ranges from about 5 to 30. This is probably too many reactant pairs to show strictly first-order kinetics. Also, the condition that the reactant pairs be confined to the vesicle is probably not fulfilled since the ZnTPPS^{3-} π -cation should be repelled from the anionic vesicle surface.

A reasonable explanation for the observed kinetics is that the recombination reaction consists of electron exchange between the ZnTPPS^{3-} π -cation in solution and C_6MV^{2+} on the vesicle surface which can rapidly supply an electron from C_6MV^+ radicals on the surface. This type of rapid electron exchange between surface bound viologens in micelles has been observed using EPR spectroscopy⁽⁵⁴⁾. This type of mechanism would be expected to show pseudo-first-order kinetics and would be first-order with respect to ZnTPPS^{3-} π -cation.

This mechanism implies initial formation of a $\text{ZnTPPS}^{3-}\text{-C}_6\text{MV}^{2+}$ ion pair followed by relatively rapid transfer of an electron from a C_6MV^+ ion. This can be expressed by the following equations:



Thus for disappearance of C_6MV^+ ,

$$-\frac{d[C_hMV^+]}{dt} = k'[ZnTPPS^{3-}-C_hMV^{2+}][C_hMV^+]$$

Next, apply the steady-state approximation to $[ZnTPPS^{3-}-C_hMV^{2+}]$,

$$\begin{aligned} \frac{d[ZnTPPS^{3-}-C_hMV^{2+}]}{dt} &= 0 = k[ZnTPPS^{3-}][C_hMV^{2+}] \\ &\quad - (k_1 + k'[C_hMV^+])[ZnTPPS^{3-}-C_hMV^{2+}] \\ [ZnTPPS^{3-}-C_hMV^{2+}] &= \frac{k[ZnTPPS^{3-}][C_hMV^{2+}]}{k_1 + k'[C_hMV^+]} \end{aligned}$$

Therefore,

$$\begin{aligned} -\frac{d[C_hMV^+]}{dt} &= k' \frac{k[ZnTPPS^{3-}][C_hMV^{2+}][C_hMV^+]}{k_1 + k'[C_hMV^+]} \\ &= k[C_hMV^{2+}][ZnTPPS^{3-}] \quad \text{when } k'[C_hMV^+] \gg k_1 \end{aligned}$$

Near the end of the reaction $k'[C_hMV^+]$ must become smaller than k_1 causing deviations from first-order behavior, but this would not be observable if the majority of the reaction is over at this point. Thus, if electron transfer is rapid relative to ion pair dissociation the overall reaction reduces to the following form:

$$\begin{aligned} -\frac{d[ZnTPPS^{3-}]}{dt} &= -\frac{d[C_hMV^+]}{dt} = \\ &k_1[C_hMV^{2+}_{(a)}][ZnTPPS^{3-}] + k_2[C_hMV^{2+}_{(b)}][ZnTPPS^{3-}] \end{aligned} \quad (4-16)$$

The two types of viologen, $C_1MV^{2+}_{(a)}$ and $C_1MV^{2+}_{(b)}$, might represent different binding configurations on the vesicle surface. Two kinetically distinct viologens were observed for the long alkyl chain ($n \geq 12$) viologens in studies of dithionite reduction of DHP-bound viologens⁽¹²⁾. Since the number of viologen radicals formed by a single laser flash only represents about 1-3% of the total viologen present, the direct reaction of $ZnTPPS^{3-}$ with C_1MV^+ could be insignificant if this rate was not significantly faster than that for the proposed recombination pathway. Also, the anionic $ZnTPPS^{3-}$ may prefer to approach the doubly charged C_1MV^{2+} over C_1MV^+ for electrostatic reasons. DHP-bound C_1MV^{2+} has been determined to have an effective negative charge of 10-12 from Debye-Hückel analysis^(1a,12). C_1MV^+ bound to DHP vesicles would be expected to have an even higher effective negative charge due to its smaller positive charge making approach of $ZnTPPS^{3-}$ more difficult.

The results from Tables 4-17 and 4-18 support this mechanism. Varying the concentration of charge-separated pairs would not affect the rate because there is no dependence on $[C_1MV^+]$ in the rate equation, and varying the concentration of $ZnTPPS^{3-}$ does not change the rate constants, just the magnitudes of the decay curves. We would, though, expect a dependence on the initial concentration of C_1MV^{2+} . Unfortunately, the results presented in Table 4-19 are ambiguous, with the rate constants sometimes increasing significantly with viologen concentration as expected and other times decreasing slightly. A more thorough study of this dependence may resolve this uncertainty.

The ionic strength results are also rather ambiguous. There is definitely a dependence upon ionic strength, which would be expected, but it appears to reach its maximum at about 50 mM Tris-HCl. The reason for this is not readily apparent from the proposed mechanism. It may be that the structure of the vesicles is being changed along with the ionic strength thus affecting the binding configurations of the viologens. The viologen may be becoming less accessible to ZnTPPS^{3-} as the ionic strength is increased offsetting the increased rates expected from the ionic strength effect. A definitive explanation will require further study of this system.

CHAPTER 5

MECHANISTIC CONCLUSIONS

The results described in the first four sections of Chapter 4 have given a fairly complete description of viologen-mediated transmembrane oxidation-reduction across DHP bilayers. We understand the dynamics of these systems well enough that we are able to predict the results for most new experiments. This section summarizes what we know about the dynamics of transmembrane redox in vesicles and will also discuss what details are still unknown.

There are two requirements for rapid transmembrane electron transfer. First, there must exist a facile pathway which allows for electron transfer itself. Secondly, there must be a membrane-permeable ion in the system which can readily translocate to dissipate the charge gradient which is generated by the electron transfer process. In the simplest system studied, containing equimolar MV^{2+} inside and outside of DHP vesicles, the electron transfer pathway appears to consist of electron exchange between inner MV^{2+} and outer MV^+ . The lipophilic ion, MV^+ , is already in the system. The overall electron transfer rate in this case appears to be limited by the electron transfer process, not the attending ion flow, since the reaction is second-order.

In the cases where C_1MV^+ is the charge-compensating ion it was observed that accumulation of viologen inside the vesicle resulted from the inward transfer of electrons. This in turn led to an increase in

the amount of dimerized viologen radical, presumably due in part to the increased effective concentration. This was most dramatic in the case of MV^{2+} . Externally localized MV^+ is probably not associated with the vesicle surface since MMB^+ , which is structurally similar to MV^+ , showed little binding by equilibrium dialysis. But after translocation into the vesicles the MV^+ radicals would be constrained to a volume approximately 1000-fold smaller. Apparently this high concentration, along with the unique environment provided by the vesicle interior, leads to high percentages of dimer. It is probable that the inner vesicle surface has a higher surface charge density and that the enclosed water is more highly ordered than in bulk solution, both of which would lead to increased binding affinity of MV^{2+} to the vesicle surface. The increased surface concentration would increase the percentage of viologen radical in the dimeric form. The longer alkyl chain viologens have a much stronger association with the DHP molecules and therefore never achieve the high percentage of dimer found in MV^{2+} systems. The strong interaction with DHP competes with the interactions which exist between the viologen radicals to form dimer.

In most cases this inward flow of viologen radical was able to compensate for transmembrane electron transfer at a sufficient rate such that it was not rate-limiting. Again, this was evidenced by the fact that the transmembrane reduction step was observed to be second-order not zero- or first-order as would be expected for ion diffusion. Only two cases were found where ion diffusion was limiting. The first case was with the longer alkyl chain viologens, near the end of the reaction. For example, DHP vesicles with equilibrium distributions of $C_{16}MV^{2+}$

inside and outside did not exhibit complete viologen reduction upon the addition of excess dithionite. The reduction of inner viologen appeared second-order at first but the tail end of the reaction deviated from the second-order curve. This was due to restriction of the flow of viologen radical into the vesicle, as was demonstrated by the fact that addition of the ionophore MMB^+ led to complete reduction of the system. The restriction of inward viologen radical migration in these cases was probably due to their strong association with the DHP bilayer. Since C_{16}MV^+ appears to be intercalated within the inner and outer monolayers of the vesicle, transfer of the radical from the outer to the inner monolayer increases the surface area of the inner leaflet relative to the outer leaflet. This translocation strains the vesicle structure. The tight packing in the inner monolayer may therefore restrict further incorporation of C_{16}MV^+ inside the vesicle.

The second case where lack of ion flow impeded electron flow was the system with MV^{2+} inside and MSPB^+ outside of DHP vesicles. In that case the MSPB^0 radical could mediate electron transfer but could not compensate for the charge gradient since it is neutral. In this system electron flow could be controlled stoichiometrically by ion flow. Addition of the ionophore MMB^+ allowed electron transfer to MV^{2+} inside to proceed.

The exact mechanism of the transmembrane redox step is still unknown. For these experiments it has been convenient to describe it as electron exchange between outer viologen radical and inner electron acceptor. This pathway is supported by the observation of second-order kinetics for this process. One might argue that catalytic amounts of

doubly reduced viologen, C_4MV^0 , could be present and that the reaction is between this molecule and the internal electron acceptor. This is unlikely since the concentration of the doubly reduced viologen is determined by the dithionite ion concentration, but the transmembrane reduction rates are independent of dithionite concentration.

Another possibility is diffusive exchange of inner electron acceptor with outer viologen radical; i.e. flip-flop. This process would be difficult to distinguish from electron exchange since the end result is the same. Intuitively though, it seems unlikely that molecules as different as MV^+ and $C_{10}MV^{2+}$ would undergo flip-flop especially since MV^+ does not appear to associate with the membrane. However, MV^+ mediates electron transfer to $C_{10}MV^{2+}$ entrapped inside DHP vesicles and to $Fe(CN)_6^{3-}$, which is not amphiphilic and therefore unable to participate in a flip-flop mechanism.

The question also arises when considering electron exchange whether or not some electron tunneling is involved. Tunneling over the whole bilayer thickness is much too slow according to theory, but if redox components diffused to within 10 angstroms or so this would be quite possible on the reaction timescale. The studies presented here provide no information on this process; no piece of experimental evidence refutes either possibility. However, the observation that the $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ systems contained a transmembrane redox pathway that was fast relative to that for C_4MV^{2+} suggests the possibility of a long-range electron tunneling mechanism. The diffusion of the longer alkyl chain viologens would be expected to be slower than the shorter chain viologens. Comparison of the slower rate components for $C_{12}MV^{2+}$ and

$C_{16}MV^{2+}$ with the transmembrane rates for MV^{2+} and C_6MV^{2+} in Table 4-16 indicate that the rates decrease with increasing alkyl chain length as expected. The faster components for $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ may represent an electron tunneling pathway not available in the MV^{2+} and C_6MV^{2+} systems because $C_{12}MV^{2+}$ and $C_{16}MV^{2+}$ are more deeply embedded in the membrane thus their distance of separation across the membrane is less and electron tunneling probabilities are correspondingly greater.

The determination of these finer points will require much more sensitive techniques, but for now our understanding is sufficient to predict the dynamics of electron and ion movement in these simple systems. Further work would be useful in the area of finding molecules which are able to undergo the electron exchange reaction more rapidly. Possibly the introduction of unsaturated alkyl chains either in the redox-active molecules or the amphiphiles which compose the bilayer would help to facilitate electron transfer.

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Appendix A

Data Transfer Program

This program allows transfer of waveform voltage data from a Nicolet 4094 mainframe oscilloscope to a DEC PRO-350 computer via a GPIB port. The computer was equipped with a Real Time Interface board which is supported by the RTI Library software. The HG.SYS driver must be loaded prior to running and the program must be linked with the HBLIB.SYS library (See pp 3-18 thru 3-20 in the RTI Library manual for details). Twelve data files can be stored on one floppy disk or thirteen if the SQUEEZE utility is run before addition of the last file. Note that if 0 is entered for the voltage at $t=0$ the analysis program will just use the highest voltage in the waveform for this parameter.

After the 5 modules were compiled with a FORTRAN IV compiler they were linked using the command file BILINK.COM by entering the command @BILINK. This file is as follows:

```
R LINK
BINK2=BINK2,HGLIB//
LIST2/O:1
DIREC2/O:1
IEEE2/O:1
READ2/O:1//
^C
```

BINK2.FOR

```

      program diskwrite
c
c   This program along with its subroutines reads in waveform data
c   from the Nicolet 4094 oscilloscope. It then puts that data onto a
c   floppy disk.
c
      byte filnam(15),dummy
      ithru=0
c
      write(5,*)'*Insert data disk in left drive.'
      write(5,*)'*Hit Return when ready. Enter Q to quit otherwise
+ run program all the way thru'
      read(5,80)dummy
      if(dummy.eq.'Q')go to 110
c
50    full=0.0
      call list(full)
      if(full.eq.2.0)write(5,*)'Disk full-Abort'           !print files
      if(full.eq.2.0)go to 110
      if(full.eq.1.0)write(5,*)'Did you SQUEEZE disk?'
      if(full.eq.1.0)read(5,80)dummy
      if(dummy.eq.'N')write(5,*)'Must SQUEEZE disk first-Abort'
      if(dummy.eq.'N')go to 110
c
60    write(5,*)'*Enter output filename DATAxx.DAT (xx=file no.)'
      read(5,70)(filnam(i),i=5,15)
      format(11a1)                                           !accept
c                                                         !filename
      write(5,72)(filnam(i),i=5,15)
      format(1x,'*Correct filename is: ',11a1,'(Y/N)?')
      read(5,74)dummy
      format(a1)
      if(dummy.eq.'N')go to 60
c
      filnam(1)=68
      filnam(2)=90                                           !create DZ0:
      filnam(3)=48                                           !DATAxx.DAT
      filnam(4)=58
c
      open(unit=10,name=filnam,type='UNKNOWN')
      close(unit=10)
      open(unit=10,name=filnam,type='OLD')
      read(10,80,end=90)dummy                                !check file
80    format(a1)
      write(5,*)'*Filename in use:Choose a new filename'
      close(unit=10)
      go to 60                                               !if in use
c                                                         !try again
90    close(unit=10)
c
      call ieee(filnam,iq,idat,ithru)                       !read data
c
      call readin(filnam,dummy,iq,idat)                     !read norm
c                                                         !from scope
      if(dummy.eq.'N')go to 100
c
      call direct(filnam)                                   !input title
c
100   write(5,*)'**** Data Read/Save Finished ****'
c
105   write(5,*)'Do you want to store another scan? (Y/N)'
      read(5,74)dummy
      if(dummy.eq.'Y')go to 50
      if(dummy.ne.'N')go to 105

```

```
c
110 write(5,*)'* Turn down screen brightness'
c
    stop
    end
```

LIST2.FOR

```

c      subroutine list(full)
c
c      This routine prints out the names of any waveforms already on disk.
c
c      byte title(32),dummy
c      integer n,filnum
c
c      open(unit=2,name='DZ0:direct.fil',type='UNKNOWN')      !checks to see
c      close(unit=2)                                           !if dir. file
c      open(unit=2,name='DZ0:direct.fil',type='OLD')           !already exists
c
c      read(2,10,end=20)dummy
c      format(a1)
c      goto 50
10
c      close(unit=2)                                           !if not then
c      write(5,*)'*New data disk'                               !print message
c      return
c
c      close(unit=2)
c      open(unit=2,name='DZ0:direct.fil',type='OLD')
c
c      read(2,60)n
c      format(i2)
c      if(n.eq.12)full=1.0
c      if(n.gt.12)full=2.0
c      write(5,*)
c      write(5,*)'                Already on Data Disk:'
c      write(5,*)'                File No.                Title'
c      write(5,*)
c      do 70 i=1,n
c          read(2,60)filnum
c          read(2,80)(title(j),j=1,32)
c          write(5,90)filnum,(title(j),j=1,32)
c
c      continue
c      format(32a1)
c      format(15x,i2,10x,32a1)
c      write(5,*)
c
c      close (unit=2)
c      return
c      end

```

DIREC2.FOR

```

      subroutine direct(filnam)
c
c   This routine keeps a directory file on the disk which
c   associates file numbers with titles.
c
c
      byte title(33),dummy,bnum(2),temp,otitle(20,32),filnam(15)
      integer n,filnum,oldfil(20),count,count1

```

```

c      itemp=filnam(10)-48
      ktemp=(filnam(9)-48)*10
      filnum=itemp+ktemp
c
c      write(5,*)
c
c      write(5,5)
5      format(1x,'*32nd char. at asterisk',8x,'*')
6      write(5,*)'*Input Title(32 char. max)'
      read(5,7)(title(i),i=1,32)
c      format(33a1)
c      if(title(33).ne.32)write(5,*)'*Title too long'
c      if(title(33).ne.32)go to 6
c
c      write(5,8)(title(i),i=1,32)
8      format(1x,'*Correct title is: ',32a1,'(Y/N)?')
9      read(5,9)dummy
9      format(a1)
9      if(dummy.eq.'N')go to 6
c
c      open(unit=2,name='DZ0:direct.fil',type='UNKNOWN')
c      close(unit=2)
c      open(unit=2,name='DZ0:direct.fil',type='OLD')
c
c      read(2,10,end=20)dummy
10     format(a1)
c      goto 50
c
c      close(unit=2)
c      open(unit=2,name='DZ0:direct.fil',type='NEW')
c      n=1
c      write(2,25)n
c      write(2,25)filnum
25     format(1x,i2)
c      write(2,30)(title(i),i=1,32)
c      format(1x,32a1)
c      close(unit=2)
c      return
c
c
c
50     close(unit=2)
c      open(unit=2,name='DZ0:direct.fil',type='OLD')
c      backspace 2
c      read(2,55)n
55     format(i2)
c      count1=0
c
c      format(32a1)
57     do 60 i=1,n
c         read(2,55)oldfil(i)
c         read(2,57)(otitle(i,j),j=1,32)
c         if(oldfil(i).lt.filnum) count1=count1+1
60     continue
c
c      close (unit=2)
c      open(unit=2,name='DZ0:direct.fil',type='NEW')
c      write(2,25)n+1
c
c      if(count1.gt.0) go to 65
c      write(2,25)filnum
c      write(2,30)(title(i),i=1,32)

```

!byte to
!integer

!read title
!check length

!check dir.
!file

!if it doesn't exist
!then create it and
!write in info.

!if dir file exists
!then read out old
!data and close.

!reopen dir file and

```

65      count=count1
      if(count1.eq.0) count=n
c
      do 70 i=1,count
        write(2,25)oldfil(i)
        write(2,30)(otitle(i,j),j=1,32)
70      continue
c
      if(count1.eq.0) go to 75
      write(2,25)filnum
      write(2,30)(title(i),i=1,32)
c
75      if(count1.eq.0.or.count1.eq.n) go to 90
      do 80 i=count+1,n
        write(2,25)oldfil(i)
        write(2,30)(otitle(i,j),j=1,32)
80      continue
c
90      close(unit=2)
c
      return
      end

```

IEEE2.FOR

```

      subroutine ieee(filnam,iq,idat,ithru)
c
      byte in(17),bpoint(2004),temp,start(5),filnam(15)
      integer*2 istat(2),ipoint(1002)
      equivalence(ipoint,bpoint(1))
c
      data in/68,44,52,44,48,44,48,48,48,48,48,44,57,57,48,44,52/
      iloop=1990
c
      if(ithru.eq.1)go to 13
c
      call ibinit(istat,,,2,1)
c
      istat=1
c
      write(5,*)'*Input quadrant no. (1-4)*
      read(5,15)iq
      format(i1)
      if(iq.lt.1.or.iq.gt.4)go to 13
c
      write(5,*)'* WAIT-WORKING'
c
      if(iq.eq.1)iadd=0
      if(iq.eq.2)iadd=1
      if(iq.eq.3)iadd=2
      if(iq.eq.4)iadd=3
c
      open(unit=10,name=filnam,type='NEW',initialsize=57)
c
      do 40 i=1,4
c
      if(i.eq.1)istart=0+iadd
      if(i.eq.2)istart=3960+iadd
      if(i.eq.3)istart=7920+iadd
      if(i.eq.4)istart=11880+iadd
c
      encode(5,20,start)istart
      format(i5)
c
      do 25 j=1,5
        if(start(j).lt.48)start(j)=48
c

```

!rewrite elements
!back into file
!inserting new data
!in proper place
!according to file
!number.

!init. GPIB

!determine
!start pt.

!integer to
!char. string

!put start pt.

```

                in(j+6)=start(j)                !into array
25      continue
c
        if(i.eq.4)in(15)=56
c
        call ibsend(istat,in,17,21,1+4,,10,14)    !send output
c
        if(i.ne.4)call ibrecv(istat,ipoint,1988,23,1,,14) !receive data
        if(i.eq.4)call ibrecv(istat,ipoint,2004,23,1,,14) !from scope
c
        if(i.eq.4)iloop=2004
        do 30 k=1,iloop,2                        !exchange high
            temp=bpoint(k)                        !and low bytes
            bpoint(k)=bpoint(k+1)
            bpoint(k+1)=temp
30      continue
c
        if(i.eq.1)idat=ipoint(3)
c
        do 35 k=0,98
            write(10,700)(ipoint(j+k*10),j=3,12)
35      continue                                !write data to
        if(i.eq.4)write(10,700)(ipoint(992+j),j=1,8) !disk file
700     format(1x,10i7)
c
40      continue
c
        close(unit=10)
c
        return
        end

```

READ2.FOR

```

        subroutine readin(filnam,dummy,iq,idat)
c
c      This routine will read the waveform parameters from the Nicolet 4094
c      oscilloscope.This information is then transfered to floppy disk.
c
        byte filnam(15),dummy,tp(10),in(60)
        real norm(17)
        integer istat(2)
c
        do 2 i=1,17
            norm(i)=0
2      continue
c
        tp(1)='N'
        tp(2)=','
c
        if(iq.eq.1)tp(3)=48
        if(iq.eq.2)tp(3)=49
        if(iq.eq.3)tp(3)=50
        if(iq.eq.4)tp(3)=51
c
        call ibsend(istat,tp,3,20,1+4,,10,14)
c
        call ibrecv(istat,in,60,21,1,,14)
c
        do 5 i=9,17
            tp(i-8)=in(i)
5      continue

```

```

        decode(9,700,tp)anorm7                                !get Unorm
        do 7 i=18,26
            tp(i-17)=in(i)
7        continue
        decode(9,700,tp)anorm8                                !get Hnorm
        do 9 i=27,32
            tp(i-26)=in(i)
9        continue
        c
        decode(6,800,tp)inorm9                                !get Uzero
700       format(e9.3)
800       format(i6)
        c
        do 11 i=33,38
            tp(i-32)=in(i)
11        continue
        decode(6,800,tp)inorm0                                !get Hzero(up)
        c
        do 13 i=39,44
            tp(i-38)=in(i)
13        continue
        decode(6,800,tp)inorm1                                !get Hzero(lo)
        c
        norm(7)=anorm7
        norm(8)=anorm8
        norm(9)=inorm9
        norm(10)=inorm0
        norm(11)=inorm1
        c
        first=float(idat-norm(9))*norm(7)
        write(5,57)first
57        format(1x,'*First data point of scan is '
+        ,f8.5,'volts? (Y/N)')
        read(5,58)dummy
58        format(a1)
        if(dummy.eq.'N')open(unit=10,name=filnam,type='OLD')
        if(dummy.eq.'N')close(unit=10,disp='DELETE')
        if(dummy.eq.'N')go to 110
        c
        write(5,*)'*Input offset voltage in mV'
        read(5,810)norm(16)
        write(5,*)'*Input Vmax in mV (True V at t=0)'
        read(5,810)norm(1)
        write(5,*)'*Input voltage at infinity in mV'
        read(5,810)norm(17)
810       format(f7.0)
        write(5,*)'*WAIT-WORKING'
        c
        filnam(12)=73
        filnam(13)=78
        filnam(14)=70
        c
        open(unit=11,name=filnam,type='NEW')
        c
        do 80 j= 1,6
            write(11,85)norm(j)
80        continue
85        format(1x,f9.2)
        c
        write(11,90)norm(7)
        write(11,90)norm(8)
90        format(1x,e10.4)
        c
        do 100 j=9,17
            write(11,85)norm(j)
100       continue
        c

```

```
close(unit=11)

c
110  if(dummy.eq.'Y')go to 120
      write(5,*)'If first data point was wrong then the wrong set'
      write(5,*)'of data has been read in. Try filling any empty'
      write(5,*)'quadrants of memory on the scope with artificial'
      write(5,*)'data and try to read your waveform again.'
c
120  continue
c
      return
      end
```

Appendix B

Kinetic Analysis Program

This program allows kinetic data obtained on the stopped-flow apparatus to be fit to one, two, or three exponential functions. The analysis is based on a non-linear fitting algorithm which originated with Levenberg and later, independently, with Marquardt. The computer algorithm used here is an adaptation of the program CURFIT by Bevington⁽⁵⁵⁾. The analysis requires the input of approximate values for the variable parameters to begin. Successive improvements continue until a convergence criterion is satisfied.

The goodness-of-fit can be evaluated quantitatively by the variance which is the average squared deviation between the data and best-fit curves. It can also be evaluated qualitatively by observing the residual which can uncover systematic deviations between the two curves which is indicative of fitting to an improper functional form. The results can then be plotted on a Hewlett-Packard 7470A two-pen plotter.

The program was run on a DEC PRO-350 running under the RT-11 operating system. It was compiled and linked as follows:

Each subroutine file, *.FOR, was compiled using the FORTRAN IV compiler with the command

FORT *.FOR/LIST/NOLINE

The 7 compiled modules were then linked in an overlay

configuration such that the different modules are swapped in and out of computer memory as they are used by the program. This was done with the following command file, SHLINK.COM, which was run using the command @SHLINK.

```
R LINK
KMAIN1=KMAIN1//
FIT1/0:1
SUP1/0:1
REDIN1/0:2
REGID1/0:2
CUR1/0:2
PICK1/0:2//
^C
```

```

      program fitplot
c
c   This program allows the user to perform a best fit analysis
c   on the voltage data by converting to absorbance then fitting
c   to the equation  $y=A_0+A_1\exp(-kt)$ ,  $y=A_0+A_1\exp(-k_1t)+A_2\exp(-k_2t)$ ,
c   or  $y=A_0+A_1\exp(-k_1t)+A_2\exp(-k_2t)+A_3\exp(-k_3t)$ . Picking the
c   section of the data considered to be good is aided by
c   on-screen graphics. The final results can then be output
c   to the plotter.
c
      byte filnam(15),dummy,title(32),bell
      virtual idat(3969),ttrace(3968)
      real rnorm(17)
      integer dp,status(2)
      double precision chisqu
      common a(7)
c
c
      lun=13
      call giopen(status,lun,0,1,6,0)           !open gidis
c
      call readin(filnam,vmax,idat,rnorm,title) !read in data
c
      call fitdat(idat,rnorm,vmax,ttrace,istart !find best fit
+ ,istop,chisqu)
c
      write(5,*)
      write(5,*)'*Do you wish to use the plotter?(Y/N) '
      read(5,190)dummy
190  format(a1)
      if(dummy.eq.'N')go to 200
c
      call super(rnorm,title,ttrace,istart,istop,chisqu) !plot waveform
c
      bell=7
      write(5,195)bell
195  format(1x,a1)
c
      call giclos(status,lun)
c
200  stop
      end

```

FIT1.FOR

```

c
c   FUNCTION FUNTN
c   FUNCTION FUNTN(I,VAR)
c   DIMENSION VAR(7)
c   FUNTN=VAR(1)+VAR(2)*EXP(-VAR(3)*I)+VAR(4)*EXP(-VAR(5)*I)
+ +VAR(6)*EXP(-VAR(7)*I)
c   END
c
c   *****
c
c   SUBROUTINE FITDAT(IDAT,RNORM,UMAX,TTRACE,ISTART,ISTOP,CHISQU)
c   A PROGRAM TO ANALYSE DATA RECORDED BY THE PROGRAM BINK.FOR.
c   THE DATA MAY BE FIT TO ONE EXPONENTIAL,
c    $Y = A_0 + A_1 \exp(-K_1 T)$ 
c   TO A SUM OF TWO EXPONENTIALS,

```

```

C      Y = A0 + A1*EXP(-K1*T) + A2*EXP(-K2*T)
C      OR TO A SUM OF THREE EXPONENTIALS,
C      Y = A0 + A1*EXP(-K1*T) + A2*EXP(-K2*T) + A3*EXP(-K3*T)
C      THE DATA FITTING IS A NON-LINEAR LEAST SQUARES METHOD OF MARQUARDT
C      AND IS AN ADAPTATION OF THE ROUTINE CURFIT FOUND IN:
C      "DATA REDUCTION AND ERROR ANALYSIS FOR THE PHYSICAL SCIENCES"
C      BY PHILIP R.BEVINGTON,MC GRAW-HILL BOOK CO. (1969)
C
C      VARIABLES
C      IDAT      THE 3968 DATA POINTS OF THE TRACE WHICH ARE READ
C                IN FROM DATAxx.DAT.
C      RATE      CONTAINS THE RECORDING RATES FOR EACH TRACE.
C      VMAX      CONTAINS THE t=0 VOLTAGE FOR THE WAVEFORM.
C      OFFSET    CONTAINS THE OFFSET VALUE FOR EACH TRACE.
C      A         IS THE ARRAY OF FITTED PARAMETERS.
C      TTRACE    IS THE ARRAY CONTAINING THE NORMALIZED REPRESENTATION
C                OF THE DATA READINGS.
C      IGUESS    IS AN ARRAY CONTAINING THE VARIABLES WHICH ARE
C                REPRESENTING THE PARAMETERS.
C
C      BYTE BELL,DECIDE,FIXX,SUB
C      DOUBLE PRECISION CHISQU,CHISQ1
C      INTEGER TRACE
C      REAL START,STOP,RNORM(17)
C      VIRTUAL TTRACE(3968),IDAT(3968)
C      COMMON A(7)
C      DIMENSION RATE(1),VOLTS(1),OFFSET(1),IGUESS(7)
C      DATA BELL/7/
C      DATA IGUESS/'A0','A1','k1','A2','k2','A3','k3'/
C
C      OFFSET(1)=RNORM(16)/1000.0
C      RATE(1)=RNORM(8)
C      HOFF=RNORM(10)*65536.0+RNORM(11)
C      TRACE=1
C      VMAX=(VMAX-RNORM(9))*RNORM(7)
C      IF(RNORM(1).NE.0)VMAX=RNORM(1)/1000.0
C      IF(RNORM(1).EQ.0)WRITE(5,*)'VMAX USED WAS CALCULATED'
C      VINP=RNORM(17)/1000.0
C      SUB=27
C
C      BOTTOM=ALOG10((VMAX+OFFSET(1))/(VINP+OFFSET(1)))
C      DO 100 I=1,3968
C        TEMP=(FLOAT(IDAT(I))-RNORM(9))*RNORM(7)
C        TOP=ALOG10((TEMP+OFFSET(1))/(VINP+OFFSET(1)))
C        TTRACE(I)=TOP/BOTTOM
100    CONTINUE
C
C      REQUEST STARTING POINT AND STEP SIZE FOR DATA ANALYSIS.
C      INPUT INITIAL VALUES FOR PARAMETERS A.IF A(4)=A(5)=0 NUMBER
C      OF UNKNOWN,NTERMS=3,WHICH RESULTS IN A SINGLE EXPONENTIAL,
C      NTERMS=5 GIVES THE SUM OF TWO EXPONENTIALS AND NTERMS=7
C      GIVES THE SUM OF THREE EXPONENTIALS.
C
C      WRITE(5,175)BELL
C      WRITE(5,*)
C      WRITE(5,*)'*Make sure printer is turned on.'
C      WRITE(5,*)
105    WRITE(5,*)'*Enter D to display ln(delta A), enter M to manually
+ input start and stop pts.'
      READ(5,110)DECIDE
110    FORMAT(A1)
      IF(DECIDE.EQ.'M')GO TO 170
      IF(DECIDE.NE.'D')GO TO 105
      CALL PICK(TTRACE,RNORM,START,STOP,HOFF)
      GO TO 185

```

```

C      THRU=0
170    WRITE(5,*)
      WRITE(5,175)BELL
175    FORMAT(1X,A1)
      TYPE 180
180    FORMAT(1X,'*Enter: Starting pt., Stop pt. (In msec)? ',*)
      READ(5,182)START,STOP
182    FORMAT(F7.0,F7.0)
185    WRITE(5,*)
      WRITE(5,*)'*Input step size(i.e. 1 for every pt., 2 for
+ every other pt., etc)'
      READ(5,187)ISTEP
187    FORMAT(I3)
      STEP=ISTEP*RNORM(8)*1000.0
      IF(START.EQ.0)START=RATE(1)*1000.0
      ISTART=IFIX(START/(RATE(1)*1000.0))+HOFF
      ISTOP=IFIX(STOP/(RATE(1)*1000.0))+HOFF
      WRITE(5,*)ISTART,ISTOP,ISTEP
C
      THRU=THRU+1
      IF(THRU.EQ.1)GO TO 193
188    WRITE(5,*)'*Do you want to use previous A and k values for
+ initial guesses?(Y/N)'
      READ(5,110)DECIDE
      IF(DECIDE.EQ.'N')GO TO 193
      IF(DECIDE.NE.'Y')GO TO 188
      NTERMS=7
      DO 189 I=1,NTERMS
        WRITE(5,330)IGUESS(I),A(I)
189    CONTINUE
      WRITE(5,*)
      GO TO 215
C
193    TYPE 195
195    FORMAT(1X,'*Fitting function=',//,5x,'Y= A0 + A1*exp-k1*t
1    +A2*exp-k2*t + A3*exp-k3*t',//,' *Initial guesses:')
      DO 200 I=1,7
        TYPE 210,IGUESS(I)
210    FORMAT(1X,A2,'=? ',*)
        ACCEPT *, A(I)
200    CONTINUE
      NTERMS=7
      IF (A(4).EQ.0.0) NTERMS=3
      IF (A(6).EQ.0.0.AND.A(4).NE.0.0) NTERMS=5
C
C      TO SIMPLIFY CALCULATIONS THE RATE PARAMETERS ARE EXPRESSED IN UNITS
C      OF 1/POINT RATHER THAN 1/SEC.IN THIS WAY THE TIME VALUE
C      CORRESPONDING TO A PARTICULAR TTRACE(I) IS JUST I.THE TIME
C      POINTS MUST BE EVENLY SPACED TO BE ABLE TO DO THIS.
C      CALCULATE CHI SQUARE AND ALLOW OPERATOR TO DECIDE BETWEEN
C      CHOOSING A NEW STARTING POINT OR PROCEEDING TO FIT THE DATA
C      POINTS BY CALLING ROUTINE CURFIT.
C
215    A(3)=A(3)*RATE(TRACE)
      A(5)=A(5)*RATE(TRACE)
      A(7)=A(7)*RATE(TRACE)
      FLAMDA=.002
      ITIMES=0
      CHISQU=0.0
      DO 220 II=ISTART,ISTOP,ISTEP
        K=II
        FTR=TTRACE(II)
        CHISQU=CHISQU + (FTR-FUNTN(K,A))**2
220    CONTINUE
      NFREE=(ISTOP-ISTART)/ISTEP

```

```

      NFREE=NFREE-NTERMS
      CHISQU=CHISQU/NFREE
      TYPE 240,BELL,CHISQU
240  +  FORMAT(1X,A1,'*var.=',E10.3,/,', *Proceed(P) or New guesses(N) '
      +  ,$,)
      ACCEPT 242,DECIDE
242  +  FORMAT(A1)
      IF(DECIDE.EQ.'N')A(3)=A(3)/RATE(1)
      IF(DECIDE.EQ.'N')A(5)=A(5)/RATE(1)
      IF(DECIDE.EQ.'N')A(7)=A(7)/RATE(1)
      IF(DECIDE.EQ.'N') GO TO 105
C
C   ROUTINE CURFIT MAKES A LEAST SQUARES FIT TO A NON-LINEAR FUNCTION
C   WITH A LINEARIZATION OF THE FITTING FUNCTION.
C
245  +  CALL CURFIT(NFREE,NTERMS,FLAMDA,CHISQ1,CHISQU,ISTART,ISTEP,
      +  TTRACE,ISTOP,FIXX,HOFF)
C
      IF(ITIMES.GT.0)GO TO 247
      WRITE(5,*)
      WRITE(5,*)'          var.          k1          k2          k3'
      WRITE(5,*)
247  +  TYPE 250,CHISQ1,A(3)/RATE(1),A(5)/RATE(1),A(7)/RATE(1),BELL
250  +  FORMAT(4X,E11.4,3(4X,E10.3),A1)
C
C   COMPARE THE NEW CHI SQUARE(CHISQ1) WITH THE OLD CHI SQUARE
C   (CHISQU).REPEAT DATA FITTING WITH NEW CHI SQUARE UNTIL
C   TWO SUCCESSIVE CHI SQUARE VALUES DIFFER FROM EACH OTHER
C   BY LESS THAN .05% OR UNTIL 10 CALLS TO CURFIT HAVE BEEN MADE.
C
      IF(CHISQU-CHISQ1 .LE. .0005*CHISQU) GO TO 300
      CHISQU=CHISQ1
      ITIMES=ITIMES+1
      IF (MOD(ITIMES,10) .NE. 0) GO TO 245
      TYPE 260, ITIMES
260  +  FORMAT(1X,'*Fit did not converge after ',I2,' iterations')
265  +  WRITE(5,*)'*ENTER C TO CONTINUE, S TO STOP'
      READ(5,270)DECIDE
270  +  FORMAT(A1)
      IF(DECIDE.EQ.'C')GO TO 245
      IF(DECIDE.NE.'S')GO TO 265
300  +  TYPE 310,BELL,BELL
310  +  FORMAT(1X,2A1,'*Final values:',/)
      CHISQU=CHISQ1
      A(3)=A(3)/RATE(TRACE)          !GIVE DIMENSIONS 1/SEC
      A(5)=A(5)/RATE(TRACE)
      A(7)=A(7)/RATE(TRACE)
      DO 320 I=1,NTERMS
          TYPE 330,IGUESS(I),A(I)          !,SIGMAA(I)
          FORMAT(1X,A2,'=',E10.3)          !,'+or-'E10.3)
330  +
320  +  CONTINUE
C
C   WRITE BEST FIT PARAMETERS ON PRINTER
      WRITE (6,*)'
      WRITE (6,*)'
      WRITE (6,336) RATE(TRACE),UMAX,CHISQU
      WRITE (6,333) START,STOP,STEP
      WRITE (6,*)
      WRITE (6,337) (IGUESS(I),I=1,7)
      WRITE (6,334) (A(I),I=1,7)
C
333  +  FORMAT(2X,'Start pt.= ',f7.1,' msec, Stop pt.= ',f7.1,'msec,
      +  Step = ',f7.2,' msec')
334  +  FORMAT(1X,7(1X,E10.3))
335  +  FORMAT(1X,'+ OR - ',5(2X,E10.3))
336  +  FORMAT(2X,' RATE=',E10.3,'SEC-1', ' UMAX=',

```

```

337 + E10.3,' VAR.=',E10.3)
C      FORMAT(3X,7(A2,9X))
C
C      DO 406 I=1,5
C      WRITE(6,405)(SUB,J=1,100)
405      FORMAT(1X,100A1,'K')
406      CONTINUE
C
410      WRITE(5,*)
C      WRITE(5,*)'*Enter R for residual, S to skip'
C      READ(5,500)DECIDE
C      IF(DECIDE.EQ.'R')GO TO 420
C      IF(DECIDE.NE.'S')GO TO 410
C      GO TO 450
420      CALL REGID(TTRACE,ISTART,ISTOP,RNORM)
C      GO TO 450
C
450      WRITE(5,*)'*Do you want to try again?(Y/N)'
C      READ(5,500)DECIDE
500      FORMAT(A1)
C      IF(DECIDE.EQ.'Y')GO TO 105
C      IF(DECIDE.NE.'N')GO TO 450
C
550      RETURN
C      END

```

187

SUP1:FOR

```

C
C      subroutine super(rnorm,title,ttrace,istart,istop,chisqu)
C
C      This routine will output the normalized data to the plotter
C      superimposed over the best-fit curve from the analysis.
C      The residual is plotted on the reverse side of the paper.
C      (Logical unit 8 is assigned to XC: in the startup file.)
C
C      virtual ttrace(3968)
C      byte title(32),dummy,bell,xon,esc
C      real rnorm(17)
C      double precision chisqu
C      common a(7)
C
C      offset=rnorm(16)/1000.0
C      Vinf=rnorm(17)/1000.0
C      hoff=rnorm(10)*65536.0+rnorm(11)
C      residl=0
C      bell=7
C      xon=17
C      esc=27
C
C      write(5,*)'*MAKE SURE plotter is on, has paper, 2 pens.'
C      write(5,*)'*Switch junc. box to A.'
C      write(5,*)
C      write(5,*)'*Hit return when ready'
C      read(5,3)dummy
3      format(a1)
C
4      write(5,*)'*Computer will beep when plot is finished.'
C
C      write(8,*)'IN;'
C      write(8,800)esc
C      !initialize
800      format(1x,a1,'.H80;0;17:')
C      write(8,810)esc

```

```

810      format(1x,a1,'.N;19:')                                !handshake init.
      write(8,820)xon
820      format(1x,a1)
      if(residl.eq.0)write(8,*)'SP2;'                        !grab right pen
      if(residl.eq.1)write(8,*)'SP1;'                        !grab left pen
c
      write(5,*)'*Starting plot'
c
      if(residl.eq.1)go to 6
      write(8,*)'PU;PA3000,7540;DT*;LBTITLE:*'              !print title
      write(8,5)(title(i),i=1,32)
      format(1x,'LB',32a1,'*SP1')
5
c
6      write(8,*)'PU;PA1450,7200;PD;PA1450,450;'            !draw window
      write(8,*)'PA9850,450,9850,7200,1450,7200;PU;'
      write(8,*)'PA5200,100;PD;DT*;LBTIME(sec);PU'          !label x axis
      write(8,7)rnorm(8)*(istart-hoff)
7      format(1x,'PA1350,100;DTs;LB',f8.4,'sPA9000,100;')
      write(8,10)rnorm(8)*(istop-hoff)
10     format(1x,'DTs;LB',f9.4,'sPU;')
c
      if(residl.eq.0)go to 16
      write(8,*)'PU;PA4900,7525;DT*;LB*'                    !title for residual
      write(8,*)'LB RESIDUAL *'
c
16     if(residl.eq.1)go to 20
c
20     write(8,*)'PA1500,450;PD;XT;'                          !increment x axis
      do 30 i=1,10
         write(8,*)'PU;PR830,0;PD;XT;'
         k=0.02
30     continue
c
      if(residl.eq.1)go to 120
c
      write(8,*)'PU;PA25,7325;DT;';
      if(a(6).eq.0)write(8,*)'PA500,7325;';
      if(a(6).eq.0.and.a(4).eq.0)write(8,*)'PA2000,7325;';
      write(8,35)(a(i),i=1,3)
35     format(1x,'LB A0=',f7.4,' A1=',f7.4,' k1=',f7.4,'(1/s)')
      if(a(4).eq.0)go to 45
      write(8,40)(a(i),i=4,5)
40     format(1x,'LB A2=',f7.4,' k2=',f7.4,'(1/s)')
      if(a(6).eq.0)go to 45
      write(8,43)(a(i),i=6,7)
43     format(1x,'LB A3=',f7.4,' k3=',f7.4,'(1/s)')
c
45     write(8,*)'PU;PA1000,7540;DT;';                        !write var & rate
      write(8,47)chisqu, rnorm(8)*1000.0
47     format(1x,'LB Var.=',e10.4,'
      +      Rate=',f8.3,'(msec/pt)')
c
      write(8,*)'PU;PA3500,7000;PD;PA3700,7000;PU;'
      write(8,*)'PA3700,6975;DT.;LB Data Curve.'
      write(8,*)'SP2;PA7500,7000;PD;PA7700,7000;PU;'
      write(8,*)'PA7700,6975;LB Best Fit.SP1;'
c
      dmax=-1.0E30
      dmin=1.0E30
      do 50 i=istart,istop,10
         value=ttrace(i)
         if(value.gt.dmax)dmax=value
         if(value.lt.dmin)dmin=value
50     continue
c
      write(8,*)'PU;PA300,450;DT*;LB'

```

```

55      write(8,55)dmin,dmax                                     !label y axis
      format(1x,f9.5,'*';PA300,7100;LB',f9.5,'*')
      write(8,*)'PU;PA1450,7150;PD;YT;PU;
      write(8,*)'PA1450,500;PD;YT;PU;PU;
c
      times=0
c
60      width=float(istop-istart)
      shift=((9800-1500)*float(istart))/width
      icount=0
      do 100 i=istart,istop,10                                !istart to istop
          icount=icount+1
          time=((9800.0-1500.0)/width)*i-shift+1500.0
          t=rnorm(8)*(i-hoff)
          best=a(1)+a(2)*exp(-a(3)*t)+a(4)*exp(-a(5)*t)+
+ a(6)*exp(-a(7)*t)
          if(times.eq.1)data=best
          if(times.eq.0)data=ttrace(i)
          yplot=((7150.0-500.0)/(dmax-dmin))*(data-dmin)+500.0
          if(i.ne.istart+10)go to 75
          write(8,*)'PD;
75      write(8,70)time,yplot                                  !plots waveform data
70      format(1x,'PA',f6.0,',',f6.0,',') !on plotter
          if(times.eq.1)go to 100
          if(mod(icount,10).ne.0.and.i.ne.istop)go to 100
100     continue
c
          if(times.eq.1)go to 110
          write(8,*)'SP2;PU;                                     !switch pens
c
          times=times+1
          go to 60                                              !plot best fit
c
110     write(8,*)'PU;SP;PA0,0;
          do 112 i=1,30
              write(8,*)';;;;;;;;;;;;;;;' !flush buffer
112     continue
c
          write(5,115)bell
115     format(1x,a1)
          write(5,*)'*Turn over paper in plotter for residual'
          write(5,*)'*Hit Return when ready'
          read(5,3)dummy
          residl=1
          go to 4
c
120     dmax=0.0325
          dmin=-0.0325
          write(8,*)'IN;SP2;
c
          write(8,*)'PU;PA1800,7250;DT.;
          write(8,*)'LB Y-AXIS:ONE INCH = ONE HUNDRETH UNIT.'
          write(8,*)'PU;LT2;PA1450,3825;PD;PA9850,3825;PU;LT;
c                                                         !draws zero
c                                                         !line
          write(8,*)'PU;
c
          icount=0
          width=float(istop-istart)                            !plot residual
          shift=((9800-1500)*float(istart))/width
          do 200 i=istart,istop,10
              icount=icount+1
              time=((9800.0-1500.0)/width)*i-shift+1500.0
              t=(i-hoff)*rnorm(8)
              dat=ttrace(i)
              resid=dat-(a(1)+a(2)*exp(-a(3)*t)+a(4)*exp(-a(5)*t)+
+ a(6)*exp(-a(7)*t))

```

```

        yplot=((7150.0-500.0)/(dmax-dmin))*(resid-dmin)+500.0
        write(8,180)time,yplot
180      format(1x,'PA',f6.0,' ',f6.0,';PD;')
200      continue
        write(8,*)'PU;SP;PA0,0;'
        write(8,*)';;;;;;;;;;;;;;'
        write(8,*)';;;;;;;;;;;;;;' !flush buffer
c
        return
        end

```

REDIN1.FOR

```

        subroutine readin(filnam,vmax,idat,rnorm,title)
c
c   This subroutine allows input of a data file name to be analyzed.
c   The data file is then read into idat and rnorm.
c
        byte filnam(15),dummy,title(32),bell
        virtual idat(3969),ttrace(3968)
        real rnorm(17)
c
60      write(5,*)'*To analyse file data,'
        write(5,*)'*Enter filename DATAxx.DAT (xx=file no.)'
        read(5,70)(filnam(i),i=5,15)
70      format(11a1)                                !accept filename
c
        write(5,72)(filnam(i),i=5,15)
72      format(1x,'*Correct filename is: ',11a1,'(Y/N)?')
        read(5,74)dummy
74      format(a1)
        if(dummy.eq.'N')go to 60
c
        write(5,*)'*WAIT-WORKING'
c
        filnam(1)=68
        filnam(2)=90                                !create DZ0:DATAxx.DAT
        filnam(3)=48
        filnam(4)=58
c
        open(unit=10,name=filnam,type='UNKNOWN')
        close(unit=10)
        open(unit=10,name=filnam,type='OLD')
        read(10,80,end=90)dummy                      !check file
80      format(a1)
        go to 100
90      write(5,*)'*File doesn't exist:Choose a new filename'
        close(unit=10)
        go to 60                                    !if empty try again
c
100     rewind 10
c
        vmax=-32000.0
c
        do 102 i=0,395
            read(10,106)(idat(i*10+j),j=1,10)
102     continue
106     format(10i7)
c
        read(10,106)(idat(3960+i),i=1,8)
        close(unit=10)
c
        do 108 i=1,3968

```

```

108      if(idat(i).gt.vmax)vmax=idat(i)
      continue
c
      filnam(12)=73
      filnam(13)=78
      filnam(14)=70
      filnam(15)=0
c
      open(unit=11,name=filnam,type='OLD')
c
      do 110 i=1,6
        read(11,115)rnorm(i)
110      continue
115      format(f9.2)
c
      read(11,120)rnorm(7)
      read(11,120)rnorm(8)
120      format(e10.4)
c
      do 130 i=9,17
        read(11,115)rnorm(i)
130      continue
      close(unit=11)
c
      itemp=filnam(10)-48
      ktemp=(filnam(9)-48)*10
      inum=itemp+ktemp
      open(unit=20,name='DZ0:direct.fil',type='OLD')
      read(20,131)n
131      format(i2)
      ifile=0
      do 135 j=1,n
        if(ifile.eq.inum)go to 135
        read(20,131)ifile
        read(20,132)(title(i),i=1,32)
132      format(32a1)
135      continue
      close(unit=20)
c
      return
      end

```

191

!change .DAT to .INF

!read in title

REGID1.FOR

```

      subroutine regid(ttrace,istart,istop,rnorm)
c
c   This subroutine will display the residual for a quick check of
c   fit acceptability. It can also superimpose the data curve and
c   the best-fit curve.
c
      byte dummy,bell
      virtual ttrace(3968)
      real rnorm(17)
      integer status(2),mess(10)
      common a(7)
c
      bell=7
      sup=0
      hoff=rnorm(10)*65536.0+rnorm(11)
c
      write(5,*)
      write(5,*)' Y axis +/- .03 units, X axis every 10th pt.
+ shown, Hit Return to cont.'

```

```

40      if(sup.eq.1)write(5,*)'          Data and best-fit overlaid.'
      if(sup.eq.1)write(5,*)'
+      Hit Return to cont.'
      if(sup.eq.0)write(5,*)
      do 50 i=1,19
          write(5,*)
50      continue
      c
      dmax=0.03
      dmin=-0.03
      c
      write(5,60)((istart-hoff)*rnorm(8),(istop-hoff)*rnorm(8))
60      format(1x,f8.4,27x,'Time(sec)',24x,f8.4)
      c
      c
      lun=13
      c
      mess(1)=2308
      mess(2)=1000
      mess(3)=300
      mess(4)=10300
      mess(5)=7200
      call giwrit(status,lun,mess,5)
      c
      mess(1)=7426
      mess(2)=1000
      mess(3)=800
      call giwrit(status,lun,mess,3)
      mess(1)=6408
      mess(2)=1000
      mess(3)=7000
      mess(4)=11300
      mess(5)=7000
      mess(6)=11300
      mess(7)=800
      mess(8)=1000
      mess(9)=800
      call giwrit(status,lun,mess,9)
      if(sup.eq.1)go to 450
      c
      mess(1)=7426
      mess(2)=1000
      mess(3)=3900
      call giwrit(status,lun,mess,3)
      mess(1)=6402
      mess(2)=11300
      mess(3)=3900
      call giwrit(status,lun,mess,3)
      c
450      do 500 i=1000,11300,1030
          mess(1)=7426
          mess(2)=i
          mess(3)=6950
          call giwrit(status,lun,mess,3)
          mess(1)=6402
          mess(2)=i
          mess(3)=7050
          call giwrit(status,lun,mess,3)
500      continue
      if(sup.eq.0)go to 710
      c
600      dmax=-1.0E30
      dmin=1.0E30
      do 700 i=istart,istop,10
          t=(i-hoff)*rnorm(8)
          b=a(1)+a(2)*exp(-t*a(3))+a(4)*exp(-t*a(5))+a(6)*exp(-t*a(7))

```

192

!set window

!draw window

!draw zero
!residual

!increment
!X axis

```

        value=b+10*(ttrace(i)-b)
        if(value.gt.dmax)dmax=value
        if(value.lt.dmin)dmin=value
700      continue
c
710      width=float(istop-istart)
      shift=((11300-1000)*float(istart))/width
      do 1000 i=istart,istop,10
        time=((11300.0-1000.0)/width)*i-shift+1000.0
        t=(i-hoff)*rnorm(8)
        dat=ttrace(i)
        best=a(1)+a(2)*exp(-a(3)*t)+a(4)*exp(-a(5)*t)+
+ a(6)*exp(-a(7)*t)
        resid=dat-best
        if(sup.eq.1)resid=best
        if(sup.eq.2)resid=dat
        yplot=((7000.0-800.0)/(dmin-dmax))*(resid-dmax)+800.0
        if(i.gt.istart)go to 900
        mess(1)=7426
        mess(2)=ifix(time)
        mess(3)=ifix(yplot)
        call giwrit(status,lun,mess,3)
        go to 1000
c
900      mess(1)=6402
        mess(2)=ifix(time)
        mess(3)=ifix(yplot)
        call giwrit(status,lun,mess,3)
1000     continue
c
        mess(1)=7168
        call giwrit(status,lun,mess,1)
        mess(1)=6144
        call giwrit(status,lun,mess,1)
c
        sup=sup+1
        if(sup.eq.2)go to 710
c
        read(5,1010)dummy
1010     format(a1)
c
        if(sup.eq.3)go to 1100
        write(5,*)'*Do you want to see data and best curve super
+imposed?(Y/N) '
        read(5,1010)dummy
        write(5,*)
        write(5,*)
        if(dummy.eq.'Y')go to 40
c
1100     return
        end

```

193

!plot

!2nd and 3rd

!time thru

!plot both

!actual

!plotting

!flush

!buffers

CURL.FOR

```
C
C      SUBROUTINE CURFIT ADAPTED FROM BEVINGTON(1969).MAKES A LEAST-SQUARES
C      FIT TO A NON-LINEAR FUNCTION WITH A LINEARIZATION OF THE FITTING
C      LINE.
C
C      VARIABLES.
C          I          ARRAY OF DATA POINTS FOR INDEPENDENT VARIABLE.
C          TTRACE     ARRAY OF DATA POINTS FOR DEPENDENT VARIABLE.
C          NTERMS     NUMBER OF PARAMETERS.
C          A          ARRAY OF PARAMETERS.
C          FLAMDA     PROPORTION OF GRADIENT SEARCH INCLUDED.
C
C      SUBROUTINE FDERIV CALCULATES THE DERIVATIVES OF THE FITTING
C      FUNCTION FOR THE I TH TERM WITH RESPECT TO EACH PARAMETER.
C      SUBROUTINE MATINV INVERTS A SYMMETRIC TWO DIMENSIONAL MATRIX
C      OF DEGREE NTERMS AND CALCULATES ITS DETERMINANT.
C
C      SUBROUTINE CURFIT(NFREE,NTERMS,FLAMDA,CHISQ1,CHISQU,ISTART,
+  ISTEP,TTRACE,ISTOP,FIXX,HOFF)
C
C      BYTE FIXX
C      DOUBLE PRECISION CHISQU,CHISQ1
C      COMMON A(7)/BLK/ARRAY(7,7)
C      VIRTUAL TTRACE(3968)
C      DIMENSION ALPHA(7,7),BETA(7),DERIV(7),B(7)
C      NO WEIGHTING USED
31  DO 34 J=1,NTERMS
C          BETA(J)=0.0
C          DO 34 K=1,J
C              ALPHA(J,K)=0.0
34  CONTINUE
41  DO 50 I=ISTART,ISTOP,ISTEP
C          KT=I-HOFF
C          CALL FDERIV(KT,NTERMS,A,DERIV)
C          DO 46 J=1,NTERMS
C              FTR=TTRACE(I)
C              BETA(J)=BETA(J)+(FTR-FUNTN(KT,A))*DERIV(J)
C              DO 46 K=1,J
C                  ALPHA(J,K)=ALPHA(J,K) + DERIV(J)*DERIV(K)
46  CONTINUE
50  CONTINUE
51  DO 53 J=1,NTERMS
C          DO 53 K=1,J
C              ALPHA(K,J)=ALPHA(J,K)
53  CONTINUE
C
C      EVALUATE CHISQU AT STARTING POINT OUTSIDE SUBROUTINE.
C      INVERT MODIFIED CURVATURE MATRIX TO FIND NEW PARAMETERS.
C
71  DO 74 J=1,NTERMS
C          DO 73 K=1,NTERMS
C              ARRAY(J,K)=ALPHA(J,K)/(SQRT(ALPHA(J,J)*ALPHA(K,K)))
73  CONTINUE
C          ARRAY(J,J)=1.0+FLAMDA
74  CONTINUE
80  CALL MATINV(NTERMS,DET,TTRACE)
81  DO 84 J=1,NTERMS
C          B(J)=A(J)
C          DO 84 K=1,NTERMS
C              B(J)=B(J)+BETA(K)*ARRAY(J,K)/SQRT(ALPHA(J,J)
+  *ALPHA(K,K))
84  CONTINUE
C
C      IF CHISQU INCREASED INCREASE FLAMDA AND TRY AGAIN
C
88  CHISQ1=0.0
```

```

DO 92 I=ISTART,ISTOP,ISTEP
      KT=I-HOFF
      FTR=TTRACE(I)
      CHISQ1=CHISQ1 + (FTR-FUNTN(KT,B))*2
92  CONTINUE
      CHISQ1=CHISQ1/NFREE
      IF (CHISQ1 .LE. CHISQU) GO TO 101
      FLAMDA=FLAMDA*10.
      GO TO 71

C
C      EVALUATE PARAMETERS, ERRORS AND RETURN
C
101  DO 103 J=1, NTERMS
      A(J) = B(J)
103  CONTINUE
      FLAMDA=FLAMDA/10
110  RETURN
      END

C
C *****
C
C      FDERIV CALCULATES THE DERIVATIVES OF THE FITTING FUNCTION FOR
C      THE I-TH TERM WITH RESPECT TO EACH PARAMETER.
C
      SUBROUTINE FDERIV(I, NTERMS, A, DERIV)
      DIMENSION A(7), DERIV(7)
      DERIV(1)=1
      DERIV(2)=EXP(-A(3)*I)
      DERIV(3)=-I*A(2)*DERIV(2)
      DERIV(4)=0.
      DERIV(5)=0.
      DERIV(6)=0.
      DERIV(7)=0.
      IF (NTERMS.GE.5) DERIV(4)=EXP(-A(5)*I)
      IF (NTERMS.GE.5) DERIV(5)=-I*A(4)*DERIV(4)
      IF (NTERMS.EQ.7) DERIV(6)=EXP(-A(7)*I)
      IF (NTERMS.EQ.7) DERIV(7)=-I*A(6)*DERIV(6)
      RETURN
      END

C
C *****
C
C      SUBROUTINE MATINV INVERTS A SYMMETRIC TWO-DIMENSIONAL MATRIX OF
C      DEGREE NTERMS AND CALCULATES ITS DETERMINANT.
C
C      PARAMETERS: ARRAY-INPUT MATRIX REPLACED BY ITS INVERSE
C                  NORDER-DEGREE OF MATRIX
C                  DET-DETERMINANT OF ARRAY
C      VALID FOR MATRIX UP TO ORDER 6
C
C      FIND LARGEST ELEMENT

      SUBROUTINE MATINV(NORDER, DET, TTRACE)
      VIRTUAL TTRACE(3968)
      DIMENSION IK(7), JK(7)
      COMMON A(7)/BLK/ARRAY(7,7)
      DET=1.
10  DO 100 K=1, NORDER
11      AMAX=0.
          ! FIND LARGEST ELEMENT
21      DO 30 I=K, NORDER
          DO 30 J=K, NORDER
23          IF (ABS(AMAX)-ABS(ARRAY(I,J))) 24,24,30
24          AMAX=ARRAY(I,J)
          IK(K)=I
          JK(K)=J
30      CONTINUE
C

```

196

```

C      INTERCHANGE ROWS & COLUMNS TO PUT AMAX IN ARRAY(K,K)
C
31      IF (AMAX) 41,32,41
32      DET=0.
      GO TO 140
41      I=IK(K)
      IF(I-K) 21,51,43
43      DO 50 J=1,NORDER
          SAVE=ARRAY(K,J)
          ARRAY(K,J)=ARRAY(I,J)
          ARRAY(I,J)=-SAVE
50      CONTINUE
51      J=JK(K)
      IF(J-K) 21,61,53
53      DO 60 I=1,NORDER
          SAVE=ARRAY(I,K)
          ARRAY(I,K)=ARRAY(I,J)
          ARRAY(I,J)=-SAVE
60      CONTINUE
C
C      ACCUMULATE ELEMENTS OF INVERSE MATRIX
C
61      DO 70 I=1,NORDER
          IF (I-K) 63,70,63
63      ARRAY(I,K)=-ARRAY(I,K)/AMAX
70      CONTINUE
71      DO 80 I=1,NORDER
          DO 80 J=1,NORDER
              IF (I-K) 74,80,74
              IF (J-K) 75,80,75
              ARRAY(I,J)=ARRAY(I,J)+ARRAY(I,K)*ARRAY(K,J)
74      CONTINUE
75      CONTINUE
80      DO 90 J=1,NORDER
          IF (J-K) 83,90,83
83      ARRAY(K,J)=ARRAY(K,J)/AMAX
90      CONTINUE
      ARRAY(K,K)=1.0/AMAX
      DET=DET*AMAX
100     CONTINUE
C
C      RESTORE ORDERING OF MATRIX
C
101     DO 130 L=1,NORDER
          K=NORDER-L+1
          J=IK(K)
          IF (J-K) 111,111,105
105     DO 110 I=1,NORDER
          SAVE=ARRAY(I,K)
          ARRAY(I,K)=-ARRAY(I,J)
          ARRAY(I,J)= SAVE
110     CONTINUE
111     I=JK(K)
          IF (I-K) 130,130,113
113     DO 120 J=1,NORDER
          SAVE=ARRAY(K,J)
          ARRAY(K,J)=-ARRAY(I,J)
          ARRAY(I,J)=SAVE
120     CONTINUE
130     CONTINUE
140     RETURN
      END

```

PICK1.FOR

```
      subroutine pick(ttrace,rnorm,start,stop)
c
c   This routine will display ln delta A and magnify it which will allow
c   the user to input start and stop points for the analysis.
c
      virtual ttrace(3968),rdat(3968)
      real rnorm(17)
      integer status(2),isect(2),mess(9)
      byte dummy
c
      lun=13
      calc=0
      hoff=rnorm(10)*65536.0+rnorm(11)
c
      write(5,*)'*Wait 30 seconds for plot'
10      do 20 i=1,17
         write(5,*)
20      continue
         write(5,30)(i,i=1,10)
30      format(2x,10(5x,i2))
         write(5,31)-hoff*rnorm(8),(3968.0-hoff)*rnorm(8)
31      format(5x,f9.2,21x,'Time(sec)',24x,f9.2)
         write(5,*)
         write(5,*)
         write(5,*)
         write(5,*)'*Enter the X-axis section number(1-10) that you
+ want magnified'
c
      mess(1)=2308
      mess(2)=1000
      mess(3)=300
      mess(4)=10300
      mess(5)=5200
      call giwrit(status,lun,mess,5)
c
      mess(1)=7426
      mess(2)=1500
      mess(3)=1000
      call giwrit(status,lun,mess,3)
      mess(1)=6408
      mess(2)=1500
      mess(3)=5000
      mess(4)=9800
      mess(5)=5000
      mess(6)=9800
      mess(7)=1000
      mess(8)=1500
      mess(9)=1000
      call giwrit(status,lun,mess,9)
c
      do 100 i=1500,9800,830
         mess(1)=7426
         mess(2)=i
         mess(3)=950
         call giwrit(status,lun,mess,3)
         mess(1)=6402
         mess(2)=i
         mess(3)=5050
         call giwrit(status,lun,mess,3)
100      continue
         if(calc.eq.1)go to 210
c
      dmax=-1.0E30
      dmin=1.0E30
c
```

```

do 200 i=1,3968
  b=tttrace(i)
  if(b.le.0)rdat(i)=-1000.0
  if(b.le.0)go to 200
  rdat(i)=alog(b)
  if(rdat(i).gt.dmax)dmax=rdat(i)
  if(rdat(i).lt.dmin)dmin=rdat(i)
200 continue
c
210 init=1
c
do 300 i=1,3968,10
  time=((9800.0-1500.0)/3968.0)*i+1500.0
  if(rdat(i).eq.-1000.0)go to 300
  yplot=((5000.0-1000.0)/(dmin-dmax))*(rdat(i)-dmax)+1000.0
  if(init.eq.0)go to 220
    mess(1)=7426
    mess(2)=ifix(time)
    mess(3)=ifix(yplot)
    call giwrit(status,lun,mess,3)
    init=0
    go to 300
c
220 mess(1)=6402
    mess(2)=ifix(time)
    mess(3)=ifix(yplot)
    call giwrit(status,lun,mess,3)
300 continue
c
    mess(1)=7168
    call giwrit(status,lun,mess,1)
c
    read(5,1000)isect(1)
1000 format(i2)
    write(5,*)isect(1)
c
    isect(2)=0
    width=400
    offset=0
    iscold=isect(1)
    call draw(rdat,isect,rnorm,width,offset,start,stop,hoff)
c
    if(isect(2).eq.0)width=40
    if(isect(2).ne.0)width=80
    offset=(iscold-1)*400
    call draw(rdat,isect,rnorm,width,offset,start,stop,hoff)
    calc=1
c
1005 write(5,*)'*Hit Return to find another pt., Enter D if done
+ finding stop/start pts.'
    read(5,1010)dummy
1010 format(a1)
    if(dummy.eq.'D')go to 1020
    go to 10
c
1020 return
end
c
c *****
c
c subroutine draw(rdat,isect,rnorm,width,offset,start,stop,hoff)
c
c This subroutine draws the appropriate section.
c
c virtual rdat(3968)
c real rnorm(17)

```

```

byte dummy
integer status(2),isect(2),mess(10)

c
lun=13
ioff=ifix(offset)

c
if(width.eq.400)mult=400
if(width.ne.400)mult=40

c
ibegin=ioff+(isect(1)-1)*mult
iend=ibegin+ifix(width)

c
if(width.eq.40.or.width.eq.80)go to 40
do 20 i=1,20
  write(5,*)
20  continue
  write(5,23)(i,i=1,10)
23  format(2x,10(5x,i2))
  write(5,25)(ibegin-hoff)*rnorm(8)*1000,(iend-hoff)*rnorm(8)*1000
25  format(1x,f9.2,27x,'Time(msec)',23x,f9.2)
  write(5,27)rnorm(8)*1000
  write(5,*)
27  format(22x,'Time per pt. is ',f8.4,' msec')
  do 30 i=1,2
    write(5,*)
30  continue
  go to 100

c
40  tick=(ibegin-hoff)*rnorm(8)*1000
  write(5,47)(tick+(2.0*j+1)*width*100.0*rnorm(8),j=0,4)
47  format(5x,5(4x,f9.2))
  do 50 i=1,16
    write(5,*)
50  continue
  write(5,60)(tick+(2.0*j*(width*100.0*rnorm(8))),j=0,5)
60  format(3x,f9.2,5(4x,f9.2))
  write(5,*)
  write(5,65)rnorm(8)*1000,ifix(width)/10
65  format(22x,f7.3,'msec per point,',i1,' points per section')
  write(5,*)
  write(5,*)'*Enter A### for start or Z### for stop pt.'
+ ('### is no. in msec')
  write(5,*)'*Enter S to skip start/stop pt. entry'

c
100 mess(1)=7426
    mess(2)=1500
    mess(3)=1000
    call giwrit(status,lun,mess,3)
    mess(1)=6408
    mess(2)=1500
    mess(3)=5000
    mess(4)=9800
    mess(5)=5000
    mess(6)=9800
    mess(7)=1000
    mess(8)=1500
    mess(9)=1000
    call giwrit(status,lun,mess,9)

c
do 150 i=1500,9800,830
  mess(1)=7426
  mess(2)=i
  mess(3)=950
  call giwrit(status,lun,mess,3)
  mess(1)=6402
  mess(2)=i

```

!draw window

!increment
!x axis

```

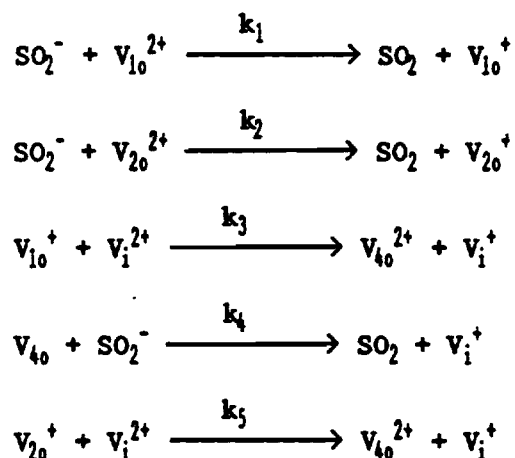
        mess(3)=5050
        call giwrit(status,lun,mess,3)
150    continue
c
        init=1
c
        dmax=-1.0E30
        dmin=1.0E30
c
        do 170 i=ibegin+1,iend+1
            if(i.gt.3968)go to 170
            if(rdat(i).eq.-1000)go to 170
            if(rdat(i).gt.dmax)dmax=rdat(i)
            if(rdat(i).lt.dmin)dmin=rdat(i)
170    continue
c
        do 200 i=ibegin+1,iend+1
            if(i.gt.3968)go to 200
            time=((9800.0-1500.0)/width)*(i-(ibegin+1))+1500.0
            if(rdat(i).eq.-1000.0)go to 200
            yplot=((5000.0-1000.0)/(dmin-dmax))*(rdat(i)-dmax)+1000.0
            if(init.eq.0)go to 190
                mess(1)=7426
                mess(2)=ifix(time)
                mess(3)=ifix(yplot)
                call giwrit(status,lun,mess,3)
                init=0
                go to 200
c
190        mess(1)=6402
        mess(2)=ifix(time)
        mess(3)=ifix(yplot)
        call giwrit(status,lun,mess,3)
200    continue
c
        mess(1)=7168
        call giwrit(status,lun,mess,1)
c
        if(width.eq.400)go to 210
        go to 203
202    write(5,*)'*Enter A**** for start or Z**** for stop pt.(****
+ is no. in msec)'
        write(5,*)'*Enter S to skip start/stop pt. entry'
203    read(5,205)dummy,point
205    format(a1,f7.0)
        if(dummy.eq.'S')go to 206
        if(dummy.ne.'A'.and.dummy.ne.'Z')write(5,*)'*You forgot prefix'
        if(dummy.ne.'A'.and.dummy.ne.'Z')go to 202
        if(dummy.eq.'A')start=point
        if(dummy.eq.'Z')stop=point
206    write(5,207)start,stop
207    format(5x,'start= ',f9.2,' msec. stop= ',f9.2,' msec')
        write(5,*)'*Hit Return to continue, Enter R to reenter
+ start/stop point'
        read(5,208)dummy
208    format(a1)
        if(dummy.eq.'R')go to 202
        go to 250
c
210    write(5,*)'*Enter 1 or 2 sect. #'s(If 2 separate w/ comma)'
        read(5,220)isect(1),isect(2)
220    format(i2,i2)
        if(isect(2).eq.0)go to 250
        if(((isect(2)-isect(1)).ne.1)write(5,*)'*Sections must be
+ consecutive'
        if(((isect(2)-isect(1)).ne.1)go to 210
c
250    return
        end

```

Appendix C

Kinetic Simulation Program

This program allows actual voltage data from the stopped-flow apparatus to be compared to simulated data from any given mechanism consisting of the series of reaction equations. These equations must be edited into the program subroutine SIM4.FOR. The equations in the program record the changes in concentration of each chemical species over some small increment of time, dt . For example, given the mechanism shown in Figure 4-14:



from these equations we can write

$$\begin{aligned}
 d[\text{V}_{10}^{2+}] &= (-k_1[\text{SO}_2^-][\text{V}_{10}^{2+}])dt \\
 d[\text{V}_{20}^{2+}] &= (-k_2[\text{SO}_2^-][\text{V}_{20}^{2+}])dt \\
 d[\text{V}_{40}^{2+}] &= (-k_4[\text{SO}_2^-][\text{V}_{40}^{2+}] + k_3[\text{V}_i^{2+}][\text{V}_{10}^+] + k_5[\text{V}_i^{2+}][\text{V}_{20}^+])dt \\
 d[\text{V}_{10}^+] &= (-k_3[\text{V}_i^{2+}][\text{V}_{10}^+] + k_1[\text{SO}_2^-][\text{V}_{10}^{2+}])dt \\
 d[\text{V}_{20}^+] &= (-k_5[\text{V}_{20}^+][\text{V}_i^{2+}] + k_2[\text{SO}_2^-][\text{V}_{20}^{2+}])dt
 \end{aligned}$$

$$d[V_i^{2+}] = (-k_3[V_{10}^{+}][V_i^{2+}] - k_3[V_{20}^{+}][V_i^{2+}])dt \quad 202$$

$$d[V_i^{+}] = (k_3[V_{10}^{+}][V_i^{2+}] + k_4[V_{40}^{2+}][SO_2^{-}] + k_3[V_{20}^{+}][V_i^{2+}])dt$$

where dt is some small time increment.

This program was run on a DEC PRO-350 running under the RT-11 operating system. It was compiled and linked as follows:

Each subroutine file, *4.FOR, was compiled using the FORTRAN IV compiler with the command

FORT *4.FOR/LIST/NOLINE

The 7 compiled modules were then linked in an overlay configuration such that the different modules are swapped in and out of computer memory as they are used by the program. This was done with the following command file, SHLIN4.COM, which was run using the command @SHLIN4.

```
R LINK
KMAIN1=KMAIN4//
FIT4/O:1
SUP4/O:1
SIM4/O:2
REDIN4/O:2
REGID4/O:2
PICK4/O:2//
^C
```

KMAIN4.FOR

```

c      program fitplot
c
c      This is the control subroutine for a simulation program that
c      will accept any number of equations describing a given reaction
c      mechanism. The simulated C/Co curve can then be compared to an
c      actual data curve to assess the validity of the mechanism.
c
c      byte filnam(15),dummy,title(32),bell
c      virtual idat(3969),ttrace(3968),sim(3968)
c      real rnorm(17),vars(10)
c      integer dp,status(2)
c
c
c      lun=13
c      call giopen(status,lun,0,1,6,0)          !open gidis
c
c      call readin(filnam,vmax,idat,rnorm,title) !read in data
c
c      call fitdat(idat,rnorm,vmax,ttrace,istart !find best fit
+ ,istop,vars,sim)
c
c      write(5,*)
c      write(5,*)'*Do you wish to use the plotter?(Y/N) '
c      read(5,190)dummy
190  format(a1)
c      if(dummy.eq.'N')go to 200
c
c      call super(rnorm,title,ttrace,istart,istop,vars,sim) !plot waveform
c
c      bell=7
c      write(5,195)bell
195  format(1x,a1)
c
c      call giclos(status,lun)
c
c
200  stop
      end

```

FIT4.FOR

```

c      SUBROUTINE FITDAT(IDAT,RNORM,UMAX,TTRACE,ISTART,ISTOP,VAR
+ ,SIM)
c
c      VARIABLES
c
c      IDAT      THE 3968 DATA POINTS OF THE TRACE WHICH ARE READ
c                IN FROM DATAxx.DAT.
c      RATE      CONTAINS THE RECORDING RATES FOR EACH TRACE.
c      UMAX      CONTAINS THE t=0 VOLTAGE FOR THE WAVEFORM.
c      OFFSET    CONTAINS THE OFFSET VALUE FOR EACH TRACE.
c      TTRACE    IS THE ARRAY CONTAINING THE NORMALIZED REPRESENTATION
c                OF THE DATA READINGS.
c      SIM       IS THE ARRAY CONTAINING THE SIMULATED WAVEFORM.
c
c      BYTE BELL,DECIDE,SUB
c      DOUBLE PRECISION CHISQU
c      REAL START,STOP,RNORM(17),K(5),VAR(10)
c      VIRTUAL TTRACE(3968),IDAT(3968),SIM(3968)
c      DATA BELL/7/

```

```

C      OFFSET=RNORM(16)/1000.0
                                           204
      RATE=RNORM(8)
      VMAX=(VMAX-RNORM(9))*RNORM(7)
      HOFF=RNORM(10)*65536.0+RNORM(11)
      IF(RNORM(1).EQ.0)WRITE(5,*)'VMAX USED WAS CALCULATED'
      VINP=RNORM(17)/1000.0
      SUB=27

C      WRITE(5,*)'*Input sum of Ai factors'
                                           !NORMALIZATION
      READ(5,95)SUMA
95      FORMAT(F6.4)
      WRITE(5,*)'*WAIT-WORKING'

C      BOTTOM=ALOG10((VMAX+OFFSET)/(VINP+OFFSET))
      DO 100 I=1,3966
          TEMP=(FLOAT(IDAT(I+2))-RNORM(9))*RNORM(7)
          TOP=ALOG10((TEMP+OFFSET)/(VINP+OFFSET))
          TTRACE(I)=(TOP/BOTTOM)/SUMA
100     CONTINUE

C      WRITE(5,106)BELL
106     FORMAT(1X,A1)

C      WRITE(5,*)'*Enter D to display data, I to input start,stop
+ points'
      READ(5,500)DECIDE
      IF(DECIDE.EQ.'I')WRITE(5,*)'*Input start,stop in msec'
      IF(DECIDE.EQ.'I')READ(5,112)START,STOP
112     FORMAT(F8.0,F8.0)
      IF(DECIDE.EQ.'I')GO TO 115
      CALL PICK(TTRACE,RNORM,START,STOP,HOFF)
115     IF(START.LT.RATE*1000)START=RATE*1000.0
      ISTART=IFIX(START/(RATE*1000.0))+HOFF
      ISTOP=IFIX(STOP/(RATE*1000.0))+HOFF
      WRITE(5,*)ISTART,ISTOP
      WRITE(5,*)

C      START=TTRACE(ISTART)
      CALL SIMUL(RATE,VARS,START,ISTOP,FAST,SIM)

C      CHISQU=0.0
      COUNT=0.0
      DO 250 I=ISTART,ISTOP
          COUNT=COUNT+1.0
          CHISQU=CHISQU+(TTRACE(I)-SIM(I))*2
250     CONTINUE
      CHISQU=CHISQU/COUNT
      WRITE(5,*)''
      WRITE(5,260)CHISQU
      WRITE(5,*)''
260     FORMAT(1X,'VAR=',E10.3)

C      WRITE BEST FIT PARAMETERS ON PRINTER
C      WRITE (6,*)'
      WRITE (6,*)'
      WRITE (6,336) RATE,VMAX,CHISQU
      WRITE (6,333) START,STOP
      WRITE (6,*)
      WRITE(6,334)(I,I=1,5)
      WRITE(6,337)(VARS(I),I=1,5)

C      333     FORMAT(2X,'Start pt.= ',f7.1,' msec, Stop pt.= ',f8.1,'msec')
      334     FORMAT(7X,5('k',I1,10X))
      335     FORMAT(1X,'+ OR - ',5(2X,E10.3))
      336     FORMAT(3X,'RATE=',E10.3,'SEC-1', ' VMAX=',E10.3, ' VAR=',E10.3)
      337     FORMAT(1X,5(2X,E10.3))

```

```

C      DO 406 I=1,5
      WRITE(6,405)(SUB,J=1,100)
405    FORMAT(1X,100A1,'K')
406    CONTINUE
C
410    WRITE(5,*)
      WRITE(5,*)'*Enter R for residual, S to skip'
      READ(5,500)DECIDE
      IF(DECIDE.EQ.'R')GO TO 420
      IF(DECIDE.NE.'S')GO TO 410
      GO TO 450
420    CALL REGID(TTRACE,ISTART,ISTOP,RNORM,SIM)
      GO TO 450
C
450    WRITE(5,*)'*Do you want to try again?(Y/N)'
      READ(5,500)DECIDE
500    FORMAT(A1)
      IF(DECIDE.EQ.'Y')GO TO 110
      IF(DECIDE.NE.'N')GO TO 450
C
      VARS(10)=CHISQU
C
      RETURN
      END

```

SUP4.FOR

```

C      subroutine super(rnorm,title,ttrace,istart,istop,vars,sim)
C
C      This routine will output the normalized data to the plotter
C      superimposed over the best-fit curve from the simulation.
C      The residual is plotted on the reverse side of the paper.
C      (Logical unit 8 is assigned to XC: in the startup file.)
C
C      virtual ttrace(3968),sim(3968)
C      byte title(32),dummy,bell,xon,esc
C      real rnorm(17),vars(10)
C
C      offset=rnorm(16)/1000.0
C      Vinf=rnorm(17)/1000.0
C      hoff=rnorm(10)*65536.0+rnorm(11)
C      resid1=0
C      bell=7
C      xon=17
C      esc=27
C
C      write(5,*)'*MAKE SURE plotter is on, has paper, 2 pens.'
C      write(5,*)'*Switch junc. box to A.'
C      write(5,*)
C      write(5,*)'*Hit return when ready'
C      read(5,3)dummy
3      format(a1)
C
4      write(5,*)'*Computer will beep when plot is finished.'
C
C      write(8,*)'IN;'
C      write(8,800)esc
800    format(1x,a1,'.H80;0;17:')
C      write(8,810)esc
810    format(1x,a1,'.N;19:')

```

!initialize

!handshake init.

```

write(8,820)xon
820 format(1x,a1)
    if(residl.eq.0)write(8,*)'SP2;'          !grab right pen
    if(residl.eq.1)write(8,*)'SP1;'          !grab left pen
c
write(5,*)'*Starting plot'
c
    if(residl.eq.1)go to 6
    write(8,*)'PU;PA3000,7540;DT*;LBTITLE:*'    !print title
    write(8,5)(title(i),i=1,32)
    format(1x,'LB',32a1,'*SP1')
5
c
6    write(8,*)'PU;PA1450,7200;PD;PA1450,450;'    !draw window
    write(8,*)'PA9850,450,9850,7200,1450,7200;PU;'
    write(8,*)'PA5200,100;PD;DT*;LBTIME(sec);PU'    !label x axis
    write(8,7)norm(8)*(istart-hoffB)
7    format(1x,'PA1350,100;DTs;LB',f8.4,'sPA9000,100;')
    write(8,10)norm(8)*(istop-hoff)
10   format(1x,'DTs;LB',f9.4,'sPU;')
c
    if(residl.eq.0)go to 16
    write(8,*)'PU;PA4900,7525;DT*;LB*'          !title for residual
    write(8,*)'LB RESIDUAL *'
c
16   if(residl.eq.1)go to 20
c
20   write(8,*)'PA1500,450;PD;XT;'          !increment x axis
    do 30 i=1,10
        write(8,*)'PU;PR830,0;PD;XT;'
        k=0.02
30   continue
c
    if(residl.eq.1)go to 120
c
    write(8,*)'PU;PA25,7325;DT/;'
    write(8,35)(vars(i),i=1,5)
35   format(1x,'LB k1=',e8.2,' k2=',e8.2,' k3=',e8.2,' k4=',e8.2,
+   ' k5=',e8.2,'/')
    write(8,*)'PU;PA6900,7325;DT%;'
    write(8,43)vars(6),vars(7)*100.0
43   format(1x,' LB V2t=',e8.2,' Perc. Out=',f4.1,'%')
c
45   write(8,*)'PU;PA1000,7540;DT);'          !write var & rate
    write(8,47)vars(8),norm(8)*1000.0
47   format(1x,'LB S204=',e10.4,'
+   Rate=',f8.3,'(msec/pt)')
    write(8,*)'PU;PA5500,6300;DT%;'
    write(8,48)vars(9)*100.0
48   format(1x,'LB Percent fast outer reduction =',f5.1,'%')
    write(8,49)vars(10)
49   format(1x,'PU;PA6000,5800;DT);LB ( Var.=',e8.2,')')
c
    write(8,*)'PU;PA3500,7000;PD;PA3700,7000;PU;'
    write(8,*)'PA3700,6975;DT.;LB Data Curve.'
    write(8,*)'SP2;PA7500,7000;PD;PA7700,7000;PU;'
    write(8,*)'PA7700,6975;LB Simulation.SP1;'
c
    dmax=-1.0E30
    dmin=1.0E30
    do 52 j=1,2
    do 50 i=istart,istop,10
        if(j.eq.1)value=ttrace(i)
        if(j.eq.2)value=sim(i)
        if(value.gt.dmax)dmax=value
        if(value.lt.dmin)dmin=value
50   continue
52   continue

```

```

c      write(8,*)'PU;PA300,450;DT*;LB'
      write(8,55)dmin,dmax                                !label y axis
55      format(1x,f9.5,'*';PA300,7100;LB',f9.5,'*')
      write(8,*)'PU;PA1450,7150;PD;YT;PU;'
      write(8,*)'PA1450,500;PD;YT;PU;PU;'

c      times=0

c
60      width=float(istop-istart)
      shift=((9800-1500)*float(istart))/width
      icount=0
      do 100 i=istart,istop,10                            !istart to istop
          icount=icount+1
          time=((9800.0-1500.0)/width)*i-shift+1500.0
          if(times.eq.1)data=sim(i)
          if(times.eq.0)data=ttrace(i)
          yplot=((7150.0-500.0)/(dmax-dmin))*(data-dmin)+500.0
          if(i.ne.istart+10)go to 75
          write(8,*)'PD;'
75      write(8,70)time,yplot                                !plots waveform data
70      format(1x,'PA',f6.0,',',',',f6.0,',')             !on plotter
          if(times.eq.1)go to 100
          if(mod(icount,10).ne.0.and.i.ne.istop)go to 100
100     continue

c      if(times.eq.1)go to 110
      write(8,*)'SP2;PU;'                                    !switch pens

c      times=times+1
      go to 60                                              !plot best fit

c
110     write(8,*)'PU;SP;PA0,0;'
      do 112 i=1,30
          write(8,*)';;;;;;;;;;;;;;;'                    !flush buffer
112     continue

c      write(5,115)bell
115     format(1x,a1)
      write(5,*)'*Turn over paper in plotter for residual'
      write(5,*)'*Hit Return when ready'
      read(5,3)dummy
      resid1=1
      go to 4

c
120     dmax=0.1
      dmin=-0.1
      write(8,*)'IN;SP2;'

c      write(8,*)'PU;PA1800,7250;DT.;'
      write(8,*)'LB Y-AXIS:ONE INCH = THREE HUNDRETH UNIT.'
      write(8,*)'PU;LT2;PA1450,3825;PD;PA9850,3825;PU;LT;' !draws zero
c                                                         !line
      write(8,*)'PU;'

c      icount=0
      width=float(istop-istart)                            !plot residual
      shift=((9800-1500)*float(istart))/width
      do 200 i=istart,istop,10
          icount=icount+1
          time=((9800.0-1500.0)/width)*i-shift+1500.0
          dat=ttrace(i)
          best=sim(i)
          resid=dat-best
          yplot=((7150.0-500.0)/(dmax-dmin))*(resid-dmin)+500.0

```

```

180      write(8,180)time,yplot
190      format(1x,'PA',f6.0,',',f6.0,',';PD;')
200      continue
210      write(8,*)'PU;SP;PA0,0;'
220      write(8,*)';;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;'
230      write(8,*)';;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;' !flush buffer
240
250      return
260      end

```

SIM4.FOR

```

SUBROUTINE SIMUL(RATE,VARS,START,ISTOP,FAST,SIM)
C
C      THIS SUBROUTINE CALCULATES THE SIMULATED CONCENTRATION
C      CURVE FROM THE MECHANISTIC EQUATIONS WHICH ARE EDITED INTO
C      THE PROGRAM.  THE KINETIC CONSTANTS, WHICH CAN BE VARIED FROM
C      WITHIN THE PROGRAM, ARE STORED IN THE FILE CONST.DAT.
C
C      BYTE VAR(5),DECIDE
C      REAL K(5),VARS(10),FAST
C      VIRTUAL SIM(3968)
C
C      OPEN(UNIT=10,NAME='CONST.DAT',TYPE='OLD')
C      READ(10,102)(K(I),I=1,5)
102  FORMAT(5(E10.3,2X))
C      READ(10,104)V2T,PEROUT,S204,FAST,TRAD
104  FORMAT(E10.3,2X,F5.3,2X,E10.3,2X,F5.3,2X,E10.3)
C      CLOSE(UNIT=10)
C
C      ***** SIMULATION *****
C
122  WRITE(5,*)
C      DO 130 I=1,5
C          WRITE(5,125)I,K(I)                                !RATE CONSTS.
125  FORMAT(1X,'k',I1,'=',E10.3)
130  CONTINUE
C      WRITE(5,*)
C      WRITE(5,132)V2T                                         !TOTAL VIOL.
132  FORMAT(1X,'Total viologen conc.(TOT) =',E10.3)
C      WRITE(5,134)PEROUT                                       !% VIOL. OUT
134  FORMAT(1X,'Fraction of viologen outside(PERO) =',F5.3)
C      WRITE(5,136)S204                                         !DITHIONITE
136  FORMAT(1X,'Dithionite conc.(DIT) =',E10.3)
C      WRITE(5,137)FAST                                         !% FAST RED.
137  FORMAT(1X,'k1 fraction of outer reduction(FAST) =',F5.3)
C      WRITE(5,138)TRAD                                         !TOT. RADICAL
138  FORMAT(1X,'Total conc. of radical produced(TRAD) =',E10.3)
C      WRITE(5,*)
C
140  WRITE(5,*)'Do you wish to change a variable? (Y/N)'
C      READ(5,145)DECIDE
145  FORMAT(A1)
C      IF(DECIDE.EQ.'N')GO TO 200
C      IF(DECIDE.NE.'Y')GO TO 140
C
C      ***** CHANGE VARIABLE *****
C
C      WRITE(5,*)'Enter variable name'
C      READ(5,150)(VAR(I),I=1,5)
150  FORMAT(5A1)
C      WRITE(5,*)'Enter value'
C      READ(5,160)TEMP
160  FORMAT(E10.3)

```

```

C      THIS SUBROUTINE CALCULATES THE SIMULATED CONCENTRATION
C      CURVE FROM THE MECHANISTIC EQUATIONS WHICH ARE EDITED INTO
C      THE PROGRAM.  THE KINETIC CONSTANTS, WHICH CAN BE VARIED FROM
C      WITHIN THE PROGRAM, ARE STORED IN THE FILE CONST.DAT.
C
C      BYTE VAR(5),DECIDE
C      REAL K(5),VARS(10),FAST
C      VIRTUAL SIM(3968)
C
C      OPEN(UNIT=10,NAME='CONST.DAT',TYPE='OLD')
C      READ(10,102)(K(I),I=1,5)
102    FORMAT(5(E10.3,2X))
C      READ(10,104)V2T,PEROUT,S204,FAST,TRAD
104    FORMAT(E10.3,2X,F5.3,2X,E10.3,2X,F5.3,2X,E10.3)
C      CLOSE(UNIT=10)
C
C      ***** SIMULATION *****
C
122    WRITE(5,*)
C      DO 130 I=1,5
C        WRITE(5,125)I,K(I)                                !RATE CONSTS.
125      FORMAT(1X,'k',I1,'=',E10.3)
130    CONTINUE
C      WRITE(5,*)
C      WRITE(5,132)V2T                                        !TOTAL VIOL.
132    FORMAT(1X,'Total viologen conc.(TOT) =',E10.3)
C      WRITE(5,134)PEROUT                                    !% VIOL. OUT
134    FORMAT(1X,'Fraction of viologen outside(PERO) =',F5.3)
C      WRITE(5,136)S204                                      !DITHIONITE
136    FORMAT(1X,'Dithionite conc.(DIT) =',E10.3)
C      WRITE(5,137)FAST                                      !% FAST RED.
137    FORMAT(1X,'k1 fraction of outer reduction(FAST) =',F5.3)
C      WRITE(5,138)TRAD                                      !TOT. RADICAL
138    FORMAT(1X,'Total conc. of radical produced(TRAD) =',E10.3)
C      WRITE(5,*)
C
140    WRITE(5,*)'Do you wish to change a variable? (Y/N)'
C      READ(5,145)DECIDE
145    FORMAT(A1)
C      IF(DECIDE.EQ.'N')GO TO 200
C      IF(DECIDE.NE.'Y')GO TO 140
C
C      ***** CHANGE VARIABLE *****
C
C      WRITE(5,*)'Enter variable name'
C      READ(5,150)(VAR(I),I=1,5)
150    FORMAT(5A1)
C      WRITE(5,*)'Enter value'
C      READ(5,160)TEMP
160    FORMAT(E10.3)

```

C

```

IF(VAR(2).EQ.'1')K(1)=TEMP
IF(VAR(2).EQ.'2')K(2)=TEMP
IF(VAR(2).EQ.'3')K(3)=TEMP
IF(VAR(2).EQ.'4')K(4)=TEMP
IF(VAR(2).EQ.'5')K(5)=TEMP
IF(VAR(2).EQ.'0')V2T=TEMP
IF(VAR(2).EQ.'E')PEROUT=TEMP
IF(VAR(2).EQ.'I')S204=TEMP
IF(VAR(2).EQ.'A')FAST=TEMP
IF(VAR(2).EQ.'R')TRAD=TEMP
GO TO 122

```

209

C

C

C

200

```

***** START SIMULATION CALCULATIONS *****
DT=RATE/5.0                                !VALUES ARE CALCULATED EVERY
EQK=1.4E-09                                !ONE FIFTH OF THE TIME PER
S02=(EQK*S204)**0.5                        !POINT FOR THE DATA CURVE.
V2I=(1-PEROUT)*V2T
V201=(V2T-V2I)*FAST
V202=(V2T-V2I)*(1.0-FAST)
V101=0.0
V102=0.0
V1I=0.0
V204=0.0

```

C

```

DO 210 I=1,(ISTOP+1)*5
  J=I
  DV2I=(-K(3)*V101*V2I-K(5)*V2I*V102)*DT
  DV201=(-K(1)*S02*V201)*DT
  DV202=(-K(2)*S02*V202)*DT
  DV101=(K(1)*S02*V201-K(3)*V2I*V101)*DT
  DV102=(K(2)*S02*V202-K(5)*V2I*V102)*DT
  DV1I=(K(3)*V101*V2I+K(5)*V2I*V102+K(4)*V204*S02)*DT
  DV204=(K(3)*V101*V2I+K(5)*V2I*V102-K(4)*S02*V204)*DT
  V2I=V2I+DV2I
  V201=V201+DV201
  V202=V202+DV202
  V101=V101+DV101
  V102=V102+DV102
  V1I=V1I+DV1I
  V204=V204+DV204
  IF(MOD(J,5).NE.0)GO TO 210
  TEMP=(TRAD-(V101+V102+V1I))/TRAD
  SIM(J/5)=TEMP
  IF(MOD(J,500).EQ.0)WRITE(5,205)J*DT,TEMP
205  FORMAT(1X,F5.1,' sec. C/Co=',F5.3)
210  CONTINUE

```

210

C

```

OPEN(UNIT=10,NAME='CONST.DAT',TYPE='NEW')
WRITE(10,510)(K(I),I=1,5)
510  FORMAT(1X,5(E10.3,2X))
WRITE(10,520)V2T,PEROUT,S204,FAST,TRAD
520  FORMAT(1X,E10.3,2X,F5.3,2X,E10.3,2X,F5.3,2X,E10.3)
CLOSE(UNIT=10)

```

C

```

DO 530 I=1,5
  VARS(I)=K(I)
530  CONTINUE
VARS(6)=V2T
VARS(7)=PEROUT
VARS(8)=S204
VARS(9)=FAST
VARS(10)=TRAD

```

C

```

RETURN
END

```

REDIN4.FOR

```

      subroutine readin(filnam,vmax,idat,rnorm,title)
c
c   This subroutine allows input of a data file name to be simulated.
c   The data file is then read into idat and rnorm.
c
      byte filnam(15),dummy,title(32),bell
      virtual idat(3969),ttrace(3968)
      real rnorm(17)
c
c
60    write(5,*)'*To analyse file data,'
      write(5,*)'*Enter filename DATAxx.DAT (xx=file no.)'
      read(5,70)(filnam(i),i=5,15)
70    format(11a1)                                !accept filename
c
      write(5,72)(filnam(i),i=5,15)
72    format(1x,'*Correct filename is: ',11a1,'(Y/N)?')
      read(5,74)dummy
74    format(a1)
      if(dummy.eq.'N')go to 60
c
      write(5,*)'*WAIT-WORKING'
c
      filnam(1)=68
      filnam(2)=90                                !create DZ0:DATAxx.DAT
      filnam(3)=48
      filnam(4)=58
c
      open(unit=10,name=filnam,type='UNKNOWN')
      close(unit=10)
      open(unit=10,name=filnam,type='OLD')
      read(10,80,end=90)dummy                      !check file
80    format(a1)
      go to 100
90    write(5,*)'*File doesn''t exist:Choose a new filename'
      close(unit=10)
      go to 60                                     !if empty try again
c
100   rewind 10
c
      vmax=-32000.0
c
      do 102 i=0,395
         read(10,106)(idat(i*10+j),j=1,10)
102    continue
106    format(10i7)
c
      read(10,106)(idat(3960+i),i=1,8)
      close(unit=10)
c
      do 108 i=1,3968
         if(idat(i).gt.vmax)vmax=idat(i)
108    continue
c
      filnam(12)=73
      filnam(13)=78                                !change .DAT to .INF
      filnam(14)=70
      filnam(15)=0
c

```

```

      open(unit=11,name=filnam,type='OLD')
c
      do 110 i=1,6
        read(11,115)rnorm(i)
110      continue
115      format(f9.2)
c
      read(11,120)rnorm(7)
      read(11,120)rnorm(8)
120      format(e10.4)
c
      do 130 i=9,17
        read(11,115)rnorm(i)
130      continue
      close(unit=11)
c
      itemp=filnam(10)-48
      ktemp=(filnam(9)-48)*10
      inum=itemp+ktemp
      open(unit=20,name='DZ0:direct.fil',type='OLD')
      read(20,131)n
131      format(i2)
      ifile=0
      do 135 j=1,n
        if(ifile.eq.inum)go to 135
        read(20,131)ifile
        read(20,132)(title(i),i=1,32)
132      format(32a1)
135      continue
      close(unit=20)
c
      return
      end

```

!read in title

REGID4.FOR

```

      subroutine regid(ttrace,istart,istop,rnorm,sim)
c
c   This subroutine will display the residual for a quick check of
c   the validity of the mechanism with the current kinetic constants.
c   The data and simulated curves can then be overlaid.
c
      byte dummy,bell
      virtual ttrace(3968),sim(3968)
      real rnorm(17)
      integer status(2),mess(10)
c
      bell=7
      sup=0
      hoff=rnorm(10)*65536.0+rnorm(11)
c
      write(5,*)
      write(5,*)'   Y axis +/- .10 units, Resid.= Data - Sim.
+ , Hit Return to cont.'
40      if(sup.eq.1)write(5,*)'           Data and simulation superimposed
+ respectively'
      if(sup.eq.1)write(5,*)'
+           Hit Return to cont.'
      if(sup.eq.0)write(5,*)
      do 50 i=1,19
        write(5,*)
50      continue
c

```

```

dmax=0.1
dmin=-0.1
c
write(5,60)((istart-hoff)*rnorm(8),(istop-hoff)*rnorm(8)
60 format(1x,f8.4,27x,'Time(sec)',24x,f8.4)
c
c
lun=13
c
mess(1)=2308
mess(2)=1000
mess(3)=300
mess(4)=10300
mess(5)=7200
call giwrit(status,lun,mess,5)
c
mess(1)=7426
mess(2)=1000
mess(3)=800
call giwrit(status,lun,mess,3)
mess(1)=6408
mess(2)=1000
mess(3)=7000
mess(4)=11300
mess(5)=7000
mess(6)=11300
mess(7)=800
mess(8)=1000
mess(9)=800
call giwrit(status,lun,mess,9)
if(sup.eq.1)go to 450
c
mess(1)=7426
mess(2)=1000
mess(3)=3900
call giwrit(status,lun,mess,3)
mess(1)=6402
mess(2)=11300
mess(3)=3900
call giwrit(status,lun,mess,3)
c
450 do 500 i=1000,11300,1030
      mess(1)=7426
      mess(2)=i
      mess(3)=6950
      call giwrit(status,lun,mess,3)
      mess(1)=6402
      mess(2)=i
      mess(3)=7050
      call giwrit(status,lun,mess,3)
500 continue
      if(sup.eq.0)go to 710
c
600 dmax=-1.0E30
      dmin=1.0E30
      do 705 j=1,2
      do 700 i=istart,istop,10
        if(j.eq.1)b=ttrace(i)
        if(j.eq.2)b=sim(i)
        if(b.gt.dmax)dmax=b
        if(b.lt.dmin)dmin=b
700 continue
705 continue
c
710 width=float(istop-istart)
      shift=((11300-1000)*float(istart))/width
      do 1000 i=istart,istop,10

```

!set window

!draw window

!draw zero
!residual

!increment
!X axis

```

time=((11300.0-1000.0)/width)*i-shift+1000.0
dat=ttrace(i)
best=sim(i)
resid=dat-best
if(sup.eq.1)resid=dat
if(sup.eq.2)resid=best
yplot=((7000.0-800.0)/(dmin-dmax))*(resid-dmax)+800.0
if(i.gt.istart)go to 900
mess(1)=7426
mess(2)=ifix(time)
mess(3)=ifix(yplot)
call giwrit(status,lun,mess,3)
go to 1000
c
900    mess(1)=6402
        mess(2)=ifix(time)
        mess(3)=ifix(yplot)
        call giwrit(status,lun,mess,3)
1000   continue
c
        mess(1)=7168
        call giwrit(status,lun,mess,1)
        mess(1)=6144
        call giwrit(status,lun,mess,1)
c
        sup=sup+1
        if(sup.eq.2)go to 710
c
        read(5,1010)dummy
1010   format(a1)
c
        if(sup.eq.3)go to 1100
        write(5,*)'*Do you want to see data and sim. curves super
+imposed?(Y/N) '
        read(5,1010)dummy
        write(5,*)
        write(5,*)
        if(dummy.eq.'Y')go to 40
c
1100   return
        end

```

PICK4.FOR

```

subroutine pick(ttrace,rnorm,start,stop)
c
c   This routine will display C/Co and magnify it which will allow
c   the user to input start and stop points for the analysis.
c
        virtual ttrace(3968)
        real rnorm(17)
        integer status(2),isect(2),mess(9)
        byte dummy
c
        lun=13
        calc=0
        hoff=rnorm(10)*65536.0+rnorm(11)
c
        write(5,*)'*Wait for plot - Make sure printer is on'
10    do 20 i=1,17
            write(5,*)
20    continue

```

```

30      write(5,30)(i,i=1,10)
      format(2x,10(5x,i2))
      write(5,31)-hoff*rnorm(8),(3968.0-hoff)*rnorm(8)
31      format(5x,f9.2,21x,'Time(sec)',24x,f9.2)
      write(5,*)
      write(5,*)
      write(5,*)
      write(5,*)'*Enter the X-axis section number(1-10) that you
+ want magnified'
c
      mess(1)=2308
      mess(2)=1000
      mess(3)=300
      mess(4)=10300
      mess(5)=5200
      call giwrit(status,lun,mess,5)
c
      mess(1)=7426
      mess(2)=1500
      mess(3)=1000
      call giwrit(status,lun,mess,3)
      mess(1)=6408
      mess(2)=1500
      mess(3)=5000
      mess(4)=9800
      mess(5)=5000
      mess(6)=9800
      mess(7)=1000
      mess(8)=1500
      mess(9)=1000
      call giwrit(status,lun,mess,9)
c
      do 100 i=1500,9800,830
      mess(1)=7426
      mess(2)=i
      mess(3)=950
      call giwrit(status,lun,mess,3)
      mess(1)=6402
      mess(2)=i
      mess(3)=5050
      call giwrit(status,lun,mess,3)
100  continue
      if(calc.eq.1)go to 210
c
      dmax=-1.0E30
      dmin=1.0E30
c
      do 200 i=1,3968
      b=ttrace(i)
      if(b.gt.dmax)dmax=b
      if(b.lt.dmin)dmin=b
200  continue
c
210  init=1
c
      do 300 i=1,3968,10
      time=((9800.0-1500.0)/3968.0)*i+1500.0
      if(ttrace(i).eq.-1000.0)go to 300
      yplot=((5000.0-1000.0)/(dmin-dmax))*(ttrace(i)-dmax)+1000.0
      if(init.eq.0)go to 220
      mess(1)=7426
      mess(2)=ifix(time)
      mess(3)=ifix(yplot)
      call giwrit(status,lun,mess,3)
      init=0
      go to 300
c

```

```

220      mess(1)=6402
      mess(2)=ifix(time)
      mess(3)=ifix(yplot)
      call giwrit(status,lun,mess,3)
300  continue
c
      mess(1)=7168
      call giwrit(status,lun,mess,1)
c
c
      read(5,1000)isect(1)
1000  format(i2)
      write(5,*)isect(1)
c
      isect(2)=0
      width=400
      offset=0
      iscold=isect(1)
      call draw(ttrace,isect,rnorm,width,offset,start,stop,hoff)
c
      if(isect(2).eq.0)width=40
      if(isect(2).ne.0)width=80
      offset=(iscold-1)*400
      call draw(ttrace,isect,rnorm,width,offset,start,stop,hoff)
      calc=1
c
1005  write(5,*)'*Hit Return to find another pt., Enter D if done
      + finding stop/start pts.'
      read(5,1010)dummy
1010  format(a1)
      if(dummy.eq.'D')go to 1020
      go to 10
c
1020  return
      end
c
c *****
c
      subroutine draw(ttrace,isect,rnorm,width,offset,start,stop,hoff)
c
c This subroutine draws the appropriate section.
c
      virtual ttrace(3968)
      real rnorm(17)
      byte dummy
      integer status(2),isect(2),mess(10)
c
      lun=13
      ioff=ifix(offset)
c
      if(width.eq.400)mult=400
      if(width.ne.400)mult=40
c
      ibegin=ioff+(isect(1)-1)*mult
      iend=ibegin+ifix(width)
c
      if(width.eq.40.or.width.eq.80)go to 40
      do 20 i=1,20
        write(5,*)
20      continue
      write(5,23)(i,i=1,10)
23      format(2x,10(5x,i2))
      write(5,25)(ibegin-hoff)*rnorm(8)*1000,(iend-hoff)*rnorm(8)*1000
25      format(1x,f9.2,27x,'Time(msec)',23x,f9.2)
      write(5,27)rnorm(8)*1000
      write(5,*)
27      format(22x,'Time per pt. is ',f8.4,' msec')

```

```

do 30 i=1,2
  write(5,*)
30  continue
  go to 100
c
40  tick=(ibegin-hoff)*rnorm(8)*1000
  write(5,47)(tick+(2.0*j+1)*width*100.0*rnorm(8),j=0,4)
47  format(5x,5(4x,f9.2))
  do 50 i=1,16
    write(5,*)
50  continue
  write(5,60)(tick+(2.0*j*(width*100.0*rnorm(8))),j=0,5)
60  format(3x,f9.2,5(4x,f9.2))
  write(5,*)
  write(5,65)rnorm(8)*1000,ifix(width)/10
65  format(22x,f7.3, 'msec per point','i1,' points per section')
  write(5,*)
  write(5,*)'*Enter A#### for start or Z#### for stop pt.
+ (#### is no. in msec)'
  write(5,*)'*Enter S to skip start/stop pt. entry'
c
100 mess(1)=7426
    mess(2)=1500
    mess(3)=1000
    call giwrit(status,lun,mess,3)
    mess(1)=6408
    mess(2)=1500
    mess(3)=5000
    mess(4)=9800
    mess(5)=5000
    mess(6)=9800
    mess(7)=1000
    mess(8)=1500
    mess(9)=1000
    call giwrit(status,lun,mess,9)
c
do 150 i=1500,9800,830
  mess(1)=7426
  mess(2)=i
  mess(3)=950
  call giwrit(status,lun,mess,3)
  mess(1)=6402
  mess(2)=i
  mess(3)=5050
  call giwrit(status,lun,mess,3)
150 continue
c
  init=1
c
  dmax=-1.0E30
  dmin=1.0E30
c
do 170 i=ibegin+1,iend+1
  if(i.gt.3968)go to 170
  if(ttrace(i).gt.dmax)dmax=ttrace(i)
  if(ttrace(i).lt.dmin)dmin=ttrace(i)
170 continue
c
do 200 i=ibegin+1,iend+1
  if(i.gt.3968)go to 200
  time=((9800.0-1500.0)/width)*(i-(ibegin+1))+1500.0
  if(ttrace(i).eq.-1000.0)go to 200
  yplot=((5000.0-1000.0)/(dmin-dmax))*(ttrace(i)-dmax)+1000.0

```

```

        if(init.eq.0)go to 190
        mess(1)=7426
        mess(2)=ifix(time)
        mess(3)=ifix(yplot)
        call giwrit(status,lun,mess,3)
        init=0
        go to 200
c
190      mess(1)=6402
        mess(2)=ifix(time)
        mess(3)=ifix(yplot)
        call giwrit(status,lun,mess,3)
200      continue
c
        mess(1)=7168
        call giwrit(status,lun,mess,1)
c
        if(width.eq.400)go to 210
        go to 203
202      write(5,*)'*Enter A*** for start or Z*** for stop pt.(***
+ is no. in msec)'
        write(5,*)'*Enter S to skip start/stop pt. entry'
203      read(5,205)dummy,point
205      format(a1,f7.0)
        if(dummy.eq.'S')go to 206
        if(dummy.ne.'A'.and.dummy.ne.'Z')write(5,*)'*You forgot prefix'
        if(dummy.ne.'A'.and.dummy.ne.'Z')go to 202
        if(dummy.eq.'A')start=point
        if(dummy.eq.'Z')stop=point
206      write(5,207)start,stop
207      format(5x,'start= ',f9.2,' msec. stop= ',f9.2,' msec')
        write(5,*)'*Hit Return to continue, Enter R to reenter
+ start/stop point'
        read(5,208)dummy
208      format(a1)
        if(dummy.eq.'R')go to 202
        go to 250
c
210      write(5,*)'*Enter 1 or 2 sect. #'s(If 2 separate w/ comma)'
        read(5,220)isect(1),isect(2)
220      format(i2,i2)
        if(isect(2).eq.0)go to 250
        if(((isect(2)-isect(1)).ne.1)write(5,*)'*Sections must be
+ consecutive'
        if(((isect(2)-isect(1)).ne.1)go to 210
c
250      return
        end

```

BIOGRAPHICAL NOTE

Brian Clay Patterson was born on August 13, 1962 in New Orleans, Louisiana. He was adopted there by James T. and Beverly I. Patterson. In 1965 his family moved to Issaquah, Washington and in 1980 he graduated from Issaquah High School. The author received his B.A. degree in chemistry from Reed College in 1984. He then worked at Pacific Chemical Labs in Albany, Oregon synthesizing primarily purine and pyrimidine derivatives. In June 1985 he was hired as a technician at the Oregon Graduate Institute by Dr. James Hurst. In September 1986 he became a graduate student at O.G.I. under the advisement of Dr. Hurst.