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Photoinduced electron transfer, energy transfer and polymerization reactions in phospholipid membranes

Armitage, Bruce Alan, Ph.D.
The University of Arizona, 1993
PHOTOINDUCED ELECTRON TRANSFER, ENERGY TRANSFER AND POLYMERIZATION REACTIONS IN PHOSPHOLIPID MEMBRANES

by

Bruce Alan Armitage

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF CHEMISTRY
In Partial Fulfillment of the Requirements
For the Degree of
DOCTOR OF PHILOSOPHY
In the Graduate College
THE UNIVERSITY OF ARIZONA

1993
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Bruce Alan Armitage entitled "Photoinduced Electron Transfer, Energy Transfer and Polymerization Reactions in Phospholipid Membranes" and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director
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SIGNED: Bruce Amittage
ACKNOWLEDGMENTS

The completion of this dissertation is due to the support, guidance and encouragement of a number of faculty, students and "lay people". Warmest thanks go to Prof. David O'Brien, whose ideas and philosophy have dominated this research from the start. The remainder of my committee, Profs. Atkinson, Mash, Polt and Smith have provided valuable and diverse perspectives on a number of issues. I have learned much from each of them. In particular, Prof. George Atkinson's interest in both my present and my future has touched me deeply.

The work in the laboratory has depended on the participation of a number of students. Thanks go to Todd Sells, Henry Lamparski, Eric Oblinger and Doyle Bennett for synthesis and supply of the polymerizable lipids used in this research. I've also had the pleasure of working with a number of talented undergraduates over the past two years who have made important contributions to the research: Jennifer Retterer (Chapter III), Katrina Crawford (Chapter IV), Patty Roosa (Chapter V), Paul Klekotka and Neva Howard (Chapter VI). Most rewarding from these interactions was watching as the students began to learn, appreciate and grow from their experiences in the laboratory. I wish them all the best in the future.

Special thanks go to fellow graduate student Paula Grimes who will be continuing much of this research in the future. In addition to being eager to learn and dedicated to doing the work, Paula is quite simply the nicest person I've ever met. Good luck, Grimers.

Finally, I must acknowledge the support and encouragement of my family. They've worked hard to understand what I do and why I do it, despite the fact that most of them have never had a chemistry class. The regular phone calls and letters from Mom, Debbie, Tom and Jennifer have meant so much to me. I'm particularly thankful for rediscovering my West Coast family, Sue and Alex.

All of these people share in this work. I hope they are proud.

Bruce Armitage

1993
I recently saw a promotional advertisement for an upcoming television program in which "home" was defined not as the place of one's birth but rather as the place where one discovers one's identity. That place for me is Rochester, New York where I went to college.

Three people were most influential during that period. Prof. David Whitten gave me my first laboratory job, even after a less-than-stellar "B" performance in his organic chemistry class. Over the ensuing two years, Dave became a mentor, friend and role model for me. He was primarily interested in my development as a student and person; research results were secondary. It was a particularly selfless attitude.

I spent my last two summers in Rochester working as an intern at Eastman Kodak under the supervision of Drs. Ian Gould and Samir Farid. Ian showed me that chemistry can still be a source of excitement and pleasure when it's a job rather than a major. Samir impressed me with his sheer brilliance and dignity. Both took the time to teach me the theoretical basis for the experiments in addition to which buttons to press.

These three people have been constant sources of inspiration and encouragement, remaining powerful influences on my life. They have kept me focused on learning and teaching when cynicism has started to creep in. With this in mind, I dedicate this dissertation to the ideal of education, personified in Dave Whitten, Samir Farid and Ian Gould.

"The challenge of adulthood is retaining one's idealism after losing one's innocence." B. Springsteen
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ABSTRACT

The differential physical properties found at different depths of a phospholipid membrane permit design of systems for vectorial reactions (which are not possible in isotropic solution). In the system described in Chapter IV, a hydrophobic electron donor (triphenylbenzylborate) binds to the membrane interior while a hydrophilic electron acceptor (a cyanine dye) binds to the surface. Irradiation with red light leads to vectorial electron flow via photoinduced electron transfer (PET), as monitored by fluorescence quenching and photobleaching techniques. The PET reaction efficiency is enhanced over the case where the donor and acceptor are present in water without membranes. In that case, strong dimeric complexes are formed which reduce the efficiency of PET by enhancing nonradiative decay modes for the dye (Chapter III).

Addition of a porphyrin to the membrane surface extends the sensitivity of the system to blue light (Chapter V). Excitation of the porphyrin at 417 nm and subsequent energy transfer to the cyanine trigger the same PET chemistry observed with red-light irradiation. Furthermore, the electron- and energy-transfer reactions are enhanced on polymerized, phase-separated membranes (Chapter VI).

In these applications, membranes are media for chemical reactions. Membranes also make interesting substrates for chemical reactions, in this case, polymerization. A system consisting of a membrane-bound, amphiphilic cyanine dye and molecular oxygen is described in Chapter VII which effectively initiates the polymerization of vesicles upon irradiation with visible light. Potential utility in drug delivery applications is discussed.
CHAPTER I

INTRODUCTION TO PHOSPHOLIPID MEMBRANES

I. A. BIOLOGICAL MEMBRANES

A membrane may be defined as a structure which effectively separates two phases thereby limiting their communication with one another; the membrane must necessarily be insoluble in the two phases. Nature has developed closed membranes which envelop an aqueous phase, separating it from another external aqueous phase. For example, in animal cells the plasma membrane protects the internal cytoplasmic compartment containing the nucleus and other organelles from the extracellular medium, vital to sustenance of the organism. Indeed, the development of membrane-enclosed media is regarded as one of the most critical steps in the origin of life on Earth.¹

Biological membranes should not be thought of as solid walls, impervious to all substances which are not part of the actual membrane structure. Rather natural membranes act as semipermeable barriers with careful regulation of the flow of most molecules and ions across the membrane being essential to normal metabolic activity. Additionally, many vital regulatory proteins are bound to membranes and function as mediators of transmembrane biochemical processes such as photosynthesis and respiration.² Recently, the importance of membrane-bound carbohydrates in intercellular signal transduction has also become evident.³
The early assertions that amphiphilic molecules known as lipids are the fundamental structural building blocks for membranes and that these molecules are assembled in a bilayer array\(^4-6\) form the foundation for current models of biological membranes. The most well-known of these is the fluid-mosaic model of Singer and Nicolson\(^7\) which depicts membrane-bound proteins penetrating to various depths within a fluid lipid bilayer. With some refinements, this model has successfully described many of the structural and functional properties of biological membranes.
I. B. AGGREGATION BEHAVIOR OF LIPIDS

The aggregation of amphiphilic molecules in aqueous media to form assemblies such as bilayer membranes is not unique to \textit{in vivo} systems. That is, aggregation is a property of the molecules, not of the organism. Thus in most cases dispersion of lipids in water leads to spontaneous formation or self-assembly of the same types of supramolecular architectures found in natural systems, most notably closed bilayer membranes. (Such structures are termed "supramolecular" because the individual components are held together by noncovalent forces.)

A schematic of a lipid molecule is shown in Figure I-1. This type of molecule is amphiphilic, consisting of a polar, hydrophilic component and one or more nonpolar, hydrophobic moieties. The polar component is termed the head group while the nonpolar is the tail or tails. Dispersion of lipids in water as isolated molecules leads to favorable solute-solvent interactions for the lipid head groups but not for the tails. Aggregation of the lipids in a tail-to-tail assembly preserves the favorable water-head group interactions and virtually eliminates the unfavorable water-tail interactions.

\[ \text{Polar, Hydrophilic} \\
\text{"Head Group"} \\
\text{Nonpolar, Hydrophobic} \\
\text{"Tails"} \]

\textbf{Figure I-1.} Schematic of a lipid. Typically, } n = 1 \text{ or 2.
The magnitude of the enthalpic contribution to the free energy of the water/lipid system is determined primarily by head group solvation. This factor is at least qualitatively similar for both the monomer and the aggregate. Therefore aggregation is an entropically driven process. While there is a significant decrease in the entropy of the lipids incorporated into the aggregate, the increase in the entropy of the water molecules freed from solvation of the tails can overcome this. The resulting positive entropy provides the driving force for aggregation. This is the basis of the hydrophobic effect elucidated by Tanford.\(^8\)

The preceding discussion leads to the prediction that the aggregation behavior of lipids in water should be critically dependent on the relative sizes of the hydrophilic and hydrophobic components of the molecule. For example, single-chain amphiphiles should aggregate at significantly higher concentrations (CMC or critical micelle concentration) than their double-chain analogues as fewer unfavorable solvent-solute interactions are present in the former. Thus fewer water molecules will be released upon aggregation of each single-chain lipid molecule. The resulting smaller driving force translates into self-assembly at higher concentrations for single-chain lipids.

The significant difference in molecular shapes of single- and double-chain amphiphiles should lead to formation of different types of aggregates. The relationship between molecular shape and aggregate morphology has been developed by Israelachvili.\(^9\) The important conclusions for single- and double-chain lipids are summarized here. Single-chain lipids usually have an approximately conical shape, owing to the larger cross-sectional area of the head group relative to the tail. Double chain amphiphiles on the other hand
frequently have similar cross-sectional areas for the head group and the tails, imparting a quasi-cylindrical shape to these molecules. Single-chain molecules should pack most favorably into spherical micellar aggregates whereas double-chain analogues should aggregate into a bilayer morphology in which curvature is minimized as illustrated in Figure 1-2. (Examples of double-chain amphiphiles which do not favor bilayer morphologies will be discussed below.) Moreover, the aggregation numbers (i. e. the number of lipids per aggregate) for these two types of assemblies should be quite different. The conical shape for single-chain lipids requires packing into an aggregate with very high curvature, severely limiting the number of lipids which can be accommodated in a given micelle. The small aggregation number is also favorable entropically. The positive entropy change for release of water from solvation of the tails should be dependent only on the total number of lipid molecules incorporated into aggregates, not on the number of aggregates formed. However, the negative entropy change associated with lipid aggregation should be of smaller magnitude for formation of assemblies with smaller aggregation numbers since more aggregates will be formed.
Figure I-2. Dependence of aggregate morphology on molecular shape. A) Aggregation of single-chain lipids to form spherical micelles. B) Aggregation of double-chain lipids to form bilayer membranes.

For the cylindrical double-chain lipids, packing into a planar bilayer with zero curvature is more favorable than packing into a spherical micelle with high curvature. However, since there is a finite number of lipids available, some lipids, specifically those at the ends of the bilayer would be exposed to water. In order to avoid unfavorable interactions between water and the exposed lipid tails, the edges of the bilayer spontaneously curve around to form a closed
structure known as a liposome or vesicle. The nonzero curvature inherent in the liposomal architecture leads to weaker intermolecular interactions within the aggregate, a potentially destabilizing effect which can be minimized by forming large aggregates since increasing the number of lipids in the assembly will decrease its curvature. Thus, whereas aggregation of single-chain amphiphiles into spherical micelles should favor smaller aggregation numbers, formation of liposomes from double-chain amphiphiles should favor larger aggregation numbers.

Each of the predictions made above has been verified experimentally. Table I-1 gives a comparison of sodium dodecyl sulfate (SDS), a typical anionic single-chain amphiphile with dipalmitoylphosphatidylcholine (DPPC, see Figure I-3 for structures), a well-studied zwitterionic double-chain lipid. For DPPC, 100 nm diameter unilamellar (single-walled) liposomes are considered for simplicity. However, simple dispersion of DPPC in water will lead to spontaneous formation of large (>1 μm diameter) multilamellar liposomes. Such structures will contain on the order of ten million lipids in the outermost bilayer alone. Unilamellar liposomes can be prepared from multilamellar systems using straightforward methods (see Section IV-B).
Table I-1. Comparison of single- and double-chain amphiphiles.

<table>
<thead>
<tr>
<th>Amphiphile</th>
<th>CMC</th>
<th>Type of Aggregate</th>
<th>Aggregation Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDS</td>
<td>8 mM$^{10}$</td>
<td>Spherical Micelle</td>
<td>62</td>
</tr>
<tr>
<td>DPPC</td>
<td>0.4 nM$^{11}$</td>
<td>Bilayer</td>
<td>80,000$^{*}$</td>
</tr>
</tbody>
</table>

$^{*}$ Calculated for a 100 nm diameter liposome.

Figure I-3. A) Sodium Dodecyl Sulfate (SDS), a single-chain lipid and B) Dipalmitoylphosphatidylcholine (DPPC), a double-chain lipid.
While the shape model described above is useful for predicting the actual morphology adopted by a given lipid aggregate, it has important shortcomings. In particular, mixtures of lipids cannot be analyzed using the model since a molecular shape can only be defined for a single lipid at a single temperature, pressure and concentration. A more general model developed by Gruner\textsuperscript{12-14} is formulated primarily in terms of the properties of the aggregate and secondarily on the molecular properties. Known as the intrinsic curvature hypothesis, this model rationalizes observed aggregate morphologies by considering the inherent tendency of a lipid layer to curl. The propensity of a lipid layer to curl, known as the intrinsic curvature, is determined by the lateral interactions among the lipids in the layer. These interactions include van der Waals' and electrostatic forces as well as hydrogen bonding. If these forces are equal in the head group and tail regions then the layer will prefer to be planar but any inequality will drive the aggregate toward a curved morphology where the actual curvature is equal to the intrinsic curvature. The intrinsic curvature is thus a property of the aggregate and can be defined regardless of the number of components in the lipid layer, a significant improvement on the shape model.

The degree to which a lipid layer actually does curl is determined by the interplay of many factors. The curvature free energy is minimized when the actual and intrinsic curvatures are equal. However, packing strain in the lipid hydrocarbon chains may also play a role. In order for a particular morphology to be observed, the reduction in the curvature free energy must at least compensate for any increase in free energy due to unfavorable packing of the chains. The competition between curvature and packing strain has been studied by Gruner and coworkers in systems exhibiting transitions from the disordered
lamellar ($L_\alpha$) phase to the inverted hexagonal ($H_{\Pi}$) phase (Figure 1-4; the phase behavior of phospholipids will be considered in more detail in Section I. D. 1). Lipids which feature larger cross-sectional areas for the tails than for the head groups such as unsaturated phosphatidylethanolamines (PEs, see Figure I-7 and Table I-2 for general structure) undergo the $L_\alpha$-to-$H_{\Pi}$ phase transition at relatively low temperatures. PE head groups are smaller than PC head groups for two reasons: 1) the PE ammonium is unsubstituted whereas the PC ammonium is quaternized with methyl groups and 2) PEs are capable of hydrogen bonding to other lipids in the layer, effectively reducing the head group size. Meanwhile, unsaturation in the tails produces kinked chains which will tend to splay outward from the head group. Shape theory predicts that these lipids favor curved morphologies, verified by the observation that dioleoylphosphatidylethanolamine (DOPE) possesses a $L_\alpha$-$H_{\Pi}$ phase transition at 6 °C. The depiction of the $H_{\Pi}$ phase in Figure I-4 suggests that some of the lipids, particularly those lipids whose chains are directed toward the vertices of the hexagon, are under greater packing strain than the others. The intrinsic curvature hypothesis predicts that any mechanism which relieves this packing strain should facilitate the $L_\alpha$-$H_{\Pi}$ phase transition, thereby driving it to lower temperature. Gruner and coworkers have verified this by adding small amounts of dodecane to aqueous dispersions of N-methyl-DOPE. This lipid exhibits a $L_\alpha$-$H_{\Pi}$ phase transition temperature at 60 °C but upon addition of 5 mol % dodecane to the system, the transition temperature occurs below 0 °C. Because it does not have a polar head group, the dodecane is free to fill in the interstitial spaces among the lipid cylinders, relieving the packing strain on the lipid chains. Thus the morphology adopted by the N-methylated DOPE aggregate is
determined primarily by the curvature free energy which drives the system into the $H_{\parallel}$ phase at lower temperature in the presence of the dodecane.

Figure 1-4. Illustration of disordered lamellar and inverted hexagonal phases. The $L_\alpha$ phase is viewed along the bilayer edge; the $H_{\parallel}$ phase is viewed from above. Adapted from ref. 14.
In closing, it should be pointed out that other single- and double-chain amphiphiles are capable of forming more exotic morphologies such as rodlike micelles, tubules and cubic phases under appropriate conditions. These assemblies will not be considered further here.
I. C. TYPES OF BILAYER-FORMING LIPIDS

Figure I-1 and the subsequent discussion suggest that a very wide range of amphiphilic molecules should be capable of forming bilayer membranes in water. Clearly natural systems offer a huge number of bilayer-forming lipids (although not all biological lipids form bilayers by themselves). Natural lipids will be discussed further below.

Kunitake has shown that simple dialkyl(dimethyl)ammonium salts (Figure I-5) spontaneously aggregate into bilayers in water.21 This work has had far-reaching effects since it demonstrated that totally synthetic molecules could be designed which would assemble to form aggregates of the same fundamental structure as biological membranes. The ability to form such "biomimetic" assemblies from unnatural components has permitted the design of bilayer membranes exhibiting physical properties beyond what would be possible using only natural lipids. For example, the aforementioned ammonium amphiphiles permit construction of membranes bearing positive surface charge whereas only neutral and anionic lipids are found in nature.

Figure I-5. Didodecyldimethylammonium bromide, a synthetic bilayer-forming amphiphile.
A tremendously diverse and rapidly-expanding catalog of synthetic, bilayer-forming amphiphiles has been developed\textsuperscript{22} including the structures shown in Figure 1-6. The natural phospholipid DPPC (I) has been included for comparison. The three primary components of DPPC are the phosphocholine headgroup, glycerol backbone and saturated acyl chains. The other lipids shown in Figure 1-6 employ amino acid backbones (II)\textsuperscript{23}, crown ether head groups (III)\textsuperscript{24} and azobenzene (IV)\textsuperscript{25} or conjugated polyene tails (V)\textsuperscript{26}. These examples are not meant to show systematic variation but rather the drastic structural departures from natural lipids which can be tolerated while maintaining the ability to form bilayer structures.
Figure 1-6.
Nature has selected phospholipids as the primary components for most biological membranes. This class of lipids generally consists of a substituted phosphate head group and two long-chain hydrocarbons linked to a glycerol backbone (Figure I-7). Most often the hydrocarbon tails are connected to the backbone by acyl linkages as shown in Figure I-7 but numerous lipids, particularly those in bacterial membranes use ether rather than ester linkages.

\[
\begin{align*}
\text{O} & \quad \text{P} & \quad \text{O}^- \\
\text{R}_1 & \quad \text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} & \quad \text{O} \\
\text{R}_2 & \quad \text{R}_3
\end{align*}
\]

\textbf{Figure I-7.} Generic phospholipid structure. See Table I-2 for R\textsubscript{1}; R\textsubscript{2} and R\textsubscript{3} are long-chain hydrocarbons.

Considerable structural diversity exists in natural phospholipids. The phosphate may be esterified with choline, ethanolamine, serine, inositol or glycerol (Table I-2). These lipids are either zwitterionic or anionic; other negatively charged lipids include the free phosphatidic acid and cardiolipin, an intriguing dimeric phospholipid found in the membranes of mitochondria, chloroplasts and some bacteria.\textsuperscript{2}
Table I-2. Phospholipid head groups

<table>
<thead>
<tr>
<th>$R_1$</th>
<th>Lipid (Phosphatidyl-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CH$_3$)$_3$N$^+$</td>
<td>choline</td>
</tr>
<tr>
<td>H$_3$N$^+$</td>
<td>ethanolamine</td>
</tr>
<tr>
<td>H$_3$N$^+$CO$_2^-$</td>
<td>serine</td>
</tr>
<tr>
<td></td>
<td>inositol</td>
</tr>
<tr>
<td></td>
<td>glycerol</td>
</tr>
</tbody>
</table>
These structural possibilities are multiplied by variation of the length and degree of unsaturation in the fatty acids which are connected to the glycerol backbone. These chains range from 12 to 24 carbons in length (only even numbers) and may contain up to six nonconjugated double bonds. Furthermore, the two chains of a single lipid will often have different length and degree of unsaturation with the sn-2 chain (the chain attached to the secondary hydroxyl of glycerol, see Figure I-10) typically bearing greater unsaturation and longer length than the sn-1 chain. Although biological membranes generally contain many different lipids, model membranes used for in vitro experiments rarely consist of more than two or three lipids since this is nearly always sufficient to bestow a desired physical property upon the membranes.
I. D. PROPERTIES OF MEMBRANES

The aggregate properties of lipid bilayers will now be described. The membrane is an ordered, two-dimensional medium. However, for the case of lipid bilayers, the definition for membranes given above requires considerable elaboration. A lipid bilayer cannot be thought of as merely a slab of hydrocarbon separating two aqueous phases for just as a suspension of lipid bilayers is a heterogeneous mixture, a single lipid bilayer is a microheterogeneous medium. This is because the bulk properties of the membrane are variable at different depths within the membrane (i.e., at different distances from the aqueous interface). For example, the local dielectric constant decreases from that of bulk water ($\varepsilon=80$) to that of hydrocarbon ($\varepsilon=2-3$) and then increases back up to the aqueous value upon traversing the bilayer. This permits binding of hydrophobic molecules to the interior of the bilayer and hydrophilic molecules at or near the surface.

Four properties of lipid bilayers will be discussed in greater detail: 1) membrane fluidity or microviscosity, 2) surface charge, 3) hydration forces and 4) electric dipole potential which are all important factors in the experiments described below.

I. D. 1. Membrane Fluidity

Aggregation of lipids into a bilayer results in formation of an ordered, two-dimensional structure in which the constituent lipids are packed fairly close together. At low temperatures, the lipid tails are found in a completely extended conformation permitting maximal van der Waals interactions with neighboring
lipids and very close packing. This state is known as the solid-analogous or gel phase, given the symbol \( L_b \). At high temperatures, the lipid chains have greater conformational freedom leading to kinks in the chains due to formation of gauche rotamers. This diminishes the interlipid van der Waals attractions and results in a more loosely-packed membrane. This state is known as the liquid-analogous or liquid crystalline phase, given the symbol \( L_\alpha \).

The transition from \( L_b \) to \( L_\alpha \) does not occur continuously. Rather it is a first-order phase transition occurring over a fairly narrow temperature range. In analogy to the behavior of crystals, the lipid chains can be thought of as "melting" upon heating through the phase transition. The physical properties of the membrane are highly dependent on whether the bilayer is in the \( L_b \) or \( L_\alpha \) phase. For example, the effective area of the lipid chains increases approximately 20% while the bilayer thickness decreases approximately 15% upon transition into the \( L_\alpha \) phase.\(^{28}\) Both effects can be attributed to the higher population of gauche rotamers in the lipid tails above the phase transition.

Some phospholipids exhibit two distinct gel phases. This is true of the saturated phosphatidylcholines shown in Figure 1-8.\(^{29}\) These lipids exhibit a low temperature \( L'_b \) phase where the prime symbol indicates that not only are the chains completely extended as in the \( L_b \) phase but they are also tilted with respect to the bilayer normal, maximizing chain packing. At higher temperatures the bilayer enters the \( P'_b \) phase. This structure is termed the "ripple" phase because of its undulatory surface texture as observed in electron microscopy. The chains remain tilted in this phase. At still higher temperatures, the lipid chains abruptly melt, yielding the \( L_\alpha \) phase. As shown in Table 1-3, the
temperatures for both of these transitions increase with increasing chain length due to the greater van der Waals interactions for the longer chains.

Figure 1-8. Saturated phosphatidylcholines (n=10-19).
Table I-3. $L_{\beta'}$-to-$P_{\beta'}$ and $P_{\beta'}$-to-$L_{\alpha}$ phase transition temperatures for saturated phosphatidylcholines.

<table>
<thead>
<tr>
<th>n</th>
<th>$L_{\beta'}$-to-$P_{\beta'}$ ($^\circ$C)</th>
<th>$P_{\beta'}$-to-$L_{\alpha}$ ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>-</td>
<td>-2.1</td>
</tr>
<tr>
<td>11</td>
<td>-0.8</td>
<td>13.7</td>
</tr>
<tr>
<td>12</td>
<td>14.3</td>
<td>23.9</td>
</tr>
<tr>
<td>13</td>
<td>24.8</td>
<td>34.7</td>
</tr>
<tr>
<td>14</td>
<td>34.2</td>
<td>41.4</td>
</tr>
<tr>
<td>15</td>
<td>43.0</td>
<td>49.8</td>
</tr>
<tr>
<td>16</td>
<td>50.7</td>
<td>55.3</td>
</tr>
<tr>
<td>17</td>
<td>57.8</td>
<td>61.8</td>
</tr>
<tr>
<td>18</td>
<td>63.7</td>
<td>66.4</td>
</tr>
<tr>
<td>19</td>
<td>68.7</td>
<td>71.1</td>
</tr>
</tbody>
</table>

Perhaps the one property most affected by the membrane phase is the fluidity. Lateral diffusion of lipids in the bilayer can be studied using the fluorescence recovery after photobleaching (FRAP) technique. The diffusion coefficients extracted from these experiments typically exhibit a 100-fold decrease in the gel phase ($D=10^{-10}$ cm$^2$/s) relative to the liquid crystalline phase ($D=10^{-8}$ cm$^2$/s).30

The close packing of lipids can have an additional effect on small molecules bound to the membrane. In the gel phase, small molecules may be unable to penetrate as deeply into the membrane due to the closer packing of
the lipids below the phase transition. This has been shown to lead to extrusion of small molecules out of the hydrocarbon region into the aqueous medium.\textsuperscript{31}

Finally, the fluidity within the membrane is not constant at different depths. The acyl chains are significantly more constrained close to the glycerol backbone than at the center of the bilayer. Thus fluidity is expected to be greater in the center of the bilayer relative to near the interface, a prediction which has been verified experimentally by \textsuperscript{2}H-NMR\textsuperscript{32} and ESR\textsuperscript{33} measurements. The molecular order parameter $S_{\text{mol}}$ measured by \textsuperscript{2}H-NMR spectroscopy is related to the conformational mobility of the molecule. Qualitatively, $S_{\text{mol}}$ increases with decreasing conformational freedom. Dipalmitoylphosphatidylcholine (DPPC, Figure I-8, $n=14$) was selectively deuterated at nine different sites along the hydrocarbon chains.\textsuperscript{32} Measurement of the order parameter at each site by \textsuperscript{2}H-NMR spectroscopy allowed construction of a profile of membrane disorder as a function of depth within the bilayer. At the $P_{\beta'}$ to $L_{\alpha}$ phase transition ($T=41.4^\circ C$), $S_{\text{mol}}$ was essentially constant for carbons 1 to 10 (the acyl carbon is numbered 1) but then decreased by 50% between C-10 and C-15. At higher temperatures ($T=50^\circ C$, 57 $^\circ C$) the conformational restriction did not propagate as deeply into the bilayer as $S_{\text{mol}}$ began to decrease between C-5 and C-9. Additionally, $S_{\text{mol}}$ decreased with increasing temperature at each of the nine positions. Thus, these experiments determined that at a given temperature fluidity increases with increasing depth in the membrane and that the fluidity at a given depth increases with increasing temperature.
I. D. 2. Membrane Surface Charge

Virtually all biological membranes are negatively charged due to the presence of anionic phospholipids and sialic acid residues in the membrane. Model membranes may be endowed with anionic or cationic surface charge simply by constructing the membranes from charged lipids. These systems may be composed either entirely of the charged lipid or of a mixture of the charged lipid with an electrically neutral lipid. The latter configuration permits systematic variation of the amount of charge associated with a given liposome. Two advantages of charged versus uncharged liposomes arise from electrostatic effects: 1) Uncharged liposomes will often aggregate and precipitate within one day of preparation. Charged liposomes electrostatically repel one another, inhibiting aggregation. 2) Charged hydrophilic molecules may be bound to the surface of liposomes bearing the opposite charge provided the aqueous medium is of sufficiently low ionic strength to avoid screening of the surface charge. This is especially useful for studies which employ lipid bilayers as media for chemical reactions.

A more subtle method for imparting electrical charge to otherwise neutral liposomes is to bind hydrophobic ions to the interior of the membrane. This class of ions is composed of molecules having charge which is either highly delocalized or surrounded by hydrophobic groups causing the ion to partition from water into the membrane interior. Binding to neutral membranes thus imparts electrical charge to the liposome and should permit binding of water-soluble ions of the opposite charge to the membrane surface.
I. D. 3. The Effect of Hydration Forces on Membrane Fusion

Hydration of lipid head groups has been found to be an important factor controlling interaction of separate membrane surfaces.\textsuperscript{34,35} These interactions are of considerable interest since many biological processes such as viral infection and fertilization require fusion of two initially distinct and spatially separated membranes. The hydration force results from the ordering of water molecules at a hydrophilic surface. For lipid bilayers, this ordering is due to solvation of the lipid head groups. In order for membrane fusion to occur, two membranes must be brought into contact (apposition). This requires removal of the ordered water layers at the membrane surfaces. Since solvation of the head groups diminishes the free energy of the system, this dehydration process requires energy input. Thus fusion of two hydrated membranes is not a spontaneous process.

In addition to the energetic cost of water removal from the interfaces, a more subtle barrier to membrane apposition arises from dipolar repulsion of the two surfaces. Even for membranes composed of electrically neutral lipids, repulsion can arise between the ordered water layers of the membrane surfaces being brought together.\textsuperscript{34-36} To a first approximation, these two layers should be oriented antiparallel to one another, creating an electrostatic repulsion.

Rand and Parsegian proposed that the hydration force is actually the net result of a competition between repulsive and attractive components.\textsuperscript{37} As illustrated in Figure I-9, the dipolar nature of the lipid head groups will cause antiparallel ordering of the water molecules at a single interface. If another membrane approaches with a complementary ordering of the water molecules,
a net attraction will result. Hydrogen bonded networks of water molecules in the intervening space between the two surfaces will augment the electrostatic attraction. However, if the two interfaces are brought together such that the water dipoles oppose one another, a repulsive interaction will result.

While this model presents an intriguing and potentially important advance in the understanding of interbilayer interactions, neither theoretical nor experimental evidence exists for a situation in which the hydration force is net attractive. Additionally, a net attractive force based on the intervening water molecules would bring the two membranes to an energy minimum at a distance of separation which is greater than direct contact. At closer separation, water would have to be removed, increasing the energy of the system. Thus hydration attraction is most likely unimportant in membrane fusion.

**Figure 1-9.** Illustration of hydration forces. In hydration attraction (A) water dipoles and hydrogen bonding are complementary. In hydration repulsion (B) ordered water layers repel one another electrostatically and cannot form bridging hydrogen bonds. Adapted from ref. 37.
The extent to which water is ordered by a membrane surface is dependent on the composition of the membrane. For example, PC membranes are hydrated roughly twice as much as PE membranes based on the number of water molecules present per lipid at maximum hydration. This difference has been attributed to the ability of the PE lipids to form intermolecular hydrogen bonds to neighboring lipids via the free ammonium in the head group, stabilizing the membrane. PC lipids lack this capability since the head group ammonium is quaternized (Table 1-2). Thus PC membranes are stabilized by increased hydration. The lower hydration of PE membranes leads to a smaller hydration repulsion. The practical implication of this result is that PE membranes often undergo fusion events under conditions where PC membranes are stable. The application of this distinction between PC and PE membranes to visible light triggered fusion processes will be discussed in Chapter VII.

I. D. 4. Membrane Dipole Potential

An electric dipole potential has been found to exist across phosphatidylcholine membrane interfaces. This potential is approximately 200-250 mV in magnitude with the positive component residing within the membrane interior. The potential is believed to result primarily from the ester carbonyl linking the acyl chain to the sn-2 position of the glycerol backbone. The x-ray structure of dimyristoylphosphatidylcholine (DMPC, structure in Figure I-10) has shown this carbonyl to be oriented nearly perpendicular to the membrane surface with the oxygen pointing out toward the aqueous phase. This results in the positive end of the carbonyl dipole oriented into the membrane thus contributing to the observed potential. In comparison,
the carbonyl of the sn-1 chain is very nearly parallel to the membrane surface and should make virtually no contribution to the dipole potential. While the x-ray crystal structure is performed on a lipid crystal and may not accurately reflect the structure of the lipids in a hydrated membrane, other experiments in aqueous systems indicate that the overall lipid conformation illustrated in Figure 1-10 is preserved in the bilayer aggregate.43-45

![Diagram of DMPC structure](image)

Figure 1-10. Structure of DMPC illustrating different orientations of carbonyl groups connecting sn-1 and sn-2 chains to the glycerol backbone.

The magnitude of the carbonyl contribution to the dipole potential has been investigated using DPPC and its ether-linked analog, dihexadecylphosphatidylcholine (DHPC).31 The potential was measured by ion conductances through planar bilayers and yielded values of 227 mV for DPPC and 118 mV for DHPC. While the results clearly demonstrate the importance of
the carbonyls for the dipole potential, the fact that DHPC exhibited a significant potential of its own was interpreted as due to the ordering of water molecules at the membrane surface. The presence or absence of carbonyls has no detectable effect on the ordering of these water molecules since the hydration force (see Section I. D. 3) was the same for DHPC and DPPC bilayers. This ordering is the result of dipolar and hydrogen bonding interactions between the water molecules and the zwitterionic PC headgroup.

Based on the discussion in Section I. D. 3, one might predict a smaller dipole potential for PE membranes than for PC membranes because PE membranes induce less ordering of water. Since the sn-2 carbonyl for PE lipids has been found to have a similar orientation to that of PCs, the dipole potential would be expected to follow the order: PC (ester) > PE (ester) > PC (ether) > PE (ether). Experimental verification of this prediction has not been reported.

The dipole potential is believed to be the origin of dramatically different permeabilities and binding constants observed for structurally similar anions and cations with lipid bilayers. For example, chloride anion can cross lipid bilayers with a permeability coefficient ($P$) of $10^{-11}$ cm/s whereas for sodium cation $P=10^{-13}-10^{-14}$ cm/s.$^{46,47}$ While both numbers are quite low, the difference of two to three orders of magnitude is significant. Similar comparisons can be made for the structurally analogous tetraphenylborate anion (Ph$_4$B$^-$) and tetraphenylphosphonium cation (Ph$_4$P$^+$). For Ph$_4$B$^-$, the permeability coefficients and binding constants ($K$) are $P=10^{-1}$ cm/s and $K=10^5-10^6$ M$^{-1}$ while the corresponding values for Ph$_4$P$^+$ are $P=10^{-7}$ cm/s and $K=10^2-10^3$ M$^{-1}$. The particularly high binding constant of Ph$_4$B$^-$ makes this anion an attractive agent
for imparting negative charge to otherwise uncharged phosphatidylcholine liposomes as described in Section I. D. 2.
I. E. POLYMERIZED VESICLES

While dipolar forces and van der Waals' attractions help stabilize lipid bilayers, biological membranes feature additional stabilization systems. These typically consist of polymeric networks which are associated with the membrane surface. For example, bacteria and plant cells possess exterior polysaccharide cell walls which are anchored to the cell membrane by proteins. On the other hand, animal cells have a flexible cytoskeleton on the interior of the cell membrane comprised of water-soluble proteins linked to one another and anchored to the membrane by association with integral proteins. This allows the cell to reversibly deform as is required, for example, when an erythrocyte must pass through tiny capillaries.

Polymer chemistry has also been employed in synthetic systems to enhance the stability of phospholipid membranes. Polymerizable groups have been incorporated into bilayer-forming amphiphiles by chemical synthesis. Subsequent formation of vesicles yields a two-dimensional array of the polymerizable groups. Several configurations can be envisioned for such systems: in principle, the polymerizable moiety can be positioned anywhere along the lipid tails or linked to the head group covalently or electrostatically (Figure I-11). Polymerization leads to covalent linkage of the lipids in the bilayer, resulting in increased stability of the assembly.
Figure I-11. Configurations for formation of polymerized vesicles featuring polymerization of A) lipid tails, B) lipid head groups, and C) electrostatically associated gegenions. Adapted from ref. 46.

Each of the configurations illustrated in Figure I-11 has been realized; the remainder of this discussion will focus on case A where the polymerizable groups are in one or both of the lipid tails, as these are representative of the systems used in Chapters VI-VIII.
I. E. 1. Polymer Chemistry in Phospholipid Membranes

A wide variety of polymerizable groups have been incorporated into lipids including (but not restricted to) styryl,\textsuperscript{52-54} diacetylenyl,\textsuperscript{55-61} dienoyl,\textsuperscript{55,63} sorbyl,\textsuperscript{62} acryloyl,\textsuperscript{64} and methacryloyl\textsuperscript{65-67} (Figure 1-12) moieties. Lipids having each of these groups undergo polymerization when irradiated with UV light. Thermal azo initiators such as AIBN have also been used to polymerize dienoyl, acryloyl and methacryloyl lipids.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{polymerizableGroups.png}
\caption{Examples of polymerizable groups which have been incorporated into bilayer-forming amphiphiles.}
\end{figure}

Many of the same principles which govern polymerization in three dimensions (isotropic solution or bulk systems in the melt) also apply to the two-dimensional environment of lipid bilayers.\textsuperscript{68} For example, monofunctional lipids (as illustrated in Figure 1-11) will form linear polymers whereas bifunctional lipids which contain a polymerizable group in each tail will yield cross-linked polymer networks.
Two of the factors controlling the polymer chain length in conventional three-dimensional polymerizations are the ratio of monomer to initiator ([M]/[I]) and the monomer concentration. In particular, it has been found that decreasing either of these quantities leads to formation of shorter polymer chains. Recent work in vesicles indicates the same relationships apply to two-dimensional polymerizations. For AIBN-initiated polymerizations of a mono-acryloyl-substituted PC, the polymer chain length was found to decrease with decreasing [M]/[I]. Moreover, decreasing the monomer concentration (by mixing into the vesicles variable amounts of a nonpolymerizable lipid) also led to shorter polymer chains. Finally, termination of the growing polymer chains was found to occur by primary termination, a process involving coupling of an initiator radical with the reactive polymer terminus. This termination mechanism is operative under conditions which are not favorable to the standard termination modes commonly observed in solution polymerizations involving coupling and disproportionation of two polymer chains. These conditions include high initiator concentration and viscosity, both of which were likely applicable to the study in bilayers.
I. E. 2. Effect of Polymerization on the Bilayer

Electron microscopy \(^{54,56,60,69}\) and laser light scattering \(^{52}\) have been used to demonstrate that polymerization does not significantly alter the shape or diameter of spherical vesicles. However, polymerization of a vesicle can have dramatic effects on the physical properties of the bilayer, particularly when the polymerizable groups are located in the lipid tails. Polymerization leads to a decrease in the fluidity of the membrane, as indicated by retarded lateral diffusion of the lipids within the bilayer.\(^{70}\) Modest reductions are observed for monofunctional lipids, but diffusion is almost completely stopped by polymerization of bifunctional lipids. This is analogous to the effect of cross-linking in three-dimensional polymerizations in which gelation occurs after a certain extent of polymerization. Polymerization of the lipids in essence causes the membrane to gel. (Of course the aqueous medium is still fluid, permitting diffusion of the vesicles throughout the suspension.)

Polymerization of the lipid tails usually leads to abolition (or at least attenuation) of the main phase transition of the bilayer.\(^{55,71}\) Covalent linkage of the lipid tails inhibits formation of the gauche rotamers and cooperativity typically observed as unpolymerized bilayers pass through the phase transition. The loss of the phase transition is correlated to an increase in the chemical and physical stability of vesicles after polymerization. Polymerized vesicles generally exhibit enhanced stability to organic solvents and detergents,\(^{55,65,72}\) longer shelf-life,\(^{52,54}\) and decreased permeability to water-soluble molecules.\(^{67}\)

The results described above demonstrate that polymerization is in fact an effective means of stabilizing lipid bilayers. Other applications of polymerized
vesicles as media for photochemical reactions and as potential drug delivery vehicles will be described in Chapters VI and VII-VIII, respectively.

This introduction has been designed to give a description of natural, synthetic and polymerized lipid bilayers at the molecular and aggregate level. The remaining chapters describe two fairly distinct areas of research but both draw heavily on the principles outlined above. The first area (Chapters II-VI) details experiments involving the use of phospholipid membranes as media for chemical reactions. In particular, photoinduced energy and electron transfer between membrane-bound donors and acceptors has been studied. The second area (Chapters VII and VIII) details experiments in which phospholipid membranes have been used as substrates for chemical reactions, specifically the polymerization of lipid bilayers triggered by visible light. Whereas this introduction has been intended to convey the structural diversity of lipid bilayers, the remainder of this dissertation will hopefully illuminate the functional diversity of these systems.
CHAPTER II

INTRODUCTION TO PHOTOCHEMISTRY IN PHOSPHOLIPID MEMBRANES

II. A. SUPRAMOLECULAR ASSEMBLIES AS MEDIA FOR PHOTOCHEMICAL REACTIONS

Microheterogeneous or organized media are systems which exhibit bulk homogeneity but local heterogeneity. For example, the environment within a spherical micelle is clearly nonuniform given the hydrophobic core and hydrophilic surface of the micelle (see Figure I-2). However, the micelles themselves are distributed homogeneously throughout the aqueous medium. With the exception of those water molecules involved in hydration of the micellar surface, the bulk water is largely unperturbed by the presence of the micelles. Thus molecules dissolved in the aqueous medium which do not bind to the micelles should exhibit similar physicochemical properties as would be observed in pure aqueous solutions. However, molecules bound to the micelle are likely to show drastically altered behavior since any solvent-dependent properties will reflect the micellar medium rather than the aqueous medium.

Supramolecular assemblies form a subset of microheterogeneous media which consist of a collection of molecules held together by noncovalent forces such as hydrogen bonding, electrostatic attraction and solvophobic interactions. Micelles and lipid bilayers, which are stabilized by hydrophobic interactions fit this definition. An example of a microheterogeneous medium which is not a supramolecular assembly is a cyclodextrin (CD) molecule. A CD is a single
cyclic polysaccharide in which the hydroxyl groups are located primarily on the exterior of the ring. This gives the CD a hydrophilic surface and a hydrophobic interior. Small nonpolar molecules of the appropriate dimensions can be occluded within the interior of CDs providing a method of suspending otherwise insoluble organic molecules in aqueous media. Since a CD is a single macromolecule it cannot be characterized as a supramolecular assembly.

One of the advantages in using supramolecular assemblies such as lipid bilayers rather than macromolecular systems such as CDs as media for photochemical reactions arises from the greater flexibility available in design of systems involving the former. The biggest difference between the two media comes from the binding modes available within the structure of the media. The covalent linkage of the saccharide units in CDs precludes binding of molecules within the ring itself. Moreover, the small dimensions of the internal hydrophobic space (4.7-8.3 Å diameter depending on the number of saccharide units) limits binding to one or two molecules per CD. On the other hand, the lack of covalent bonding between the lipids in a bilayer permits binding of hydrophobic molecules within the membrane interior. Furthermore, hundreds of molecules may be bound to a single liposome, affording a much higher density than in CD-based systems, an important concern for practical applications of these systems. The relative ease with which the membrane properties can be varied (see Section I. D.) leads to manifold possible configurations for membrane-based chemistry.
II. B. WHY PHOTOCHEMISTRY?

Photochemical reactions are often studied in supramolecular assemblies due to the nonperturbing effect of light on the medium.\textsuperscript{10,74} As described in section I. D. 1., the physical state of the bilayer is temperature dependent with the different phases often existing over much narrower temperature ranges than homogeneous solvents such as water or methanol. Thus application of sufficient heat to trigger a thermal reaction in a lipid bilayer may alter the phase of the hydrated lipids. Moreover the activation barrier to a thermal reaction may preclude study of the reaction in the low-temperature phase(s). Conversely a reaction with a very low barrier cannot be employed since the system must have time to reach an equilibrium distribution of the reactants throughout the membrane. For example, hydrophobic molecules may require time to partition into the membrane interior. Reaction before this equilibrium is reached can lead to ambiguous conclusions. These concerns place significant limitations on the scope of studies involving thermal reactions in lipid bilayers. Since one of the goals of this work was to study the influence of the medium on membrane-based reactions, photochemical reactions were chosen for investigation in lipid bilayers.

Many photochemical reactions exhibit negligible activation barriers because light absorption typically produces not only electronically excited states but also vibrationally excited states.\textsuperscript{75,76} Reaction to form ground state products generally occurs from excited state minima. Except in cases where the excited state must undergo severe nuclear distortions before ground state products can be formed, relaxation to these minima will be exergonic. The practical
implications for this work are that different membrane phases may be accessed thermally with very little change in the energetics of the photochemical reactions under study. Thus medium effects due to variation of the physical state of the bilayer can be studied unambiguously.
II. C. MEMBRANE-BASED PHOTOCHEMISTRY

The bulk properties of a lipid bilayer such as dielectric constant and fluidity are variable at different depths within the membrane but constant (on average) at a given depth. Thus one can design a particular configuration for a membrane-based photochemical system with considerable facility. Four possible configurations can be identified (Figure II-1): 1) Surface reactions occur between molecules localized at a single surface of the membrane. 2) Interior reactions occur between two molecules bound within the hydrophobic interior of the membrane. The binding depths of A and B overlap in this case. 3) Vectorial reactions occur when A and B are bound at nonoverlapping depths. One such case has A bound to the surface and B bound in the interior of the membrane. 4) A special case of vectorial systems is the transmembrane configuration, where A and B are bound at different surfaces of the bilayer. (Recall that the membranes are actually closed structures, the two surfaces of which may be differentiated using chromatographic techniques.) In each of these configurations, the membrane acts as both a reaction vessel and a solvent.
Figure II-1. Possible configurations for bimolecular reaction in a lipid bilayer medium: (a) surface reaction, (b) interior reaction, (c) vectorial reaction, and (d) transmembrane reaction.

The first two configurations represent two-dimensional analogues of bimolecular reactions in homogeneous media where the reactants are distributed isotropically in three dimensions. The ordered environment of the bilayer creates the possibility of ordering reactants in the membrane. The ability to bind molecules at different depths within the bilayer permits design of vectorial systems. These systems exhibit directionality of reactivity, either toward the membrane surface or toward the interior. Vectorial reactions cannot occur in isotropic media since it is not possible to organize reactants in a completely disordered environment.
A wide variety of photochemical reactions have been studied in phospholipid membranes. Investigations of photoinduced electron transfer (PET) and electronic energy transfer (EET) reactions is the subject of the first part of this dissertation. Some basic principles for these two reactions and their importance in photosynthesis will now be described.
II. D. PHOTINDUCED ELECTRON TRANSFER REACTIONS

Photoinduced electron transfer reactions have received considerable attention for their practical application in photography and Xerography™ as well as for their role in photosynthesis in phototrophic plants and bacteria. The generality of PET reactions lies in the fact that both donor and acceptor molecules exhibit enhanced redox capabilities in the excited state relative to the ground state. For example, the ground state ionization potential (which is directly related to the oxidation potential) for some donor D is simply the energy required to remove an electron from the highest occupied molecular orbital (HOMO, Figure II-2A). In the excited state, the ionization potential measures the energy required to remove an electron from what was initially the lowest unoccupied molecular orbital (LUMO). The lower ionization potential in the excited state is manifested as a lower oxidation potential, showing that electronically excited species are typically more powerful electron donors than their ground-state analogues. Similar arguments based on electron affinities and reduction potentials illustrate that molecules are also better electron acceptors in the excited state than in the ground state (Figure II-2B).
Figure 11-2. Ground and excited state ionization potentials (IP) and electron affinities (EA).
The enhanced redox capabilities of excited state molecules can be used to design systems which are stable in the dark but undergo PET reactions upon irradiation. In principle, either the donor or the acceptor can be irradiated to give the same set of redox products (Figure II-3).

**Figure II-3.** Photoinduced electron transfer involving either an excited donor (D) or an excited acceptor (A).
Much of the interest in PET reactions has come from their potential for energy conversion and storage.\textsuperscript{78,79} The light energy used to excite either D or A in Figure II-3 is converted to chemical potential by the electron transfer reaction. Some of the excitation energy is lost in the exergonic electron transfer while the remainder is stored in the redox products. The amount of energy stored in the system is determined by the thermodynamics of the reaction. Large energy differences between the HOMO of the donor and the LUMO of the acceptor maximize energy storage (Figure II-4). Chemically, this means that PET reactions which are only weakly exergonic result in storage of a large percentage of the excitation energy; this is critical for the utilization of the stored energy to drive subsequent chemical transformations.
Another concern associated with the use of PET reactions for energy conversion and storage is the length of time for which the energy can be stored in the redox products. In many cases, the predominant decay path for the redox products is via back electron transfer. As shown in Figure II-5, this reaction is downhill in energy with its exergonicity varying inversely with that of the forward reaction. This leads to the unfortunate characteristic that systems which store
the most amount of energy will also have the most exergonic back reaction. While some systems\textsuperscript{80,81} exhibit back electron transfer reactions in the Marcus inverted region\textsuperscript{82-84} where reaction rates decrease with increasing exergonicity, the back reactions in most cases are extremely fast and preclude efficient utilization of the stored energy to drive subsequent chemical reactions. The stored energy is completely wasted since the back reaction simply produces the ground state starting materials. In order to minimize this inefficiency, several strategies for avoiding back electron transfer have been developed.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure11-5.png}
\caption{Exergonic back electron transfer following photoinduced electron transfer.}
\end{figure}
II. E. INHIBITION OF BACK ELECTRON TRANSFER

Four approaches to the inhibition of back electron transfer in PET systems are described below:

1) Sacrificial component: If one of the two redox products decomposes, the remaining component will be stabilized. EDTA,$^{85}$ [Co(NH$_3$)$_5$Cl]$^{2+}$ $^{86}$ and S$_2$O$_8^{2-}$$^{87}$ have filled this role in various PET systems. Recently, Schuster and coworkers described a sacrificial borate anion which decomposes upon one-electron oxidation; back electron transfer is completely eliminated in those systems resulting in an increased lifetime of the reduced acceptor.$^{88,89}$

This approach compromises energy storage for stability: unless the decomposition product(s) are useful in the system, the energy stored in the sacrificial component is lost. Moreover, the progressive depletion of one of the reactants precludes the use of this approach in a recyclable system.

2) Spin multiplicity changes: The use of a light-absorbing component which undergoes facile intersystem crossing in the excited state can lead to stable redox products. As illustrated in Figure II-6, the generation of the triplet excited state donor and subsequent electron transfer produces a triplet radical ion pair. Back electron transfer cannot occur until conversion to a singlet radical ion pair has occurred. This increases the lifetime of the charge-separated state giving more time to harvest the energy stored by PET. Rapid solvation of the radical ion pair will prevent intersystem crossing back to the singlet state within the geminate radical ion pair. This process, which occurs primarily in polar,
nonviscous solvents is beneficial in terms of separation of the redox products but causes a decorrelation of spins. Encounter between reduced acceptor and oxidized donor of opposite spin will lead to rapid back electron transfer.

Figure II-6. Generation of triplet excited state donor before electron transfer leads to stabilized radical ion pair. Back electron transfer is inhibited.

3) Supersensitization: This method utilizes multiple electron transfers to stabilize the charge-separated state. A sensitizer (S) is added to the donor(D)-acceptor(A) system (Figure II-7). This molecule is excited and is subsequently oxidized by A or is reduced by D. A second electron transfer, either from donor
to oxidized sensitizer or from reduced sensitizer to acceptor completes the charge-separation. This two step sequential electron transfer pathway results in regeneration of ground state S and production of reduced acceptor (A\textsuperscript{-}) and oxidized donor (D\textsuperscript{+}). The success of this approach should be enhanced in systems where diffusion is restricted thereby impeding encounter between D\textsuperscript{+} and A\textsuperscript{-}.
Figure II-7. Supersensitized PET. Following excitation of S, electron transfer either occurs from S to A then D to S (steps a and b) or vice versa (steps c and d).
4) Spatial Separation of D\(^+\) and A\(^-\): Electron transfer requires orbital overlap between the donor and acceptor. Thus rate constants for these reactions are exponentially dependent on the distance of separation between D and A. Increasing the average distance between the reduced acceptor and the oxidized donor will inhibit the back electron transfer.

Typically, combinations of these strategies are employed for inhibiting back electron transfer. For example, the elegant triad, tetrad and pentad molecules of Gust and Moore feature sequential electron transfers and spatial separation of redox centers.\(^{90}\) The separation was achieved by covalent connection of the redox components in the desired order by fairly rigid linkages. Willner and coworkers have utilized all four of these strategies simultaneously to enhance PET efficiency in a micelle-based system.\(^{91}\) One of the redox products was selectively bound to the micelle interface by electrostatic attraction while the other product was repelled into the aqueous medium. As described in Section II. G., the photosynthetic reaction centers of phototrophic plants and bacteria exhibit similar strategies for avoiding back electron transfer.
II. F. ELECTRONIC ENERGY TRANSFER REACTIONS

Another pathway open to excited state donor molecules in the presence of appropriate acceptor molecules is electronic energy transfer (EET). EET involves the transfer of energy (rather than electrons) from a donor to an acceptor (Figure II-8).76

![Figure II-8. Electronic energy transfer from an excited state donor (D) to a ground state acceptor (A).](image)

There are three mechanisms by which EET can occur:

1). Trivial EET: This is simply the emission of a photon by the excited donor and subsequent absorption of that photon by the acceptor.

2) Dexter or Exchange EET:92 This mechanism relies on short-range interactions requiring overlap of donor and acceptor orbitals and is thus only operative in systems where close approach of \( \cdot D \) and \( A \) is permitted (less than ten angstroms). Conceptually, this is analogous to a double-electron transfer reaction.
3) Förster or Coulombic EET: This occurs at short- or long-range. It is a resonance interaction, relying on the ability of the oscillating dipole in the donor to stimulate the same oscillation in the ground state acceptor. This resonance requires no overlap of donor and acceptor orbitals thus permitting Förster EET to occur efficiently over tens of angstroms.

The energetic requirements for EET are manifested spectrally: the donor must absorb light of higher energy than the acceptor and the donor emission spectrum must overlap the acceptor absorption spectrum, regardless of the mechanism.

There are a number of important differences between PET and EET. First, EET yields a ground state and an excited state whereas PET yields ground state products. Thus EET often leads to replacement of the donor fluorescence by that of the acceptor while PET only quenches the donor fluorescence. Second, EET can only result from excitation of the donor while PET can occur from excited donor or acceptor. Finally, since EET by the Förster mechanism requires no orbital overlap, through-space EET can occur over much larger distances (tens of angstroms) than a corresponding PET reaction (less than ten angstroms).

EET is often used to extend the sensitivity of photochemical systems to a wider range of wavelengths. Since most chromophores possess fairly narrow absorption bands, the systems will not be active at most wavelengths of irradiation. This is of considerable importance for both natural and synthetic solar-energy converting systems. Using EET to funnel shorter wavelength light into the systems effectively broadens the wavelength range over which
photochemistry can be initiated. The energy donors used to achieve this are often referred to as antenna molecules.
II. G. PET AND EET IN PHOTOSYNTHESIS$^{94,95}$

Green plants and phototrophic bacteria utilize sunlight as an energy source to drive production of ATP from ADP. The net result is the conversion of light energy to chemical potential and storage of that energy in a stable but high energy P-O bond. ATP serves as the energy source for many biochemical regulatory pathways since cleavage of a P-O bond in ATP regenerates ADP and releases approximately 12 kcal/mole in vivo. Photosynthesis produces carbohydrates (a prime food source for nonphotosynthetic organisms), removes carbon dioxide from the air and releases oxygen into the atmosphere.

In photosynthetic organisms, the machinery which converts light energy to chemical potential is contained within membrane-bound proteins.$^{96-99}$ Green plants feature two such protein-based photosystems acting in series whereas bacteria possess a single photosystem. While both plant and bacterial systems have been well-characterized functionally, considerably more structural knowledge has been acquired for some bacterial systems.

The available knowledge through 1983 for bacterial systems concerned functionality and constitutionality.$^{97,98}$ The main part of the system known as the reaction center was known to consist of three or four protein subunits depending on the organism. A variety of cofactors had been identified including four bacteriochlorophylls, two bacteriopheophytins, two quinones and a nonheme iron atom. The fourth subunit found in select organisms was determined to be a cytochrome c. Connected with reaction center proteins were pigment-protein complexes containing more bacteriochlorophylls and carotenoids.
Transient and steady-state spectroscopic techniques revealed the following sequence upon illumination of membrane-bound reaction centers: 1) Photoinduced electron transfer from an excited bacteriochlorophyll dimer (the "special pair" identified by ESR and ENDOR measurements\textsuperscript{75}) to a bacteriopheophytin occurs in less than 10 ps. 2) Secondary electron transfer from the reduced bacteriopheophytin to a quinone happens in approximately 200 ps. 3) The oxidized special pair receives an electron from a cytochrome c on the cytoplasmic side of the membrane 270 ns after excitation. 4) The reduced quinone transfers its electron to a secondary quinone acceptor in 6\,\mu s. This secondary quinone ultimately receives a second electron, then removes two protons from the periplasmic side of the membrane. The observation of coupled oxidation and reduction processes occurring on opposite sides of the membrane led Prince and Dutton to conclude that the reaction center was in fact a transmembrane protein.\textsuperscript{100} The protons from the periplasmic side of the membrane are transported across the membrane, generating the pH and electrochemical gradients which provide the driving force for ATP production in accord with the chemiosmotic hypothesis of Mitchell.\textsuperscript{101,102}

While some of the distances between cofactors could be estimated from magnetic interactions involving the nonheme iron atom, the accurate structural information vital to a complete understanding of the function of photosynthetic reaction centers was lacking until 1984, when the x-ray structure of the reaction center of \textit{Rhodopseudomonas viridis} was reported by Diesenhofer, Michel and Huber.\textsuperscript{103} The crystal structure features a near-symmetric arrangement of the cofactor chromophores in an asymmetric protein environment. Proceeding from the cytoplasmic end to the periplasmic end of the protein, the following entities
were found: a bound cytochrome c with its four heme groups, the special pair of bacteriochlorophylls, two accessory bacteriochlorophylls, two bacteriopheophytins, together with a menaquinone ($Q_A$), nonheme iron and a ubiquinone ($Q_B$) held at the same depth in the protein relative to the membrane. (See the schematic in Figure II-9). Intriguing functional questions arising from the structure regard the role of the accessory bacteriochlorophylls and why electron transfer occurs only in the L-branch (right side of Figure II-9) rather than down both branches.\textsuperscript{104,105}

![Schematic of photosynthetic reaction center of R. viridis.](image)

**Figure II-9.** Schematic of photosynthetic reaction center of *R. viridis.* Cyt C: cytochrome c; P: bacteriochlorophyll special pair; BC: accessory bacteriochlorophyll; BP: bacteriopheophytin; $Q_A$: menaquinone; Fe: nonheme iron atom; $Q_B$: ubiquinone. Adapted from ref. 104.
The vectorial nature of the PET and subsequent thermal electron transfer reactions is evident from the crystal structure. The multiple electron transfer steps ultimately produce the oxidized cytochrome c and reduced $Q_B$ within the protein. The electron and hole are separated by more than 20Å and are prevented from diffusing to one another, which effectively eliminates back electron transfer. (A further contribution to preventing back electron transfer may come from the likelihood that these reactions are in the Marcus inverted region. Entry into this domain occurs at relatively low exergonicities in low dielectric media. The nonpolar protein medium should favor this effect.) Thus the crystal structure verified the existence of all the cofactors and precisely determined their respective locations in the protein. The role played by the protein medium in localizing and orienting the cofactors is crucial to the function. Binding each of the cofactors to a single protein creates extremely high effective concentrations permitting the rapid and efficient electron transfer chain to proceed.

A second feature exhibited by the photosynthetic systems is efficient collection of light across the entire solar spectrum. The reaction centers absorb light primarily above 800 nm. The carotenoids and other bacteriochlorophyll molecules in the pigment-protein complexes associated with the reaction centers absorb light efficiently between 500-700 nm. This light is then funnelled into the reaction center by EET. These accessory systems are referred to as antenna complexes or light-harvesting complexes. It is estimated that as much as 99% of the PET reactions triggered in the reaction centers result from light energy which enters via the antenna complexes.
In summary, the protein medium of photosynthetic reaction centers provides a structured environment which organizes the cofactors both spatially and orientationally. The high effective concentrations obtained in this manner promote efficient vectorial transmembrane electron transfer triggered either by direct irradiation of the reaction center or by energy transfer sensitization via the antenna complexes.

The next four chapters illustrate some of the advantages to using lipid bilayers as media for PET reactions. Chapter III summarizes the interaction in water between the electron donors and acceptors used in Chapters IV-VI. The donors and acceptors form complexes in water which exhibit inefficient PET relative to the membrane-based systems described in the subsequent three chapters. These liposomal systems display some of the key features of photosynthetic reaction centers: vectorial PET, antenna-sensitized PET and high effective concentrations of reactants. Of course a significant difference exists between the natural and synthetic systems with regard to the medium in which the reactants are suspended: in reaction centers the protein is the medium for the electron transfer reactions while the membrane is the medium for the protein. In the synthetic systems, the protein is removed leaving the membrane as the medium for the actual chemical reactions. Chapter IV describes a system which features vectorial PET from the hydrophobic interior of the membrane to the hydrophilic surface. The effect of membrane physical state on PET efficiency is presented.

The system described in Chapter IV is sensitive to red light. In order to extend the sensitivity of the system to shorter wavelengths of light, an antenna
pigment was added which absorbs blue light and transfers the excitation energy to the primary electron acceptor thereby triggering the same chemistry observed with red-light irradiation. These results are summarized in Chapter V.

Finally, it was noted above that one of the key contributions made by the protein medium of the photosynthetic reaction centers to the highly efficient transmembrane electron transfer is to concentrate the cofactors. Membrane-based systems must achieve similarly high concentrations if comparable efficiencies are to be obtained. Chapter VI describes one strategy to accomplish this goal. Lipid bilayers were prepared consisting of a mixture of a polymerizable, neutral lipid and a nonpolymerizable, anionic lipid. Polymerization renders a large percentage of the membrane inaccessible to donors and acceptors. Enhanced EET and PET efficiencies are demonstrated in these polymerized, phase-separated assemblies due to the increased concentrations of donors and acceptors.
III. A. INTRODUCTION

Association of two or more molecules in solution can result in formation of a molecular complex.\textsuperscript{108,109} Upon diffusive encounter, two molecules will form a collision complex which immediately dissociates in most cases. However, if the associated molecules are stabilized by some mechanism, a molecular complex is formed. The lower energy state of the complex retards dissociation; the molecular complex exists as a supramolecular species displaying physical and chemical properties distinct from those of the individual components.

A complex may be stabilized by a number of different mechanisms. Electrostatic attraction and solvophobic forces often drive molecular association. Charge-transfer (CT) interaction is also a common factor in stabilization of molecular complexes.\textsuperscript{110} In this mechanism, one molecule acts as a donor by transferring some of its electron density to an acceptor molecule.\textsuperscript{111} As one might expect, CT-complex formation is strongly dependent on the redox potentials of the donor and acceptor.

Complex formation is often investigated by UV-vis absorption spectroscopy, taking advantage of the fact that in most cases, a complex absorbs light of different wavelengths than do the individual components. For example, CT complexes typically exhibit red-shifted and broadened absorption
spectra with depressed molar extinction coefficients relative to the longest wavelength-absorbing component.\textsuperscript{76,109,112}

The electron donors and acceptors used for the membrane-based PET reactions described in Chapters IV-VI form extremely strong complexes in water. Investigations of these aqueous complexes and comparison with reports of interaction between similar donors and acceptors in organic media reveals that the aqueous complexes are stabilized primarily by hydrophobic interactions rather than by charge transfer.
III. B. EXPERIMENTAL

Materials. $N,N'$-Bis[3-(trimethylammonio)propyl]thiadicarbocyanine tribromide (Cy$^{3+}$) and $N,N'$-Dimethyldimethylindocarbocyanine iodide (Cy$^{+}$) were purchased from Molecular Probes, Inc. (Eugene, OR) and used as received. The molar extinction coefficients, measured in methanol (Table III-2) matched those reported by the supplier. Rhodamine 6-G chloride (Rh$^{+}$) was purchased from Eastman Kodak Co. (Rochester, NY) and used as received. 5,10,15,20-Tetrakis[4-(trimethylammoniophenyl]-21H, 23H-porphine tetra-p-tosylate salt (P$^{4+}$), sodium tetraphenylborate, triphenylboron, benzylimagnesium bromide (2.0 M in THF), n-Butyllithium (1.6 M in hexanes) and tetramethylammonium bromide were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received except for triphenylboron which was purified by vacuum sublimation. Milli-Q water (Millipore Co.; Bedford, MA) was used in all experiments. Spectral grade methanol and acetonitrile (J. T. Baker Inc., Phillipsburg, NJ; Photrex grade) were used as received.

Synthesis of Tetramethylammonium Triphenylbenzylborate ($\text{Ph}_3\text{BnB}^-$). All manipulations were performed under argon atmosphere. Solvents were freshly distilled and purged with argon for at least 15 min before use.

Triphenylboron (0.5 g, 2.1 mmoles) was dissolved in 10 mL THF in a 50 mL two-necked, round-bottomed flask. A solution of 1.0 mL benzylimagnesium bromide (2.0 M in THF) in 10 mL THF was added dropwise, with stirring at 0 °C. Addition was complete in 10 min; the clear, colorless solution was allowed first
to stir for 30 min and then to warm to room temperature. Solvent was removed by rotary evaporator. (An argon line was connected to the pressure inlet of the evaporator to prevent exposure of the product to air which would lead to decomposition.)

The resulting colorless oil was dissolved in water. A solution of 1.0 g (6.5 mmoles) tetramethylammonium bromide in 10 mL water was added dropwise with stirring at room temperature. A white precipitate formed immediately. The mixture was stirred for 30 min, then the white solid was collected by gravity filtration. (This and all subsequent manipulations were done without inert atmosphere since the tetramethylammonium borate salts are stable to air.) The white solid was washed with water to remove excess bromide. Recrystallization from CH\textsubscript{2}Cl\textsubscript{2}/methanol gave 0.1 g (0.24 mmoles) tetramethylammonium triphenylbenzylborate (11\% yield). The white powder decomposed at 251-252 °C and gave the following ¹H-NMR (DMSO-d\textsubscript{6}): δ 7.1-7.2 (br s, 6H), 6.8-6.9 (T, 6H), 6.6-6.8 (m, 6H), 6.4-6.5 (d, 2H), 3.1 (s, 12H), 2.4 (m, 2H). (The full spectrum is in Appendix A-1.)

**Synthesis of Tetramethylammonium Triphenyl-\textit{n}-butylborate (Ph\textsubscript{3}BuB\textsuperscript{+}).** Following a modified literature procedure,\textsuperscript{113,114} 1.3 g (5.4 mmoles) triphenylboron was dissolved in benzene in a two-necked, 50 mL round-bottomed flask. A solution of 3.3 mL 1.6 M \textit{n}-butyllithium (in hexanes) in 20 mL hexanes was added dropwise with stirring at room temperature. A white precipitate formed immediately. Addition was complete in 5 min; the mixture was stirred for an additional 15 min. The white solid was collected by gravity filtration and washed with cold \textit{n}-pentane. The solid (0.6 g) was dissolved in 10
mL water and converted to the air-stable salt as described above, using 0.4 g (2.6 mmoles) tetramethylammonium bromide in 10 mL water. Recrystallization from CH₂Cl₂ yielded 0.14 g (0.38 mmoles) tetramethylammonium triphenyl-n-butyldiborate (7% yield). The white solid decomposed at 220 °C and gave the following ¹H-NMR (DMSO-d₆): δ 7.15-7.25 (br d, 6H), 6.85-6.95 (t, 6H), 6.7-6.8 (t, 3H), 3.1 (s, 12H), 1.15-1.25 (m, 2H), 0.7-0.8 (t, 3H). (The full spectrum is in Appendix A-1.)

**Spectroscopy of Aqueous Dye-Borate Complexes.** Several different dye-borate combinations were investigated (Table III-3); representative experimental procedures for the Cy³⁺-Ph₃BnB⁻ complex are given below.

UV-vis absorption spectra were acquired on a Varian DMS-200 (Varian Techtron Pty. Limited, Victoria, Australia) double beam instrument. The absorption spectrum of a 3.85 μM aqueous Cy³⁺ solution (1.3 mL) was recorded before and after the addition of successive 1.0 μL aliquots of a 1.0 mM Ph₄B⁻ stock solution (CH₃CN).

Fluorescence spectra were acquired on a Spex Fluorolog II spectrofluorometer (Spex Industries, Inc., Edison, NJ). Spectra of aqueous Cy³⁺ and Cy³⁺-Ph₃BnB⁻ complexes were obtained with excitation at 671 nm, the isosbestic point observed in the absorption spectra during complex formation.

The stoichiometry of the Cy³⁺-Ph₃BnB⁻ complex was determined using Job's method."109,115 Stock solutions employed were 1.0 mM Cy³⁺ in water and 1.0 mM Ph₃BnB⁻ in CH₃CN. The total volume of these solutions added to 1.3 mL water was held constant at 12.0 μL. Initially, 12.0 μL Cy³⁺ and 0 μL Ph₃BnB⁻ were added and the absorption spectrum recorded. The procedure was
repeated for 10:2, 8:4, 6:6, 4:8, 2:10 and 0:12 (µL Cy³⁺: µL Ph₃BnB⁻) mixtures of the two components. The optical density of the solution at 700 nm was used to assay for complex formation as the free dye has only minor absorbance at this wavelength. The data was corrected for residual free dye absorbance.

The empirical stoichiometry of the complex was extracted from the data by first plotting OD₇₀₀ versus X where

\[ X = \frac{\text{# µL Ph₃BnB}^-}{\text{# µL Cy}³⁺ + \text{# µL Ph₃BnB}^-} \]

The plot exhibits a maximum at \( X_{\text{max}} \); the stoichiometry of the complex \((\text{Cy}³⁺)ₘ⁻(\text{Ph₃BnB}⁻)ₙ\) is calculated from

\[ \frac{n}{m} = \frac{X_{\text{max}}}{1 - X_{\text{max}}} \]

**Concentration Dependence of Complex Formation.** Cy³⁺ (1.0 mM) and Ph₄B⁻ (2.0 mM) were added to 1.2 mL water in 1.2 µL increments up to 12.0 µL of each. Absorption spectra were recorded after each addition. In this manner, the 1:2 Cy³⁺:Ph₄B⁻ stoichiometry was preserved even though the overall concentrations were varied. The ratio OD₆₅₀/OD₆₇₁ was plotted versus Cy³⁺ concentration. (650 nm is the dye absorption maximum, 671 nm is the isosbestic wavelength observed during titration with Ph₄B⁻.)
Solvent Effects. The absorption spectra of Cy\textsuperscript{3+}:Ph\textsubscript{4}B\textsuperscript{-} (1:2, 7.69 \textmu M Cy\textsuperscript{3+}) were measured in a series of water-acetonitrile mixtures. The full-width at half-maximum (FWHM) was plotted versus the percent acetonitrile in the solvent.

Titration of Cy\textsuperscript{4+}-Cy\textsuperscript{3+} Mixture. An aqueous solution containing an equimolar mixture of Cy\textsuperscript{4+} and Cy\textsuperscript{3+} (3.08 \textmu M for each dye) was titrated with Ph\textsubscript{4}B\textsuperscript{-}, added in 0.75 molar equiv increments up to 3.0 equiv (relative to total dye). Absorption spectra were recorded after each addition.

Spectra for separate solutions of Cy\textsuperscript{4+} and Cy\textsuperscript{3+} were added together, then subtracted from the spectrum of an equimolar mixture of the two. Very little deviation from zero was evident (Figure III-8). Similarly, the spectra for separate solutions of Cy\textsuperscript{4+}:Ph\textsubscript{4}B\textsuperscript{-} (1:1) and Cy\textsuperscript{3+}:Ph\textsubscript{4}B\textsuperscript{-} (1:2) were summed and subtracted from the spectrum for the mixture Cy\textsuperscript{4+}:Cy\textsuperscript{3+}:Ph\textsubscript{4}B\textsuperscript{-} (1:1:3). Considerable deviation from additivity was observed (Figure III-8).

Irradiation of Cy\textsuperscript{3+}-Borate Complexes. Photosensitivity of the Cy\textsuperscript{3+}-borate complexes was studied using steady-state irradiation. Irradiations were performed using red light obtained from the filtered output of a 200W Hg(Xe) arc lamp (Oriel Corp.; Stratford, CT). CS2-62 (Corning Glass Works, \lambda > 580 nm) and neutral density (6% transmitting) filters were used. Samples were irradiated in a quartz cuvette at a distance of 22.4 cm from the lamp. Sample temperature was controlled by using a thermostatted cell holder connected to a water circulator. The lamp flux (ca. 10\textsuperscript{16} photons per second) was measured using a thermopile detector having a surface area of 0.04 cm\textsuperscript{2}. 
Samples consisting of A) 3.2 µL 1.0 mM Cy\textsuperscript{3+} in 1.3 mL H\textsubscript{2}O, B) 4.0 µL 1.0 mM Cy\textsuperscript{3+} and 4.0 µL 2.0 mM Ph\textsubscript{4}B\textsuperscript{-} in 1.3 mL H\textsubscript{2}O, and C) 4.0 µL 1.0 mM Cy\textsuperscript{3+} and 4.0 µL 2.0 mM Ph\textsubscript{3}BnB\textsuperscript{-} in 1.3 mL H\textsubscript{2}O were prepared. These three solutions had equal total optical densities in the range of irradiation. Bleaching of the dye was monitored by the loss of optical density in the visible absorption band. Samples A and B bleached uniformly (i.e. without change in the absorption profile); thus the percent bleaching was calculated simply from the change in optical density at the maximum. Sample C bleached nonuniformly; the percent bleaching was calculated as follows:

Using the titration data (see Figure III-1), the ratio OD\textsubscript{650}/OD\textsubscript{671} was plotted versus added borate (Figure III-2). After 10 min irradiation sample C had OD\textsubscript{650}/OD\textsubscript{671} = 1.60 which based on Figure III-2 corresponds to a ratio of 1:1.4 (Cy\textsuperscript{3+}:Ph\textsubscript{3}BnB\textsuperscript{-}). Interpolating from Figure III-1, OD\textsubscript{650} for this solution \textit{in the absence of Ph\textsubscript{3}BnB\textsuperscript{-}} would be 0.37; the initial OD\textsubscript{650} (before addition of Ph\textsubscript{3}BnB\textsuperscript{-}) was 0.44. Thus 15% Cy\textsuperscript{3+} was consumed during 10 min of irradiation for sample C.
Table III-1. Structures of complex-forming dyes and borates.

**Hydrophilic Dyes**

\[
\text{Hydrophilic Dyes} \\
\begin{array}{c}
\text{Cy}^3+ \\
\text{Cy}^+ \\
\text{Rb}^4+
\end{array}
\]

**Hydrophobic Borates**

\[
R = \begin{array}{ccc}
\text{Ph}_3\text{BnB}^- & \text{Ph}_4\text{B}^- & \text{Ph}_3\text{BuB}^-
\end{array}
\]
III. C. RESULTS

III. C. 1. Formation of Aqueous Complexes. The hydrophilic dyes and hydrophobic borates used in this study are shown in Table III-1. The dyes exhibit intense absorption bands in the visible (Table III-2). At the concentrations used for these experiments, the dyes are dissolved in water as monomers, that is no aggregation of the dyes is observed.

Table III-2 Spectral Properties of Dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon_{\text{max}}$ (M$^{-1}$ cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy$^{3+}$</td>
<td>650</td>
<td>130,000</td>
</tr>
<tr>
<td>Cy$^+$</td>
<td>543</td>
<td>140,000</td>
</tr>
<tr>
<td>Rh$^+$</td>
<td>528</td>
<td>105,000</td>
</tr>
<tr>
<td>P$^{4+}$</td>
<td>410</td>
<td>360,000</td>
</tr>
</tbody>
</table>

Each of the cationic dyes forms water-soluble complexes with hydrophobic borate anions. Figure III-1 shows the results of titration of Cy$^{3+}$ with Ph$_3$SnB$. The progressive broadening and red-shifting of the absorption spectrum with concomitant depression of the molar extinction coefficient as the borate concentration increases is indicative of complex formation. Clean isosbestic behavior is observed up to addition of 2.0 molar equiv of Ph$_3$BnB$^-$; further addition causes the isosbestic point to shift to the red. The association phenomenon illustrated in Figure III-1 is quite strong: successive spectra were recorded after addition of just 0.2 molar equiv of the borate. Plotting the ratio
OD$_{650}$/OD$_{671}$ versus added borate reveals a linear dependence on the borate concentration with similar behavior for Ph$_3$BnB$^-$ and Ph$_4$B$^-$. 

Figure III-1. Absorption spectra of 3.85 μM aqueous Cy$^3+$ titrated with successive additions of 0.2 molar equiv of Ph$_3$BnB$^-$. 
Figure III-2. Plot of \( \frac{OD_{650}}{OD_{671}} \) versus added borate for titration of \( Cy^{3+} \) with \( \text{Ph}_3\text{BnB}^- \) and \( \text{Ph}_4\text{B}^- \).
The tendency for a 1:2 Cy\textsuperscript{3+}:Ph\textsubscript{4}B\textsuperscript{-} solution to form complexes depends on the overall concentration of the dye and borate (Figure III-3). When the concentration of Cy\textsuperscript{3+} exceeds 4.0 μM, complex formation is essentially quantitative as suggested by the low value of OD\textsubscript{650}/OD\textsubscript{671}. This value increases rapidly at lower concentrations, indicating that incomplete complexation occurs.

Figure III-3. Plot of ratio OD\textsubscript{650}/OD\textsubscript{671} versus [Cy\textsuperscript{3+}] with 2.0 molar equiv Ph\textsubscript{4}B\textsuperscript{-}. 
In contrast to water, when the solvent is pure acetonitrile, the addition of a 500-fold excess of Ph₃BnB⁻ to Cy³⁺ causes no change in the absorption spectrum. Study of the Cy³⁺-Ph₄B⁻ complex in water-acetonitrile solvent mixtures demonstrates that no complex is formed until the water content exceeds 75 % (Figure III-4).

**Figure III-4.** Plot of FWHM for the absorption spectra of 7.69 μM Cy³⁺ in water-acetonitrile mixtures with and without 2.0 molar equiv Ph₄B⁻.
III. C. 2. Stoichiometry of Aqueous Complexes. The stoichiometry of the 
Cy$^{3+}$-Ph$_4$B$^-$ complex was determined using Job's method$^{109,115}$ (see 
experimental section for details). The maximum in the Job plot (Figure III-5) 
occurs at X=0.67, giving a stoichiometry of 1 Cy$^{3+}$ : 2 Ph$_4$B$^-$. This leaves the 
complex with an overall charge of +1. The sharp peak in the Job plot is 
indicative of very strong binding.

![Job plot for formation of the aqueous Cy$^{3+}$-Ph$_4$B$^-$ complex. Each 
data point and set of error bars represent the mean and standard deviation, 
respectively, for three separate measurements.](figure)

Figure III-5. Job plot for formation of the aqueous Cy$^{3+}$-Ph$_4$B$^-$ complex. Each 
data point and set of error bars represent the mean and standard deviation, 
respectively, for three separate measurements.
The results for a variety of other complexes studied in this manner are given in Table III-3. (Spectra and Job plots are included in Appendices A-2 and A-3, respectively.) Importantly, the Job plots for all combinations give sharp maxima and the titration results feature clean isosbestic behavior up to the point where the ratio of dye to added borate equals the stoichiometry of the complex determined from the Job plot. The 1:1 Rh⁺-Ph₃SnB⁻ complex is consistent with the 1:1 Rh⁺-Ph₄B⁻ complex reported previously.¹¹⁶

Table III-3 Stoichiometries of aqueous dye-borate complexes

<table>
<thead>
<tr>
<th>Dye</th>
<th>Borate</th>
<th>Stoichiometry (Dye:Borate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy³⁺</td>
<td>Ph₃BnB⁻</td>
<td>1:2</td>
</tr>
<tr>
<td>Cy³⁺</td>
<td>Ph₃BuB⁻</td>
<td>1:2</td>
</tr>
<tr>
<td>Cy⁺</td>
<td>Ph₄B⁻</td>
<td>1:1</td>
</tr>
<tr>
<td>Rh⁺</td>
<td>Ph₃BnB⁻</td>
<td>1:1</td>
</tr>
<tr>
<td>P⁴⁺</td>
<td>Ph₃BnB⁻</td>
<td>1:3</td>
</tr>
</tbody>
</table>
The stoichiometries obtained from the Job plots are empirical, giving only the relative amounts of the two components in the complex. Thus this method of analysis cannot distinguish between 1:2 and 2:4 Cy$^{3+}$-Ph$_4$B$^-$ complexes. This is of concern because of the large spectral perturbations observed upon titration of the dye with the borate. Recently, Schuster and coworkers have shown that the absorption spectra of Cy$^+$-Ph$_4$B$^-$ and Cy$^+$-PF$_6^-$ contact ion pairs (in benzene) are identical.$^{88,89,118}$ However, the absorption spectrum of aqueous Cy$^+$ is significantly altered by addition of Ph$_4$B$^-$ (Figure III-6).

Figure III-6. Absorption spectra of 7.63 µM aqueous Cy$^+$ titrated with successive additions of 0.1 molar equiv of Ph$_4$B$^-$. 
The spectral perturbations evident in Figures III-1 and 6 are due to overlap of the dye orbitals with either those of the borate or with those of another dye. The evidence cited above for ion pairs argues against the first possibility. The latter possibility would result from formation of higher order complexes, for example $(\text{Cy}^+\text{-Ph}_4\text{B})_2$. It is important to realize that formation of a wide range of complexes (2:2, 3:3, 4:4, etc.) is unlikely because of the preservation of the isosbestic point throughout the titration. Thus formation of dimeric cyanine-borate complexes seems most probable.

In order to test this hypothesis, a mixture of two cyanine dyes ($\text{Cy}^+$ and $\text{Cy}^{3+}$) having different spectral properties was titrated with $\text{Ph}_4\text{B}^-$ (Figure III-7).

![Figure III-7. Absorption spectra of aqueous $\text{Cy}^+-\text{Cy}^{3+}$ (3.08 $\mu$M for each dye) titrated with successive additions of 0.75 molar equiv of $\text{Ph}_4\text{B}^-$ (relative to total dye concentration).](image)
In the absence of borate, the spectrum of the equimolar mixture of the dyes is nearly equivalent to the sum of the spectra of the individual dyes. This demonstrates that the dyes do not associate with one another in the absence of borate. In the presence of 3.0 molar equiv Ph₄B⁻ (sufficient borate to completely complex both cyanine dyes) the spectrum shows considerable deviation from the sum of the individual cyanine-borate spectra (Figure III-8).

**Figure III-8.** Difference spectra for equimolar mixture of Cy⁺ and Cy³⁺ (A) alone and (B) with 3.0 molar equiv Ph₄B⁻ (relative to total dye concentration).
III. C. 3. Photosensitivity of Aqueous Complexes. Red-light irradiation of Cy³⁺ alone or complexed to either Ph₄B⁺ or Ph₃BnB⁻ was investigated (Figure III-9). In all three samples the total optical density was the same insuring the same number of excited state dyes would be initially produced in each case. All three samples exhibit photosensitivity as evidenced by bleaching of the visible absorption band.

Figure III-9. Absorption spectra of solutions of (A) 2.5 μM aqueous Cy³⁺; (B) 3.1 μM aqueous Cy³⁺-Ph₄B⁺ (1:2); and (C) 3.1 μM aqueous Cy³⁺-Ph₃BnB⁻ (1:2) before (curve a) and after (curve b) 10 min of red light irradiation.
The dye alone (A) and the Cy\textsuperscript{3+}-(Ph\textsubscript{4}B\textsuperscript{–})\textsubscript{2} complex (B) bleach slowly and uniformly over ten minutes of irradiation. However, the Cy\textsuperscript{3+}-(Ph\textsubscript{3}B\textsubscript{n}B\textsuperscript{–})\textsubscript{2} complex (C) exhibits quite different behavior. Irradiation leads to drastic changes in the absorption spectrum, with the peak shifting back to the blue and becoming much more narrow. In other words, uncomplexed dye appears to be released as the reaction proceeds. Equal loss of cyanine and borate (on a percentage basis) should lead to uniform bleaching since remaining dye would still be exposed to two equivalents of borate, but this is clearly not observed. As described in the experimental section, 15% bleaching of Cy\textsuperscript{3+} and 41% loss of Ph\textsubscript{3}B\textsubscript{n}B\textsuperscript{–} occurs in ten minutes of irradiation. (These results suggest that either the reduced dye is being oxidized back to Cy\textsuperscript{3+} or that the borate is consumed by some process in addition to direct oxidation by excited Cy\textsuperscript{3+} within the complex. This phenomenon is discussed further in Section V. E. 3.) Thus, both complexes are more photosensitive than the dye by itself, with the Ph\textsubscript{3}B\textsubscript{n}B\textsuperscript{–} complex being the less stable of the two toward red light. The irradiation results are summarized in Table III-4.

The fluorescence of aqueous solutions of Cy\textsuperscript{3+} are strongly quenched after complex formation with the borates. The emission spectra were acquired with excitation at the isosbestic point (671 nm) observed in the absorption spectra for titration of Cy\textsuperscript{3+} with either borate. Excitation at this wavelength guarantees that the same number of excited species (free dye or complex) are produced for each measurement. As seen in Table III-4, the fluorescence is quenched considerably more efficiently in the Ph\textsubscript{3}B\textsubscript{n}B\textsuperscript{–} complex.
Table III-4  Photosensitivity of aqueous Cy$^{3+}$-Ph$_3$RB$_2$ complexes

<table>
<thead>
<tr>
<th>R</th>
<th>% Photobleaching</th>
<th>% Quenching$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bn</td>
<td>15.5</td>
<td>91 (1)</td>
</tr>
<tr>
<td>Ph</td>
<td>15</td>
<td>70 (4)</td>
</tr>
</tbody>
</table>

$^a$ Mean of six measurements; standard deviation in parentheses.
III. D. DISCUSSION

The spectral effects observed upon addition of Ph₃SnS⁻ to Cy³⁺ in water, i.e. broadened, red-shifted absorbance and diminished extinction coefficient are typically ascribed to charge-transfer (CT) interaction. However, other observations argue against CT stabilization of the complex. Generally, weaker reductants form weaker CT complexes exhibiting shorter λmax. However, nearly identical spectral perturbations are observed when Cy³⁺ is titrated with either Ph₄B⁻ or Ph₃BnB⁻ (Figure III-2) even though the oxidation potentials for these anions are 1.45 V and 1.09 V, respectively. Also, CT complexes are usually only weakly associated. The binding phenomenon illustrated in Figure III-1 is quite strong, occurring with very little added borate. Thus, the complexation observed in aqueous media appears to be primarily driven by the hydrophobicity of the borate. The concentration- and solvent-dependences illustrated in Figures III-3 and 4 support this: at sufficiently low concentration or high acetonitrile content, the solubility of the borate in the solvent competes with complex formation.

The binding constants for the complexes cannot be determined by simple Benesi-Hildebrand treatment of the data since the approximations implemented in that method do not apply to strong associations. However, the titration data indicate that the complexation reactions are essentially quantitative. For example, the observations that the Cy³⁺:Ph₃BnB⁻ complex has 1:2 stoichiometry and that the clean isosbestic point observed in Figure III-1 is lost as soon as the ratio of Ph₃BnB⁻ to Cy³⁺ exceeds 2.0 indicate that there is virtually no uncomplexed borate. Rather than being in equilibrium with the
dissociated components, the aqueous complexes appear to be discrete, supramolecular species.

Of particular interest is the Cy⁺-Ph₄B⁻ complex. As described in Section III. C. 2., Schuster and coworkers investigated ion pairs of these two components in low-polarity organic solvents. In those studies, the presence of the borate as the counterion rather than an inorganic ion such as hexafluorophosphate had no effect on the absorption spectrum of the cyanine dye. The lack of spectral perturbations in contact ion pairs suggests that the spectral changes observed in the aqueous Cy⁺-Ph₄B⁻ complex and, by extension, the other complexes, are not due to dye-borate interactions. An alternative explanation would be formation of 2:2 complexes with two Cy⁺ interacting by overlap of their chromophores. Cyanine dye dimers are well-known to exhibit significantly perturbed absorption spectra relative to the monomers. It should be emphasized again, however that formation of a range of complexes (1:1, 2:2, 3:3, etc.) is highly unlikely because of the clean isosbestic behavior observed during the titration and the single maximum observed in the Job plot, indicative of formation of a single type of complex.

The observation that the absorption spectrum of a Cy⁺:Cy³⁺:Ph₄B⁻ (1:1:3) mixture deviates from the sum of the spectra for Cy³⁺:Ph₄B⁻ (1:2) and Cy⁺:Ph₄B⁻ (1:1) supports the hypothesis that the complexes are in fact dimeric. In this experiment, three types of dimers could form: (Cy⁺-Ph₄B⁻)₂, [Cy³⁺-(Ph₄B⁻)]₂ and (Cy⁺-Ph₄B⁻)[Cy³⁺-(Ph₄B⁻)]₂. If only the first two "homodimers" form, the spectra should be additive. The deviation from additivity observed in Figure III-8 is attributed to formation of the mixed dimeric complexes. Previously, West and Pearce investigated the spontaneous
dimerization of cyanine dyes in water.\textsuperscript{119} This is an important process for dyes which are more hydrophobic than Cy\textsuperscript{+} and Cy\textsuperscript{3+}. The absorption spectra of the mixed dimers formed in that study exhibited the same qualitative deviation from additivity demonstrated in Figure III-8: enhanced absorption in the red and the blue with depleted absorption in between. The key difference between this work and that of West and Pearce is that Cy\textsuperscript{+} and Cy\textsuperscript{3+} are sufficiently hydrophilic to resist dimerizing at the concentrations used in these experiments. In the equimolar mixture, the dyes are solvated monomerically. Addition of the borate appears to trigger a two-step process: First, the borate binds to the dye, driven by hydrophobic and electrostatic interactions. The resulting dye-borate complex is considerably more hydrophobic than the isolated dye; in order to remain in solution, the dye-borate complexes dimerize. The formation of mixed dimers most likely arises because the individual complexes cannot distinguish between each other. (Presumably, addition of Ph\textsubscript{4}B\textsuperscript{-} to a mixture of two dyes which have significant structural differences, such as Rh\textsuperscript{+} and Cy\textsuperscript{3+} could lead to formation of only homodimers.) Dimerization appears to be sufficient to stabilize the system as no evidence for higher order complexes has been found. Thus the spectral perturbations observed upon titration of water-soluble dyes with hydrophobic borates are attributed to borate-induced dimerization of the dyes.

The cyanine-borate complexes exhibit interesting photophysical and photochemical properties. The pathways open to the excited complexes are illustrated in the following reaction diagram (Scheme III-1) for the [Cy\textsuperscript{3+}- (Ph\textsubscript{3}BnB\textsuperscript{-})\textsubscript{2}]\textsubscript{2} complex. (The complexes are represented as monomers for simplicity.)
Four processes are considered: 1) direct reaction of the complex to yield bleached dye ($k_b$), 2) fluorescence ($k_f$), 3) nonradiative decay, which may consist of several pathways ($k_{nr}$), and 4) intracomplex electron transfer from borate to dye ($k_{et}$). Full charges are assigned to the components of the complex because there is no evidence for charge-transfer interaction (vide supra). Since the fluorescence of the dye within the complex is strongly quenched but relatively slow bleaching is observed, the first two processes must represent minor modes of deactivation for the excited complex. Excited cyanines typically decay nonradiatively by twisting about the methine bridge linking the two heterocyclic ends of the dye.\textsuperscript{120-122} However, this observation pertains to monomeric cyanine dyes rather than the dimeric structures formed here. Additional vibrational decay modes are likely to be important within these dimers, leading to enhanced quenching of the excited state. Finally, the fluorescence quenching results (Table III-4) imply that electron transfer within the complex is also a significant decay path. Stronger quenching is observed for the Ph\textsubscript{3}BnB\textsuperscript{-}
complex than for the analogous Ph₄B⁻ complex, an effect attributable to the lower oxidation potential of Ph₃BnB⁻. In fact, it is probable that the 70 % quenching observed within the Ph₄B⁻ complex is due solely to vibrational relaxation as no quenching is observed when Cy³⁺ and Ph₄B⁻ are bound to lipid bilayers (see Chapter IV for results and further discussion).

Although bleaching for either borate complex is fairly slow and represents a minor pathway, the drastically different behavior exhibited by the two complexes in this regard is noteworthy. It seems logical that bleaching would occur from a point in the mechanism where the complexes are significantly different. Within Scheme III-1, such a situation arises after electron transfer from borate to dye within the excited complex (kₑ). Schuster and coworkers have established that oxidation of this class of borates by PET¹²³,¹²⁴ produces a boranyl radical which is capable of decomposing to form triphenylboron and a radical, R⁻, by cleavage of a boron-carbon bond. When the borate is Ph₄B⁻, an unstable phenyl radical would be produced. In this case, back electron transfer is a much more favorable process than bond cleavage (kₑ > k₉). However, when the borate is Ph₃BnB⁻, the resulting resonance-stabilized benzyl radical would allow decomposition to compete effectively with back electron transfer. Clearly, the path followed after electron transfer depends very strongly on which borate is present in the complex. Thus, bleaching is believed to occur after electron transfer within the excited complex as this leads to a point in the mechanism at which the complexes are distinctly different.
In summary, formation of dimeric complexes by various hydrophilic, cationic dyes with hydrophobic, anionic borates appears to be a general phenomenon, considering the range of dye structures which can be used. The complexes are primarily stabilized by hydrophobic rather than charge-transfer interactions, as evidenced by the lack of complex formation in acetonitrile, the strength of the interaction and the lack of dependence of complex formation on oxidation potential of the borate.
CHAPTER IV

VECTORIAL PET BETWEEN PHOSPHOLIPID MEMBRANE-BOUND DONORS AND ACCEPTORS

IV. A. INTRODUCTION

As described in Section II. G., photosynthetic organisms harvest, convert and store light energy as chemical potential by an efficient transmembrane photoinduced electron transfer (PET) sequence. The ability to mimic these features of photosynthesis is of immense practical importance for applications such as solar energy conversion. However, because of the sheer structural complexity of the reaction center, simplified systems employing liposomes (vesicles)\textsuperscript{125} have been used to achieve transmembrane electron transfer.\textsuperscript{126-129} In these systems, the redox species are either bound to the surface of the membrane or within its hydrophobic interior. Transmembrane electron transfer results from a series of short, discrete hops (truly mimicking reaction centers) and/or electron transport via a lipophilic carrier. Of course a major difference between these model systems and photosynthetic reaction centers is that in the former the medium for the reaction is a bilayer membrane whereas in the latter it is a protein.

Considerably less attention has been given to studying PET reactions occurring either completely within the bilayer or across a single interface of a phospholipid membrane, despite the fact that virtually any strategy devised to achieve efficient transmembrane PET will require these types of reactions to
occur. Intrabilayer electron transfers are especially problematic because of the difficulty involved in establishing the locations of both the donor and the acceptor in the membrane. This situation is simplified to some extent by following transfer across the membrane interface, either from a donor in the bilayer to an acceptor in the aqueous phase (electron ejection) or vice versa (electron injection). In particular, Tsuchida observed a strong inverse distance dependence on the efficiency of photoinduced electron ejection from amphiphilic porphyrins bound at different depths in the bilayer to acceptors in the aqueous medium.¹³⁰

In order to optimize transmembrane PET, the relationship between the physical properties of the membrane and the efficiency of membrane-based PET reactions must be understood. For example, the surface charge of the membrane can dramatically influence both forward and back electron transfer reactions by electrostatically attracting or repelling the redox components. This is particularly important for electron transfer across the membrane-water interface.¹³¹-¹³³

A second property which may effect PET efficiency in membranes is the fluidity of the bilayer. This property is readily modified, either by incorporating cholesterol into the membrane, by varying the sample temperature with respect to the phase transition of the membrane, or by polymerization of the bilayer.⁴⁶,⁴⁸ Hiromitsu and Kevan have measured photoionization yields of dipalmitoylphosphatidylcholine (DPPC) membrane-bound chlorophyll a. (Chla) in the presence of electron scavengers in the aqueous phase with variable amounts of cholesterol in the membrane.¹³⁴ The yields decreased with increasing cholesterol concentration, in apparent contradiction with the results
obtained by Ford and Tollin, who observed that the extent of electron transfer quenching of Chla triplets in egg phosphatidylcholine liposomes increased with increasing cholesterol concentration. Hiromitsu and Kevan suggested that the conflicting results may be due to different cholesterol-lipid interactions for the two sets of experiments, but other variables include the techniques and types of samples used (steady state ESR on frozen liposome suspensions at 77K for Hiromitsu and Kevan, transient absorbance on room temperature liposome suspensions for Ford and Tollin). The broader point to be made is that while inclusion of cholesterol in the membrane more closely mimics biological membranes, it also introduces significant structural heterogeneity into the bilayer which makes data analysis difficult.

The effect of membrane fluidity on PET reactions may also be examined by controlling the fluidity thermally. Ottolenghi and coworkers followed this strategy in their study of PET from an amine donor to excited pyrene confined within DPPC liposomes. The electron transfer reaction was more efficient at temperatures above the phase transition of the membrane, where the bilayer was fluid than at lower temperatures, where the bilayer was in the solid-like gel state. Similar results were obtained by Kano and coworkers. However, in neither case were the locations of the donor and acceptor within the membrane clearly established.

In this chapter, results are presented from studies of PET reactions confined to a phospholipid membrane in which the donor and acceptor occupy reasonably well-defined binding sites. The donor, triphenylbenzylborate anion, forms extremely strong aqueous complexes with various cationic dyes (chapter III) leading to relatively inefficient PET between them. Liposomes, however,
enhance the efficiency of the electron transfer process by preventing complex formation. Many approaches have been developed which employ organized assemblies to achieve enhanced PET efficiency with respect to a homogeneous aqueous medium.\textsuperscript{10} For example, Willner and associates have used charged colloids\textsuperscript{138} and micelles\textsuperscript{91} to disrupt aqueous charge transfer complexes. The aqueous complexes exhibited inefficient PET due to rapid back electron transfer. However, more efficient PET occurred when one component was bound to the charged interface while the other was repelled into the aqueous medium since the back electron transfer was retarded due to the much larger distance between the redox products. The system described in this chapter is unique in two important ways: 1) The liposomes bind both redox components simultaneously. The donor used in this study belongs to a class of molecules known as hydrophobic ions, characterized by their ability to partition overwhelmingly from aqueous media into neutral or anionic phospholipid membranes.\textsuperscript{31,39,139} The cationic acceptor is then bound to the liposomes by electrostatic attraction. Although donor and acceptor are both bound within the membrane, they are bound at different depths owing to the polarity\textsuperscript{27} and fluidity\textsuperscript{140} gradients inherent in the bilayer. This permits efficient electron transfer to occur between the two while the bilayer interface inhibits complex formation. 2) Although anionic liposomes work quite well at disrupting dye-borate complex formation and enhancing net PET between them, the liposomes need not be charged. Binding of hydrophobic borate anions to neutral liposomes imparts sufficient negative charge to the membranes to induce binding of cationic water-soluble dyes. Moreover, the binding site of the donor (but not the acceptor) appears to be sensitive to the physical state of the
membrane, which is simply a function of temperature. Control over the efficiency of the PET reaction is thus obtained from the resulting variable distance between donor and acceptor.
IV. B. EXPERIMENTAL

Materials. $n$-octanol (HPLC grade) and ethanol, $n$-propanol, $n$-butanol (all spectral grade) were purchased from Aldrich Chemical Company (Milwaukee, WI) and used as received. $n$-Pentanol (99%) was purchased from Aldrich and purified by fractional distillation from calcium hydride immediately before use. L-α-Dipalmitoylphosphatidylcholine (DPPC), L-α-Dioleoylphosphatidylcholine (DOPC), and L-α-Dioleoylphosphatidic acid (DOPA) were purchased from Avanti Polar Lipids (Pelham, AL) and used as received. Purity was checked by thin layer chromatography (65:25:4 CHCl₃/MeOH/H₂O eluent). Lipids were stored as chloroform solutions at 5 °C.

Liposome Preparation. An appropriate amount of lipid stock solution was added to a 10 mL pear-shaped flask. Solvent was evaporated by passing a stream of argon gas over the sample, leaving a film of lipid on the wall of the flask. Residual solvent was removed by drying under vacuum for at least eight hours. The lipid film was hydrated with 4.0 mL buffer (10 mM imidazole, pH=7.0), warmed above the lipid phase transition (for DPPC) and vortexed. The sample was then subjected to ten freeze-thaw cycles, accomplished by immersion for four min in a dry ice-isopropanol bath followed by ten min in a water bath. For DPPC preparations, the sample was placed in a 60 °C bath for 1 min prior to each freeze-thaw cycle. Liposomes were formed by extrusion ten times through two 0.1 μm polycarbonate filters (Nuclepore Co.; Pleasanton, CA) at a pressure of 250 psi. The extruder (Lipex Biomembranes, Inc.; Vancouver, B. C.) was warmed to 50 °C for preparation of DPPC liposomes.
Binding of Cy§+ to DOPC/DOPA (9:1) and DOPC Liposomes.

DOPC/DOPA (9:1) liposomes (0.5 mg/mL, assuming none was lost during the extrusion procedure) were prepared as described above. Extrusion through 0.1 μm filters typically yields liposomes with an average diameter of 100 nm. Although this was not determined for these experiments, identically prepared DOPC/DOPA (9:1) liposomes used in subsequent studies (see Chapter V) were found to have this diameter. Taking the bilayer width to be 50 Å and the area per molecule to be 70 Å², the total number of lipid molecules in a single liposome \( N_L \) can be calculated as follows:

\[
N_L = \frac{4\pi (r_o^2 + r_i^2)}{70} = 8.1 \times 10^4
\]

where \( r_o = 500 \) Å and \( r_i = 450 \) Å correspond to the outer and inner radii of the liposome.

4.0 μL of a 1.0 mM aqueous Cy§+ solution were added to 1.3 mL of the liposome suspension (0.5 mg lipid/1 mL buffer). The UV-vis absorption spectrum was recorded with a cuvette containing only liposomes in the reference beam. 4.0 μL of a 2.0 mM Ph3BnB⁻ stock solution (CH₃CN, 2.0 molar equiv, relative to Cy§+) were then added and the absorption spectrum recorded. (Note: In all experiments, the volume of added organic solvent was less than 1% of the total volume.) The sample was then irradiated with red light at room temperature as described below for a total of ten minutes. Similar experiments were performed using pure DOPC liposomes. All amounts of reagents were the same as in the DOPC/DOPA experiment except that 6.0 μL of a 2.0 mM
Ph₃BnB⁻ stock solution (CH₃CN, 3.0 molar equiv relative to Cy³⁺) had to be used, as explained in the text.

**Differential Scanning Calorimetry.** DPPC (8 mg, 10.9 µmoles) was dried from a stock chloroform solution onto the wall of a 10 mL pear-shaped flask. After drying in vacuo overnight, the sample was hydrated with 4.0 mL buffer (10 mM imidazole, pH = 7.0), warmed to 50 °C and vortexed. The sample was then subjected to ten freeze-thaw cycles. DSC thermograms were recorded using a Microcal MC-2 Calorimeter (Microcal, Inc., Northampton, MA). Four consecutive scans were acquired from 20-60 °C at a heating rate of 10 °C per hour.

After completion, the sample was removed and 5.4 µL of 50.0 mM Ph₃BnB⁻ stock (5.0 mol %) was added. The sample was then put back into the calorimeter and scanned four more times as above.

In another experiment, DPPC bilayers (2 mg/mL) were prepared as described above. 5.2 µL of 50.0 mM Ph₄B⁻ stock (4.8 mol %), 3.3 µL of 10.0 mM Ph₃BnB⁻ stock (0.6 mol %) and 18.0 µL of 1.0 mM Cy³⁺ stock (0.33 mol %) were then added. The sample was scanned four times as above.

**Steady-State Irradiations.** The procedure described in Chapter III was used.

**Photoinduced Electron Transfer In DPPC Liposomes:**
**Photobleaching.** DPPC liposomes (0.5 mg/mL) were prepared as described above. The liposomes (1.3 mL in a quartz cuvette) were warmed to 50 °C
before addition of dye and borates. Then, 4.3 µL of 10.0 mM Ph₄B⁻ stock (4.8 mol %), 2.7 µL of 2.0 mM Ph₃SnB⁻ stock (0.6 mol %) and 1.8 µL of 1.0 mM Cy³⁺ stock (0.2 mol %) were added. The tetraphenylborate was included as an inert hydrophobic anion to bind the dye to the surface of the liposome. The sample was then incubated at the desired temperature for 15 min in the dark prior to irradiation. During this time, no change was observed in the absorption spectrum of the dye. Irradiation was then performed as described above. Absorption spectra were recorded at different times up to 10 min total irradiation. The sample cell holder in the instrument was kept at the same temperature as the irradiation cell holder to prevent temperature change during acquisition of the spectrum. The percent bleaching of the dye was calculated from the loss in maximum optical density. The time required to reach 50% bleaching was determined graphically and plotted versus temperature.

**Fluorescence Quenching.** Sample preparation was as described above for the photobleaching experiments except for the total amounts of reagents used. For these experiments, 3.0 mL liposome suspension (0.1 mg/mL) were used in a fluorescence cuvette. 2.0 µL of 10.0 mM Ph₄B⁻ stock (4.8 mol %) and 8.2 µL of 0.1 mM Cy³⁺ stock (0.2 mol %) were added. Variable amounts of Ph₃SnB⁻ were also added. In this way, although the concentration of vesicles is lower than in the photobleaching experiments, the number of dye and borate molecules per liposome remains consistent. The cell holder in the fluorometer was connected to a water circulator to afford temperature control. After 15 min incubation at the desired temperature, the emission spectrum (excitation at 670 nm) was acquired. A fresh sample was used for each measurement.
IV. C. DESIGN OF THE SYSTEM

The system described below features a cationic, water-soluble electron acceptor and an anionic, hydrophobic electron donor organized vectorially within a phospholipid membrane. The acceptor is bound to the surface of the membrane electrostatically. The requisite negative charge on the membranes is obtained either by incorporating an anionic lipid such as phosphatidic acid into the bilayer or by binding hydrophobic anions to the membrane interior. In the latter strategy, the bound anion imparts sufficient negative charge to the membrane to induce binding of the cationic acceptor.

The electron acceptor, Cy^{3+} (see Table III-1) is also used as the light-absorbing component in the system. The intense absorption band allows efficient excitation of the dye and detection by absorption spectroscopy at fairly low concentrations (< 10 μM). Ph_{3}BnB^- is used as the electron donor in the system. The hydrophobicity of this anion permits it to partition into the hydrocarbon interior of phospholipid membranes, in a manner similar to Ph_{4}B^- 31,39,144 This allows the borate to function simultaneously as an electron donor and a binding agent: insertion of Ph_{3}BnB^- or Ph_{4}B^- into otherwise neutral liposomes results in binding of Cy^{3+} to the liposome surface.

The other useful feature of Ph_{3}BnB^- is that it is a sacrificial donor. As discussed in Chapter III, the boranyl radical formed by PET decomposes immediately and irreversibly upon formation. 88,89,118,123,124

\[ \text{Ph}_{3}\text{BnB}^- \rightarrow \text{Ph}_{3}\text{BnB}^- \rightarrow \text{Ph}_{3}\text{B} + \text{Bn}^- \]
The bond cleavage prevents back electron transfer from occurring, allowing the reduced cyanine to live long enough to undergo secondary chemical reactions, ultimately destroying the chromophore. Experimentally, the PET reaction can be studied by both fluorescence quenching and photobleaching techniques.

The system arrangement is shown in Scheme IV-1:

Scheme IV-1

![Diagram](attachment:image.png)

- = Cy$^{3+}$

- = Ph$_3$BnB$^-$
IV. D. RESULTS

IV. D. 1. Binding of Cy\textsuperscript{3+} to DOPC/DOPA Liposomes

Addition of 3.1 µM Cy\textsuperscript{3+} to DOPC/DOPA (9:1) liposomes (0.5 mg/mL) causes a significant bathochromic shift of the absorption spectrum of the dye (Figure IV-1) indicative of binding of the dye to the liposomes. These concentrations correspond to approximately 8*10\textsuperscript{3} DOPA molecules per liposome (slightly more than half of which are accessible to the externally added Cy\textsuperscript{3+}) and 350-400 Cy\textsuperscript{3+} per liposome (assuming complete binding).

The lack of change in the dye absorption profile upon binding indicates the dye is bound monomerically. This is attributed to the relatively low dye surface concentration (less than one Cy\textsuperscript{3+} for every hundred lipids) and the electrostatic repulsion between the highly charged dye molecules at the bilayer surface. In addition, aggregation of membrane-bound dyes is typically more favorable when the membrane is in the gel state.\textsuperscript{145,146} The DOPC/DOPA liposomes used in this experiment were in the more fluid liquid crystalline state.

Information regarding the binding site of Cy\textsuperscript{3+} within the membrane can be ascertained by examining the solvatochromism of the dye. Figure IV-2 shows the dependence of the absorption maximum of Cy\textsuperscript{3+} on solvent dielectric constant\textsuperscript{147} for a series of primary alcohol solvents. The observed maximum when bound to the DOPC/DOPA liposomes (669 nm) indicates the dye is bound within a region of dielectric constant ε = 13-14. This is too high for the dye to be bound in the hydrocarbon tail region of the bilayer. Rather the dye appears to be bound in the head group region of the membrane.
Figure IV-1. Absorption spectra of 3.1 μM Cy3+ in imidazole buffer alone (curve a) and with DOPC/DOPA liposomes (curve b). Approximately 350-400 Cy3+ bound per liposome.

Figure IV-2. Plot of absorption maximum versus solvent dielectric constant for Cy3+ dissolved in a series of primary alcohols. Dielectric constants taken from ref. 147.
Binding of Cy\textsuperscript{3+} to the anionic liposomes results in an 89% increase in the fluorescence yield relative to aqueous Cy\textsuperscript{3+}. Since the predominant decay path for excited cyanines in solution is nonradiative decay facilitated by twisting about the methine bridge,\textsuperscript{120-122} placing the dye in a more rigid environment (i.e. the membrane surface) should lead to enhanced fluorescence by restricting this twisting motion. Thus, fluorescence quantum yields for cyanines are known to increase significantly with increasing solvent viscosity.\textsuperscript{148-151} In addition, Kunitake and coworkers observed fluorescence enhancement upon binding of anionic cyanines to cationic ammonium bilayer interfaces,\textsuperscript{152} while incorporation of amphiphilic cyanines into bilayers gives similar results.\textsuperscript{153}

Addition of 2 molar equiv of either Ph\textsubscript{3}SnS\textsuperscript{−} or Ph\textsubscript{4}S\textsuperscript{−} (relative to Cy\textsuperscript{3+}) to DOPC/DOPA liposomes (700-800 borates per liposome) with surface-associated Cy\textsuperscript{3+} has very little effect on the absorption spectrum, aside from a slight (<5%) decrease in the extinction coefficient. Formation of a dye-borate complex appears to be inhibited in the presence of anionic liposomes.

**IV. D. 2. Binding of Ph\textsubscript{3}BnB\textsuperscript{−} to Lipid Bilayers**

Two-dimensional \textsuperscript{1}H-NOE spectroscopy has been used to directly demonstrate that Ph\textsubscript{4}B\textsuperscript{−} binds to the hydrocarbon interior of PC membranes, among the lipid tails.\textsuperscript{144} At concentrations less than 10 mole percent, large NOE's were observed between the bulk methylene resonances of the lipid tails and Ph\textsubscript{4}B\textsuperscript{−}. Minor NOE's were observed between the N-methyls in the lipid head group and Ph\textsubscript{4}B\textsuperscript{−}. These results demonstrate that Ph\textsubscript{4}B\textsuperscript{−} is localized within the hydrophobic interior of the membrane. (Unfortunately, NOE's to individual methylenes in the tails could not be resolved, so the exact binding depth of the
borate was not determined.) Differential scanning calorimetry (DSC) experiments suggest that Ph₃BnB⁺, a close structural analog of Ph₄B⁺, also binds among the lipid tails. This technique measures the excess heat capacity of a system as it undergoes a thermotropic phase transition. Figure IV-3 shows the effect of 5 mole % Ph₃BnB⁺ on the main phase transition (Pβ-Lα) for DPPC. The broadened and depressed transition peak is consistent with binding of the borate to the lipid bilayer. The borate is incorporated into the lipid lattice, giving the typical effect of an impurity on a melting transition. In addition, the Lβ'-Pβ' "pretransition" is no longer visible after binding of the borate. The conformational changes in the lipid molecules undergoing the phase transition primarily occur in the hydrocarbon tails. Thus the large perturbation observed in the DSC thermogram upon binding of Ph₃BnB⁺ to the DPPC bilayers is consistent with a binding location among the tails rather than among the head groups.

![Figure IV-3. DSC thermograms for DPPC alone (curve a) and in the presence of 5 mol % Ph₃BnB⁺ (curve b).](image)
IV. D. 3. Ph$_3$BnB$^-$-Induced Binding of Cy$^3+$ to DOPC Liposomes

Binding of Ph$_3$BnB$^-$ to otherwise uncharged bilayers renders the membranes anionic. Therefore, Cy$^3+$ should bind to uncharged liposomes upon addition of Ph$_3$BnB$^-$ (provided Cy$^3+$ and Ph$_3$BnB$^-$ do not form an aqueous complex as described in Chapter III). Identical experiments to those described above for anionic DOPC/DOPA liposomes were performed on pure DOPC liposomes in 10 mM phosphate buffer. In the absence of borate, Cy$^3+$ does not interact with the electrically neutral liposomes as evidenced by the fact that its absorption spectrum is unchanged by the presence of the liposomes. Addition of 3 molar equiv Ph$_3$BnB$^-$ causes the absorption profile to shift to the red (Figure IV-4). Although the extinction coefficient is slightly depressed, the spectral perturbation is very different from what is observed in the absence of liposomes (see Figure III-1). The sharp, red-shifted maximum indicates that the dye is not complexed to the borate but rather is bound to the surface of the liposome.

![Figure IV-4. Absorption spectra of 3.1 μM Cy$^3+$ in the presence of DOPC liposomes before (curve a) and after (curve b) addition of 3 molar equiv of Ph$_3$BnB$^-$.

\[ \text{MAX}=650 \text{ nm} \quad \text{MAX}=669 \text{ nm} \]
The electrostatic nature of the binding of Cy\(^{3+}\) to DOPC liposomes can be verified by altering the ionic strength of the buffer. With 100 mM NaCl added to the buffer, virtually no binding of Cy\(^{3+}\) to DOPC liposomes occurs upon addition of Ph\(_3\)SnB\(^-\). The high ionic strength screens the negative potential afforded the liposomes by the bound borate thus inhibiting binding of the cationic dye.

IV. D. 4. Photoinduced Electron Transfer in DOPC/DOPA Membranes

**Fluorescence Quenching.** Quenching of Cy\(^{3+}\) fluorescence by Ph\(_3\)SnB\(^-\) occurs when both are simultaneously bound to DOPC/DOPA membranes (Figure IV-5). Quenching is not observed when Ph\(_4\)B\(^-\) is substituted for Ph\(_3\)SnB\(^-\).

![Fluorescence Quenching](image)

**Figure IV-5.** Fluorescence of DOPC/DOPA membrane-bound Cy\(^{3+}\) before (curve a) and after (curve b) addition of 2 molar equiv of Ph\(_3\)SnB\(^-\). Excitation at 669 nm.
Photobleaching. Figure IV-6 shows the results of red-light irradiation of membrane-bound Cy³⁺ in the presence of either Ph₃BnB⁻ (A) or Ph₄B⁻ (B). Bleaching only occurs when Ph₃BnB⁻ is used. Bleaching and quenching results for membranes as well as for aqueous complexes (Chapter III) are collected in Table IV-1.

Figure IV-6. Effect of 10 min of red light irradiation on the absorption spectra of DOPC/DOPA membrane-bound Cy³⁺ in the presence of 2 molar equiv of (A) Ph₃BnB⁻ and (B) Ph₄B⁻ (λₘₐₓ = 669 nm for both A and B). In each case, approximately 350-400 Cy³⁺ and 700-800 borates were bound per liposome.
Table IV-1. Percent bleaching and fluorescence quenching of Cy³⁺ in H₂O and DOPC/DOPA membranes

<table>
<thead>
<tr>
<th>borateᵃ</th>
<th>medium</th>
<th>% Quenching</th>
<th>% Bleaching</th>
<th>PET efficiencyᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>H₂O</td>
<td>-</td>
<td>6.8</td>
<td>-</td>
</tr>
<tr>
<td>none</td>
<td>DOPC/DOPA</td>
<td>-</td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>Ph₃BnB⁻</td>
<td>H₂O</td>
<td>91</td>
<td>15</td>
<td>0.16</td>
</tr>
<tr>
<td>Ph₃BnB⁻</td>
<td>DOPC/DOPA</td>
<td>32</td>
<td>74.1</td>
<td>2.31</td>
</tr>
<tr>
<td>Ph₄B⁺</td>
<td>H₂O</td>
<td>70</td>
<td>15.5</td>
<td>0.22</td>
</tr>
<tr>
<td>Ph₄B⁺</td>
<td>DOPC/DOPA</td>
<td>-5ᶜ</td>
<td>1.6</td>
<td>-</td>
</tr>
</tbody>
</table>

ᵃ2.0 molar equiv, relative to Cy³⁺ where appropriate. ᵇCalculated by dividing % Bleaching by % Quenching. See text for explanation. ᶜ5% enhancement of Cy³⁺ fluorescence upon addition of Ph₄B⁺.

IV. D. 5. Photobleaching of Cy³⁺ in DOPC Membranes

Red-light irradiation of Cy³⁺ bound to DOPC membranes by Ph₃BnB⁻ leads to photobleaching of the dye (Figure IV-7), similar to the experiments in DOPC/DOPA membranes, albeit with a hypsochromic shift. The decrease in λₘₐₓ observed in DOPC liposomes is attributed to the PET reaction, which produces an uncharged boranyl radical from a negatively charged borate. This causes the negative charge on the membrane to decrease with increasing irradiation time. Unbleached dye begins to dislodge from the membrane surface due to the reduced electrostatic attraction, giving rise to the hypsochromic shift. Cy³⁺ binds to DOPC/DOPA liposomes even in the absence of borate. Since the
PET reaction has no effect on the anionic DOPA lipids, the dye remains bound to these membranes throughout the reaction causing $\lambda_{\text{max}}$ to be invariant with irradiation time.

Figure IV-7. Effect of 15 min of red light irradiation on the absorption spectra of DOPC membrane-bound $\text{Cy}^{3+}$ in the presence of 3 molar equiv of $\text{Ph}_3\text{BnB}^-$. Approximately 350-400 $\text{Cy}^{3+}$ and 1050-1200 $\text{Ph}_3\text{BnB}^-$ were bound per liposome.
IV. D. 6. Effect of Membrane Physical State on PET Efficiency

DPPC bilayers exhibit a highly cooperative main phase transition ($T_m$) at 41.4 °C between a solid-like gel state at low temperatures and a more fluid liquid crystalline state at high temperatures. Thus, DPPC liposomes permit investigation of the influence of the membrane physical state on processes occurring within the bilayer over a fairly wide temperature range. (In these experiments and the fluorescence quenching experiments described below, an excess of Ph$_4$B$^-$ was used to keep Cy$_3^+$ bound to the liposomes for the duration of the experiment. In all cases, the dye absorption maximum and profile were consistent throughout the irradiation, indicating the dye did in fact remain bound to the liposomes during the experiment.) Photobleaching of Cy$_3^+$ by Ph$_3$BnB$^-$ was studied in DPPC liposomes from 25 °C to 55 °C. The time required to reach 50% bleaching of the dye is plotted versus temperature in Figure IV-8, which shows that the bleaching is 3.5 times faster at temperatures below $T_m$ than above. A sharp discontinuity in the plot is observed in the region of the phase transition, although somewhat below the 41.4 °C $T_m$ for DPPC.

To examine whether the discontinuity observed in Figure IV-8 corresponds to the phase transition of the liposomes, differential scanning calorimetry (DSC) was used to determine the $T_m$ for DPPC membranes in the presence of the dye and the borates. Addition of Cy$_3^+$, Ph$_3$BnB$^-$ and Ph$_4$B$^-$ in the relative amounts used in the bleaching and quenching experiments described above lowered the phase transition temperature from 41.4 °C to 39.6 °C (Figure IV-9).
Figure IV-8. Effect of temperature on the half-life rate of photobleaching of 1.38 μM Cy3+ during red light irradiation in the presence of 4.15 μM Ph3BnB− in DPPC liposomes (0.5 mg/mL). Each data point and set of error bars represent the mean and standard deviation, respectively, for three separate measurements.

Figure IV-9. DSC thermograms for DPPC alone (curve a) and in the presence of Cy3+, Ph3BnB− and Ph4B− (curve b) in the relative amounts employed in the photobleaching and fluorescence quenching experiments.
Electron transfer from Ph$_3$BnB$^-$ to excited Cy$_{3+}$ effectively quenches the fluorescence of the dye. This process was also studied in DPPC liposomes as a function of temperature. From Figure IV-10 the quenching is clearly more efficient at temperatures below the T$_m$ than above. Based on the slopes of the lines for the T=25 °C and the T=55 °C data, the process is more favorable when the membrane is in the gel state by a factor of 6.5.

**Figure IV-10.** Effect of temperature on the fluorescence quenching of 0.27 μM Cy$_{3+}$ by Ph$_3$BnB$^-$ in DPPC liposomes (0.1 mg/mL). Each data point and set of error bars represent the mean and standard deviation, respectively, for three separate measurements.
IV. E. DISCUSSION

IV. E. 1. Enhanced PET Efficiency in Membranes versus Water

As described in the introduction, complex formation between electron donors and acceptors often reduces the efficiency of PET between them. Most often, this is because of enhanced back electron transfer but it is also possible that other nonradiative decay modes may be open to the excited complex which compete with electron transfer. This is likely true for the [Cy\textsuperscript{3+}-(Ph\textsubscript{3}RB')\textsubscript{2}]\textsubscript{2} complexes described in Chapter III. Cyanine dimers are known to exhibit self-quenching of fluorescence; since the aqueous dye-borate complexes appear to contain similar cyanine dimers, it is likely that self-quenching within the complex is a major decay path. Addition of two equiv of Ph\textsubscript{4}B\textsuperscript{-} results in quenching of 70\% of the Cy\textsuperscript{3+} fluorescence in water whereas no quenching occurs in DOPC/DOPA membranes (Table IV-1). While the redox potentials for the borate and dye may be modified upon binding to the membrane, it is highly unlikely that electron transfer would be an important pathway in the complex yet negligible when bound to membranes. Furthermore, redox potentials obtained in homogeneous solution suggest that PET from Ph\textsubscript{4}B\textsuperscript{-} to Cy\textsuperscript{3+} is endergonic by nearly \(0.4\) eV.\textsuperscript{89,154} Thus electron transfer is not believed to be a significant decay path for the aqueous [Cy\textsuperscript{3+}-(Ph\textsubscript{4}B')\textsubscript{2}]\textsubscript{2} complex. These factors would be carried over to the [Cy\textsuperscript{3+}-(Ph\textsubscript{3}BnB')\textsubscript{2}]\textsubscript{2} complex, which exhibits similar spectral features. The "extra" 21\% quenching observed in this complex is attributed to electron transfer since the oxidation potential of the benzyl borate is 360 mV lower than that of Ph\textsubscript{4}B\textsuperscript{-}.\textsuperscript{89} Moreover, the observation that quenching is still significant in the DOPC/DOPA liposomes (whereas none occurred when Ph\textsubscript{4}B\textsuperscript{-}
was present) implicates electron transfer as the sole quenching pathway in membranes.

Cy$^{3+}$ clearly binds to the surface of anionic DOPC/DOPA liposomes as evidenced by the large bathochromic shift in the absorption spectrum (Fig. IV-1) and enhanced fluorescence yield from the dye in the presence of the liposomes. Kunitake has reported similar spectral evidence for binding of anionic cyanines to cationic membranes. Since the absorption profile is unchanged upon binding (aside from the red-shift), it is concluded that the dye is monomeric when bound to the liposome surface. Addition of borate has very little effect on the spectrum so complexation does not occur within the membrane. Irradiation leads to extensive bleaching of the dye only when Ph$_3$BnB$^-$ is present. If no borate or only Ph$_4$B$^-$ is present, the dye is very stable to irradiation (Table IV-1). Moreover, the fluorescence of the dye is quenched by Ph$_3$BnB$^-$ but not Ph$_4$B$^-$ when bound to liposomes. These results indicate that excitation of the dye leads to electron transfer quenching by Ph$_3$BnB$^-$ and subsequent decomposition of the boranyl radical competes favorably with back electron transfer, leading to irreversible bleaching of the dye.

Scheme IV-2 outlines the photophysical and photochemical processes likely to be operative in the membrane-based PET system. Comparison with Scheme III-1 suggests that in both membranes and the aqueous complexes, bleaching of Cy$^{3+}$ first requires electron transfer from Ph$_3$BnB$^-$. The ratio of percent bleaching to percent quenching thus gives a measure of the efficiency of PET in these systems: a lower ratio of bleaching to quenching is indicative of a lower PET efficiency. This value is not the actual quantum efficiency of the PET reaction but is reflective of how the self-quenching path open in the
aqueous complex competes with electron transfer. In the aqueous [Cy$^{3+}$-(Ph$_3$BnB')$_2$]$_2$ complex, this ratio is only 0.16 while in membranes it is 2.31. Thus the bleaching reaction is 14 times more efficient in membranes than in the complex, implying that the PET reaction is also considerably more efficient in the former. The lower efficiency in the complex is a direct result of self-quenching of the excited state before electron transfer can occur. The point should also be made that although the ratio of cyanine to borate is the same in both cases, the effective concentrations are considerably greater in the complex where the ions are presumably in contact, than in membranes where they are separated by several angstroms. Decreasing the liposome concentration while maintaining the 1:2 ratio should lead to even greater enhancements.

Scheme IV-2
IV. E. 2. Vectorial PET in Phospholipid Membranes

The electron donor and acceptor used in these studies bind to phospholipid membranes to give a vectorial configuration. Based on the solvatochromism data of Figure IV-2, the electron acceptor Cy\textsuperscript{3+} is assigned to a binding location within the lipid head group region near the hydrophilic surface of the membrane. DSC results (Figure IV-4) as well as considerable literature evidence for the structural analog Ph\textsubscript{4}B\textsuperscript{-} indicate the electron donor Ph\textsubscript{3}BnB\textsuperscript{-} is bound among the lipid tails, in the hydrophobic interior of the membrane. Red-light irradiation of the acceptor thus triggers vectorial electron transfer from the membrane interior out to the surface.

The lack of quenching and bleaching when Ph\textsubscript{4}B\textsuperscript{-} is used rather than Ph\textsubscript{3}BnB\textsuperscript{-} indicates that the former would make a useful inert binding agent, keeping Cy\textsuperscript{3+} bound to otherwise neutral liposomes even as PET depletes the Ph\textsubscript{3}BnB\textsuperscript{-}. This strategy was used in exploring how the physical properties of the bilayer affect the efficiency of the PET reaction.

As mentioned in the Introduction, the phospholipid membrane presents a medium whose properties can be varied with relative ease. Experiments involving membranes having an accessible gel-liquid crystalline phase transition temperature are particularly attractive since this permits study of membrane-bound processes in either a solid-like or liquid-like environment. Substantial differences were expected for the PET reaction between Ph\textsubscript{3}BnB\textsuperscript{-} and Cy\textsuperscript{3+} in the two phases.

DPPC was chosen for these experiments because its T\textsubscript{m} (41.4 °C) provides a convenient temperature range in which to utilize both the gel and liquid crystalline phases. The dye, when bound to DPPC liposomes by borate,
bleached rapidly, analogous to the results for DOPC liposomes. In order to prevent release of the dye as the reaction proceeded, a large excess of Ph₄B⁻ was used as an inert binding agent. This minimizes the ambiguity in the average donor-acceptor separation distance by keeping the dye bound to the liposome at all times. Efficiency of the PET reaction between Cy⁴⁺ and Ph₃BnB⁻ in DPPC liposomes was studied as a function of temperature for both bleaching and fluorescence quenching experiments. Both processes proved to be more efficient when the membrane was below its phase transition temperature (39.6 °C in the presence of the dye and the borates). Bleaching (Fig. IV-8) and quenching (Fig. IV-10) were 3.5 and 6.5 times more efficient, respectively, below the phase transition. The discontinuity observed in Fig. IV-8 establishes that the effect observed is not merely attributable to changing temperature, but rather is due to the abrupt change in the physical properties of the membrane at its phase transition. In the gel state, the membrane presents a viscous medium which retards the diffusion of molecules solubilized within the bilayer. Fayer and coworkers have recently investigated the effect of diffusion on electron transfer reactions. Their results clearly demonstrate that electron transfer is more efficient in fluid media than in solid media. However the efficiency of electron transfer from the membrane-bound borate to Cy⁴⁺ is enhanced in the less fluid gel state. Therefore this interesting effect is not attributed to restricted diffusion. Another possible explanation for the observed results is that the excited state lifetime of Cy⁴⁺ is longer in the gel state than in the liquid crystalline state. This would permit more efficient PET, reflected in a greater extent of fluorescence quenching as illustrated in Fig. IV-10. Assuming the radiative rate constant $k_r$ has the same value above and below $T_m$, an estimate for the ratio of the
lifetimes in the two states can be obtained from the ratio of the fluorescence yields as follows:

\[
\frac{\Phi_{f}^{\text{gel}}}{\Phi_{f}^{\text{lc}}} = \frac{k_{f}^{\text{gel}}}{k_{f}^{\text{lc}}} = \frac{k_{f}^{\text{gel}} \tau_{f}^{\text{gel}}}{k_{q}^{\text{lc}} \tau_{f}^{\text{lc}}} \approx \frac{\tau_{f}^{\text{gel}}}{\tau_{f}^{\text{lc}}}
\]

\(<f_1 is the experimentally determined integrated emission spectrum of Cy^{3+}.\) This ratio is 1.6, substantially less than the factor of 6.5 observed for the overall efficiency of the quenching processes. As shown below, the difference in the quenching efficiencies in the two states cannot be attributed solely to increased lifetime in the gel state. The data indicate that the rate constant for quenching is also greater below \(T_m\) than above.

\[
\frac{\Phi_{0}}{\Phi} = 1 + K\beta = 1 + k_{q}[Q]
\]

\[
\frac{K_{f}^{\text{gel}}}{K_{f}^{\text{lc}}} = 6.5 = \frac{k_{q}^{\text{gel}} \tau_{f}^{\text{gel}}}{k_{q}^{\text{lc}} \tau_{f}^{\text{lc}}} = \frac{k_{q}^{\text{gel}}}{k_{q}^{\text{lc}}} \times 1.6
\]

\[
\frac{k_{q}^{\text{gel}}}{k_{q}^{\text{lc}}} = 4.1
\]

A plausible explanation for the enhanced electron transfer quenching below \(T_m\) is a smaller donor-acceptor distance of separation in the gel phase than in the liquid crystalline phase. This could occur from a change of position of the borate alone, of the dye alone or of both the dye and the borate. The narrow absorption profile of Cy^{3+} when bound to DPPC liposomes suggests the
dye is not experiencing a variety of environments but rather occupies a well-defined binding site within the membrane. Moreover, the absorption spectrum is independent of the physical state of the membrane. As shown in Fig. IV-2, the absorption $\lambda_{\text{max}}$ of Cy$^{3+}$ is very sensitive to the dielectric of the medium. Since there is a fairly steep dielectric gradient across the membrane interface, from bulk water ($\varepsilon = 78$) to bulk hydrocarbon ($\varepsilon = 2-3$), any change in the binding site of the dye within the membrane would be accompanied by a shift in the absorption spectrum. As no shift is observed upon change of temperature, the dye is assigned the same binding site within the membrane both above and below $T_m$. Therefore, the position of the borate within the membrane must be changing as the sample temperature changes. According to this model, the borate would be closer to the surface of the membrane in the gel state than in the liquid crystalline state. Recent studies place Ph$_4$B$^-\cdot 3-5\text{Å}$ below the ester carbonyl of the more deeply penetrating chain in DPPC membranes, locating the borate in the hydrocarbon tail region of the bilayer. (The possibility of different binding sites above and below the phase transition was not considered in that study.) While the NMR study cited above cannot pinpoint the location of Ph$_4$B$^- \cdot 3-5\text{Å}$ below the ester carbonyl of the more deeply penetrating chain in DPPC membranes, locating the borate in the hydrocarbon tail region of the bilayer. Ph$_3$BnB$, because of its close structural similarity to Ph$_4$B$^-$, is expected to occupy a similar binding site. In the gel state, the lipids are packed closely together in an essentially crystalline lattice whereas the medium is considerably more fluid above $T_m$. Therefore a bulky molecule such as Ph$_3$BnB$^-$ would certainly have a more difficult time penetrating into the hydrocarbon tail region when the membrane is below the phase
transition, leading to a binding site closer to the surface of the liposome. This would result in a more efficient PET from \( \text{Ph}_3\text{BnB}^- \) to \( \text{Cy}^{3+} \) below the phase transition.

The similarity between the relative efficiencies of photobleaching and fluorescence quenching in the two states of the bilayer (3.5 and 4.1 respectively) indicates that back electron transfer is not an important pathway in the system. This is in accord with the results obtained by Schuster in which even in highly viscous solvents back electron transfer could not compete with carbon-boron bond cleavage in the oxidized borate when a stabilized radical could be produced.\(^{69}\) This has significance with respect to the question of energy storage. In the absence of back electron transfer, the energy which remains in the system after excitation and electron transfer should be harvestable with reasonable efficiency. Moreover, redox potentials obtained in homogeneous solution suggest that the forward electron transfer is only slightly exergonic.\(^{69,154}\) Thus, very little of the initial excitation energy is lost in transfer of an electron from \( \text{Ph}_3\text{BnB}^- \) to \( \text{Cy}^{3+} \). These two features indicate that the system is capable of storing a significant amount of the excitation energy for a useful amount of time.

One final point of discussion involves comparison of the results obtained in DPPC liposomes in this study with those mentioned in the Introduction. The work by Ottolenghi and associates\(^{136,156}\) as well as by Kano et al.\(^{137}\) demonstrated enhanced electron transfer above the phase transition for donor and acceptor solubilized within the bilayer whereas the membrane-bound \( \text{Cy}^{3+}-\text{Ph}_3\text{BnB}^- \) system exhibits the opposite effect. The previous results were interpreted as due to diffusion-controlled electron transfer reactions. In those
studies, it is likely that the donor and acceptor binding depths within the membrane overlapped at all temperatures. Under such circumstances both diffusion and electron transfer occur laterally within the membrane. The results reported here describe a system where the donor and acceptor are bound at different depths within the membrane. Thus, while diffusion is still primarily lateral in nature, electron transfer occurs in the transverse direction. It is the vectorial arrangement of the system which permits the distance change in passing through the phase transition to overcome the effects of diffusion, allowing the exponential dependence of the reaction on distance to become the dominant factor. This results in the opposite dependence of the PET reaction on the membrane physical state from that reported in the earlier work.

In conclusion, liposomal membranes have been used as media for photoinduced electron transfer reactions. The donor and acceptor are conveniently separated by binding at different depths within the bilayer membrane. The aqueous complexes formed by hydrophobic borate anions with water-soluble cationic dyes have intricate photophysical and photochemical pathways open to them, resulting in relatively inefficient PET. Solubilization within phospholipid membranes prevents this association, thereby reducing the number of pathways open to the excited cyanine and enhancing PET from the borate to the dye. Moreover, the donor-acceptor separation and, therefore, electron transfer efficiency is dependent on the physical state of the membrane. The strong distance dependence of PET reactions leads to dramatic effects on the photobleaching and fluorescence quenching efficiencies. In fact, these types of experiments may provide a means of accurately determining the
locations of membrane-bound donors and acceptors, provided the binding site of one of the redox partners is known and one can quantitatively account for diffusion and the variable dielectric. An additional appealing feature of the system is that back electron transfer appears to be a very minor process within the membrane. This permits the liposomal system to store the excitation energy as redox products for potentially useful amounts of time. This general strategy could in principle be used to generate a large number of reduced acceptors at or near the surface of a liposome. The stored energy could then be used to drive subsequent chemical transformations, a system which is made even more attractive by the high surface area afforded by liposomes. In fact, Willner and associates have incorporated many of these features into a colloidal-based system which effectively hydrogenates ethylene. However, in that system the chemical processes were confined to the colloid surface or bulk aqueous medium, whereas the system described here is based on redox processes occurring within the membrane itself. Finally, the results obtained in this study suggest that the design of membrane-based systems for the harvest and conversion of light energy should include investigation of the role of the lipid bilayer in promoting or inhibiting the desired photochemistry.
CHAPTER V

COUPLED EET AND VECTORIAL PET REACTIONS IN PHOSPHOLIPID MEMBRANES

V. A. INTRODUCTION

The two-component, membrane-based system described in Chapter IV featured a vectorial photoinduced electron transfer reaction initiated by absorption of red light. The system collects light efficiently in this region of the visible spectrum due to the large extinction coefficient of the dye, Cy3+. However, the system is insensitive to light from other regions of the spectrum because of the narrow absorption band of Cy3+ (FWHM = 44 nm). Other strategies are required to extend the sensitivity of the system to shorter wavelengths of light.

Photosynthetic organisms have dealt with this very problem by incorporating an energy-transfer trigger to the electron transfer chain in reaction centers,99,105,107 which by themselves would harvest blue and green light with only modest efficiency. The antenna complexes described in Section II. G. absorb light in these regions and then use electronic energy transfer processes to funnel the light into the reaction centers, greatly increasing the spectral sensitivity for solar energy conversion.

A similar approach has been adopted for extending the sensitivity of the simple two-component system described in Chapter IV. An antenna pigment is added to the system which efficiently absorbs blue light. Energy transfer to Cy3+
produces the same excited state as direct irradiation of the cyanine with red light. Vectorial electron transfer from Ph$_3$BnB$^-$ bound in the membrane interior quenches the fluorescence and bleaches the absorption band of the surface-associated Cy$^{3+}$. Thus the same chemistry involving Cy$^{3+}$ results from irradiation of this three component system using blue light as observed for direct irradiation of Cy$^{3+}$ in the two-component system using red light.
V. B. EXPERIMENTAL

Liposome Preparation. An appropriate amount of DOPC/DOPA (9:1) stock solution (in chloroform) was added to a 10 mL pear-shaped flask. The solvent was evaporated by passing a stream of argon gas over the sample, leaving a film of lipid on the wall of the flask. Residual solvent was removed by drying under vacuum for at least eight hours. The lipid film was hydrated with buffer (either 10 mM imidazole, pH=7.0 or 10 mM phosphate, pH=7.4) and vortexed. Liposomes were formed by the freeze-thaw and extrusion procedures described in Chapter IV.

Determination of Liposome Concentration. The resulting liposomes were assayed for phosphatidylcholine content (90% of the total lipid content). This was done by removing 60 μL of the liposome suspension and adding to approximately 0.5 mL spectral grade methanol in a test tube. The solvent was then removed by placing the test tube in a warm water bath and passing argon gas over the solution. 0.5 mL spectral grade chloroform was added and evaporated as above. The first step ensures that the liposomes are dissolved while the second guarantees the complete removal of methanol. This is particularly important since methanol may cause inaccurate results in the assay.

After drying, 2.0 mL spectral grade chloroform was added to the lipid film followed by 2.0 mL of the ammonium ferrothiocyanate test solution (prepared by dissolving 3.04 g ammonium thiocyanate and 2.703 g ferric chloride in 100 mL water). The test tube was vortexed for one min then allowed to stand for ten min. The optical density of the chloroform layer at 488 nm was measured and
compared to a calibration curve to determine the phosphatidylcholine content in the liposome suspension. (Phosphatidic acids are not detected in this assay, which requires a positive charge to be present in the lipid.) The entire procedure was done in triplicate. The calibration curve was generated by starting with a solution of DOPC in chloroform of known concentration (in which case the evaporation steps were unnecessary).

The liposomes were sized by dynamic laser light scattering (Brookhaven BI-8000AT correlator with a 5-mW He-Ne laser light source operating at 632.8 nm; Brookhaven Instruments Corp., Holtsville, NY) utilizing four separate analysis packages to extract the set of exponential functions which made up the autocorrelation functions. Measurements were made in triplicate at 60°, 90° and 120° scattering angles. Liposomes prepared by extrusion as outlined above were 95 +/- 5 nm in diameter.

The diameter together with the lipid concentration were then used to determine the liposome concentration. The number of lipids in the unilamellar liposomes was calculated as described in Section IV. B. The lipid concentration determined by the ammonium ferrothiocyanate assay was then divided by the number of lipids per liposome to give the liposome concentration.

**Binding of P^4+ to Liposomes.** The UV-vis absorption spectra of 3.85 μM solutions of P^4+ in the absence or presence of DOPC/DOPA liposomes (0.5 mg/mL = 0.6 mM lipid) were acquired on a Varian DMS-200 (Varian Techtron Pty. Limited, Victoria, Australia) double beam instrument. The concentration of lipid was 0.5 mg/mL.
Fluorescence spectra of 10.0 μM solutions of P⁴⁺ in the absence or presence of 1.0 nM DOPC/DOPA liposomes were acquired on a Spex Fluorolog II spectrofluorometer (Spex Industries, Inc., Edison, NJ). Excitation was done at 414 nm, which is an isosbestic point in the absorption spectra for the porphyrin in water and on DOPC/DOPA liposomes. The liposome-bound P⁴⁺ emitted with 18 % greater total intensity than the aqueous sample.

Additionally, P⁴⁺ was added to 5.0 nM DOPC/DOPA liposomes (concentration refers to liposomes, not lipid) in 10 P⁴⁺ per liposome increments, up to 100 P⁴⁺. The emission spectrum was recorded after each addition and the integrated emission intensity was plotted versus number of porphyrins per liposome to investigate possible self-quenching.

**Energy Transfer Experiments.** P⁴⁺ was added to a suspension of 5.0 nM DOPC/DOPA liposomes in a ratio of 10 porphyrins per liposome. The fluorescence emission spectrum was recorded with excitation at 417 nm, the P⁴⁺ absorption maximum. Subsequent spectra were recorded with increasing amounts of Cy³⁺ added to the liposomes in 10 Cy³⁺ per liposome increments, up to 460 Cy³⁺ per liposome. Subsequently, a sample was prepared having 460 Cy³⁺ per liposome but no P⁴⁺. The emission spectrum was then acquired under identical conditions to the porphyrin-containing sample.

**Electron Transfer Experiments: Fluorescence Quenching.** A fluorescence emission spectrum was acquired in the time base mode (excitation at 417 nm, emission at 696 nm) for a suspension of 0.6 nM DOPC/DOPA liposomes to which were added in sequence: P⁴⁺ (400 per
liposome), Cy$^{3+}$ (400 per liposome) and Ph$_3$BnB$^-$ (2000 per liposome). The dyes and borate were added as aqueous and acetonitrile solutions, respectively. At no time did the amount of added organic solvent exceed 1% of the sample volume. The sample was removed from the instrument for each addition and mixed thoroughly before returning.

In a separate experiment, P$_{4}^{4+}$ (400 per liposome) was added to 6.25 nM DOPC/DOPA liposomes. The emission intensity at 696 nm was measured with excitation at 417 nm. Ph$_3$BnB$^-$ was added in 400 borate-per-liposome increments up to 2000 Ph$_3$BnB$^-$ per liposome. The emission intensity was recorded after each addition. Similar experiments were run for Cy$^{3+}$ (400 per liposome, excitation at 669 nm) and a 1:1 mixture of P$_{4}^{4+}$ and Cy$^{3+}$ (400 each per liposome, excitation at 417 nm). The ratio of the emission intensity without and with Ph$_3$BnB$^-$ ($I_o/I$) was calculated for each addition and plotted versus the number of Ph$_3$BnB$^-$ per liposome.

**Electron Transfer Experiments: Photobleaching.** A sample containing either 12.5 nM or 6.25 nM DOPC/DOPA liposomes with P$_{4}^{4+}$, Cy$^{3+}$ and Ph$_3$BnB$^-$ (variable amounts of each per liposome) was purged in the dark with Argon gas for 15 min. The sample was then irradiated in the fluorometer sample compartment using 417 nm light with 1.0 mm (3.6 nm bandpass) slits on the excitation monochromator to filter the light from the excitation source, a 450W Xe lamp (Osram Corp.; Newburgh, NY). The sample was stirred during irradiation and the shutter in the instrument was opened and closed from the computer keyboard. UV-vis spectra were recorded every 2 min and the reaction was followed by monitoring the loss in optical density of the two pigments.
Direct photobleaching of DOPC/DOPA-bound P⁴⁺ in the presence of Ph₃BnB⁻ was also studied using a 200W Hg(Xe) lamp as the irradiation source (lamp flux = ca. 10¹⁶ photons per second). The light was passed through a Corning CS3-70 filter (λ > 490 nm). 160 porphyrins were bound per liposome and the concentration of liposomes was 8.1 nM. Variable amounts of Ph₃BnB⁻ were used. The reaction was followed by the loss in optical density of the Soret band.
V. C. DESIGN OF THE SYSTEM

The antenna pigment selected for this system should fulfill three important criteria:

1) The absorption spectrum should be well-resolved from that of Cy$^{3+}$. Aside from the obvious point that there is no need for the antenna pigment to absorb in the same regions as the cyanine, resolution of the absorption bands would permit selective excitation of either pigment in the three-component system leading to greater flexibility in probing mechanistic questions.

2) The emission spectrum of the antenna should have significant overlap with the absorption spectrum of Cy$^{3+}$. As described in Section II. F., this is a fundamental requirement for resonance energy transfer: the larger this overlap, the more efficient the transfer of energy from donor to acceptor.$^{93,158}$

3) The antenna should bind to the membrane at a reasonably well-defined location, preferably the surface. This would minimize ambiguity regarding the relative distribution of the three components in the bilayer. Moreover, surface association should minimize competition from electron transfer (PET from the borate to the excited antenna), a process which would likely be more favorable for binding in the hydrophobic interior, where the antenna would be in closer proximity to the borate.

The first two criteria, regarding the optical properties of the antenna, imply the need for a large difference between the maximum absorption and emission wavelengths. The porphyrins exhibit the necessary blue absorption$^{159}$ and red emission$^{160}$ typically a difference of over 200 nm. The large shift arises
from the fact that while the most intense absorption band (the Soret band) in the porphyrins corresponds to excitation directly to the second excited singlet state, $S_2$, whereas emission occurs from the lowest excited singlet state, $S_1$. (The $S_2$ state undergoes rapid internal conversion to $S_1$ prior to fluorescence.) Thus the porphyrins absorb most intensely near 410 nm but emit near 650 nm. A drawback to using porphyrins as energy donors is their relatively low fluorescence quantum yields ($<10\%$), which will diminish the energy transfer efficiency.

Simple porphyrins such as tetraphenylporphyrin fulfill the optical requirements stated above, but are known to not only partition into the hydrophobic interior of lipid bilayers but to do so in an aggregated state.$^{161,162}$ The water-soluble, tetracationic porphyrin $P^4+$ (see Table III-1) was chosen instead. This porphyrin has been reported to resist aggregation in water at concentrations as high as 100 $\mu$M.$^{163}$ Additionally, the electrostatic forces used to bind Cy$^{3+}$ to the surface of anionic vesicles should be applicable to binding of $P^4+$ as well.

The configuration of the liposome-based three-component system is illustrated in Scheme V-1. (Only the outer leaflet of the bilayer is shown for simplicity.) The two cationic pigments should bind to the anionic surface of the bilayer while the hydrophobic borate binds in the membrane interior. Irradiation with blue light results in selective excitation of $P^4+$. Energy transfer parallel to the membrane surface generates the cyanine excited state and returns the porphyrin to the ground state. Vectorial electron transfer from the borate to the excited cyanine completes the sequence.
Scheme V-1

- $c::J = p^{4+} = cl^+ = Ph_3BnB^-$
V. D. RESULTS

V. D. 1. Binding of P4+ to Anionic Liposomes

The water-soluble porphyrin P4+ absorbs intensely ($\varepsilon = 3.6E05 \text{ M}^{-1} \text{ cm}^{-1}$) at 411 nm in aqueous solution. Addition of P4+ to a suspension of DOPC/DOPA (9:1) liposomes causes a bathochromic shift of this peak to 417 nm (Figure V-1) with an isosbestic point at 414 nm. The FWHM increases only slightly upon binding (13.4 nm in water, 15.3 nm on DOPC/DOPA); the narrow absorption band indicates that the prophyrin is not aggregated.

![Figure V-1](image)

**Figure V-1.** UV-vis absorption spectra of 3.85 µM solutions of P4+ in A) water and B) DOPC/DOPA (9:1) liposomes (0.5 mg/mL lipid).
Fluorescence from excited P\textsuperscript{4+} yields the doubly-peaked profile characteristic of porphyrins. The emission maximum undergoes a bathochromic shift from 646 nm in water to 653 nm in the presence of DOPC/DOPA liposomes (Figure V-2). The fluorescence yield is enhanced by 18%.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fluorescence_spectra.png}
\caption{Fluorescence spectra of 20.0 nM solutions of P\textsuperscript{4+} in A) 1.0 nM DOPC/DOPA (9:1) liposomes and B) water.}
\end{figure}

The bathochromic shift of the absorption spectrum and enhanced fluorescence for P\textsuperscript{4+} in the presence of DOPC/DOPA is attributed to binding of the porphyrin to the liposomes.
V. D. 2. Energy Transfer from P⁴⁺ to Cy³⁺ on Anionic Liposomes

P⁴⁺ fulfills the optical requirements listed in Section V. C.: Its absorption spectrum is well-resolved from that of Cy³⁺ (Figure V-3) but its emission spectrum overlaps extensively with the cyanine absorption band (Figure V-4). Thus P⁴⁺ should transfer energy to Cy³⁺ on the liposome surface.

Figure V-3. UV-vis absorption spectra of A) P⁴⁺ (λ_max = 417 nm) and B) Cy³⁺ (λ_max = 669 nm) in the presence of DOPC/DOPA (9:1) liposomes (0.5 mg/mL lipid). The spectra are shown full scale to emphasize the resolution of the absorption bands; in actuality, the porphyrin absorbs approximately 2.6 times more intensely than the cyanine at their respective maxima.
Figure V-4. Overlap (shaded region) of A) P⁴⁺ fluorescence and B) Cy³⁺ absorption spectra in the presence of DOPC/DOPA (9:1) liposomes.
Figure V-5 illustrates how the fluorescence intensity from the membrane-bound porphyrin varies as its surface concentration increases. The linear increase observed as $P^{4+}$ is added to the liposomes indicates that the fluorescence is not self-quenched up to at least 100 porphyrins per liposome. (For comparison, there are approximately 40,000 lipids in the outer leaflet of a 100 nm-diameter liposome, approximately 4,000 of which are DOPA.)

Figure V-5. Dependence of $P^{4+}$ fluorescence intensity in the presence of DOPC/DOPA (9:1) liposomes (5.0 nM) as a function of porphyrin surface concentration.
Addition of Cy^3+ to membrane-bound P^{4+} effectively quenches the porphyrin emission at 650 nm, replacing it with cyanine emission at 696 nm (Figure V-6), clear evidence that energy is transferred from the excited porphyrin to the cyanine on the membrane surface.

Figure V-6. Fluorescence spectra (excitation at 417 nm) for P^{4+} (50.0 nM, 10 per liposome) in the presence of 5.0 nM DOPC/DOPA (9:1) liposomes with different amounts of Cy^3+ : A) 0, B) 40, C) 80, D) 140, E) 200, F) 260, G) 340 and H) 460 cyanines per liposome.
The ratio of the fluorescence intensities at the cyanine (696 nm) and porphyrin (653 nm) maxima (R) is related to the energy transfer efficiency. Plotting this ratio as a function of added Cy\textsuperscript{3+} reveals that R and thus energy transfer efficiency increase progressively with cyanine concentration (Figure V-7). (Note that R does not equal zero in the absence of Cy\textsuperscript{3+} because of the intrinsic emission of P\textsuperscript{4+} at 696 nm.)

![Graph](image)

**Figure V-7.** Variation of R, the ratio of fluorescence intensity at 696 nm (Cy\textsuperscript{3+} maximum) to 653 nm (P\textsuperscript{4+} maximum), with the number of Cy\textsuperscript{3+} bound per DOPC/DOPA (9:1) liposome.
Figure V-8 shows the emission spectrum of DOPC/DOPA-bound Cy$^{3+}$ with and without P$^{4+}$. The addition of just ten porphyrins per liposome results in a 60-fold increase in the Cy$^{3+}$ emission, thereby establishing that the cyanine fluorescence is sensitized by the porphyrin.

**Figure V-8.** Fluorescence spectra (excitation at 417 nm) for Cy$^{3+}$ (460 per DOPC/DOPA liposome) A) with and B) without 10 P$^{4+}$ per liposome.
V. D. 3. Ph₃BnB⁻ Quenching of Sensitized Cy³⁺ Emission

Since the Cy³⁺ excited state produced by energy transfer is the same as that which results from direct excitation using red light, addition of Ph₃BnB⁻ to the system should quench the emission at 696 nm by electron transfer. Figure V-9 shows the effect of sequential addition of the three components (P⁴⁺, Cy³⁺, and Ph₃BnB⁻) to DOPC/DOPA liposomes. The sample was excited at 417 nm while emission was monitored at 696 nm (the porphyrin absorption and cyanine emission maxima, respectively). The time axis has no kinetic relevance, it merely reflects the effect of sequential addition of each component on the emission at 696 nm. Addition of P⁴⁺ (400 per liposome) results in emission at 696 nm due to the porphyrin's intrinsic fluorescence (see Figure V-2). A large increase is observed upon introduction of an equimolar amount of Cy³⁺ due to sensitized emission from the cyanine. Finally, the emission is quenched approximately 25% upon addition of a five-fold excess (relative to either pigment) of Ph₃BnB⁻.

The quenching of liposome-bound Cy³⁺ and P⁴⁺ by Ph₃BnB⁻ was investigated by Stern-Volmer analysis. The borate quenches the emission of Cy³⁺ 2.0 times more efficiently than that of P⁴⁺ (Figure V-10). However, Ph₃BnB⁻ quenches a 1:1 mixture of the two dyes with intermediate kinetics (1.7 times as efficiently as for P⁴⁺ alone). Assuming that the kinetics observed for quenching of the Cy³⁺-P⁴⁺ system is a linear combination of the kinetics for Cy³⁺ and for P⁴⁺ alone, the data in Figure V-10 indicate that 68 % of the observed quenching is due to electron transfer to Cy³⁺ while the remaining 32 % corresponds to PET to P⁴⁺.
Figure V-9. Fluorescence intensity (excitation at 417 nm) at 696 nm for successive addition of $P^{4+}$, $Cy^{3+}$, and $Ph_3BnB^-$ to 0.625 nM DOPC/DOPA (9:1) liposomes. Numbers refer to the quantities of each component bound per liposome.

Figure V-10. Stern-Volmer analysis for quenching of DOPC/DOPA-bound $P^{4+}$, $Cy^{3+}$ and $P^{4+}:Cy^{3+}$ (1:1) by $Ph_3BnB^-$. 400 of each dye bound per liposome; [liposomes] = 6.25 nM. Excitation of $P^{4+}$-containing samples at 417 nm; sample with $Cy^{3+}$ excited at 669 nm.
V. D. 4. Sensitized Photobleaching of Cy3+ using Blue Light

The results presented in Figure V-10 indicate that some of the quenching observed in Figure V-9 is due to electron transfer from Ph3BnB' to Cy3+. Based on the results from Chapter IV, this should lead to irreversible bleaching of the cyanine absorption band. This is because the same excited state is produced whether the cyanine is excited directly using red light or by energy transfer following blue-light excitation of P4+. Indeed, irradiation of the three-component system at 417 nm does lead to bleaching of Cy3+ (Figure V-11). In the absence of P4+, bleaching is very slow using blue light but upon changing the irradiation wavelength to 669 nm, the cyanine bleaches readily (Figure V-12). This is consistent with the effect of the porphyrin on the cyanine fluorescence after excitation at 417 nm (Figure V-8): production of excited state Cy3+ only occurs via energy transfer from P4+.

![Graph showing absorbance vs. wavelength for Cy3+ absorption band before and after irradiation.](image)

**Figure V-11.** Effect of irradiation (417 nm) on Cy3+ absorption band for 6.25 nM DOPC/DOPA (9:1) liposomes with 400 Cy3+, 400 P4+, and 2000 Ph3BnB' bound per liposome. A) Before and B) after 10 min irradiation.
Figure V-12. A) Photosensitized (irradiation at 417 nm) bleaching of Cy\textsuperscript{3+} with P\textsuperscript{4+} (400 per liposome) and without P\textsuperscript{4+}. B) Effect of irradiation wavelength on direct photobleaching of Cy\textsuperscript{3+} in the absence of P\textsuperscript{4+}. [Liposomes] = 12.5 nM, 400 Cy\textsuperscript{3+} and 800 Ph\textsubscript{3}BnB\textsuperscript{-} per liposome.
The rate of sensitized Cy\textsuperscript{3+} bleaching increases with increasing Ph\textsubscript{3}BnB\textsuperscript{-} concentration (Figure V-13) but is strongly retarded if Ph\textsubscript{3}BnB\textsuperscript{-} is replaced by the weaker electron donor Ph\textsubscript{4}B\textsuperscript{-} (Figure V-14). These results demonstrate that the key step in the sensitized bleaching using blue light is the same as for direct photobleaching using red light: electron transfer.

**Figure V-13.** Dependence of photosensitized (417 nm) bleaching of Cy\textsuperscript{3+} on [Ph\textsubscript{3}BnB]. Concentrations of liposomes and Cy\textsuperscript{3+} are the same as in Figure V-12; 400 P\textsuperscript{4+} per liposome. A) 0, B) 400 and C) 800 Ph\textsubscript{3}BnB\textsuperscript{-} bound per liposome.
Figure V-14. Dependence of photosensitized (417 nm) bleaching of Cy$^{3+}$ on electron-donating ability of borate. Concentrations of liposomes, Cy$^{3+}$, P$^{4+}$, and borate (Ph$_3$BnB$^-$ or Ph$_4$B$^-$) same as in Figure V-12.
Figure V-15 emphasizes the requirement that all three components be present in order to bleach Cy$^{3+}$ with blue-light irradiation. In the absence of either the energy donor (P$^{4+}$) or the electron donor (Ph$_3$BnB$^-$), the reaction proceeds significantly slower than when both are present.

Figure V-15. Bleaching of DOPC/DOPA-bound Cy$^{3+}$ (irradiation at 417 nm) in the presence of A) Ph$_3$BnB$^-$ alone, B) P$^{4+}$ alone and C) both Ph$_3$BnB$^-$ and P$^{4+}$. [Liposomes] = 6.25 nM; 400 Cy$^{3+}$ and P$^{4+}$ (where appropriate), 2000 Ph$_3$BnB$^-$ (where appropriate) bound per liposome.
V. D. 5. Direct Photobleaching of $\text{P}^4+$

While blue-light irradiation of the three-component system stimulates bleaching of $\text{Cy}^3+$, $\text{P}^4+$ is also consumed. In the absence of $\text{Ph}_3\text{BnB}^-$, virtually no bleaching of the porphyrin occurs. Faster bleaching is observed when the borate is present but not the cyanine whereas intermediate behavior is observed when all three components are present (Figure V-16).

![Graph](image.png)

Figure V-16. Bleaching of DOPC/DOPA-bound $\text{P}^4+$ (irradiation at 417 nm) in the presence of A) $\text{Cy}^3+$ alone, B) $\text{Ph}_3\text{BnB}^-$ alone and C) both $\text{Ph}_3\text{BnB}^-$ and $\text{Cy}^3+$. Concentrations of liposomes, cyanine, porphyrin and borate are the same as in Figure V-15.
If the system is irradiated at 669 nm, only $\text{Cy}^{3+}$ bleaches (Figure V-17). This indicates that the chemistry which consumes the porphyrin is not triggered by the excited cyanine.

Figure V-17. Bleaching of DOPC/DOPA-bound $\text{P}^{4+}$ and $\text{Cy}^{3+}$ (irradiation at 696 nm) in the presence of $\text{Ph}_3\text{BnB}^-$. Concentrations of liposomes, cyanine, porphyrin and borate are the same as in Figure V-15.
Further investigation of $P^{4+}$ photobleaching suggests that it too is triggered by electron transfer from $\text{Ph}_3\text{BnB}^-$, analogous to the direct photobleaching of $\text{Cy}^{3+}$ described in Chapter IV. Figure V-18 shows that the rate of $P^{4+}$ photobleaching increases with increasing $\text{Ph}_3\text{BnB}^-$ concentration.

**Figure V-18.** Effect of $[\text{Ph}_3\text{BnB}^-]$ on photobleaching (irradiation $\lambda > 490$ nm) of DOPC/DOPA-bound $P^{4+}$ in the absence of $\text{Cy}^{3+}$. [Liposomes] = 8.1 nM and 160 $P^{4+}$ bound per liposome. A) 800, B) 400, C) 200, and D) 100 $\text{Ph}_3\text{BnB}^-$ bound per liposome.
V. D. 6. Photoinduced Consumption of Ph₃BnB⁻

Bleaching of both pigments is triggered by photoinduced electron transfer from Ph₃BnB⁻. However, the borate appears to be consumed at a significantly faster rate than the pigments. This results in incomplete bleaching of the pigments, even when the borate is present in significant excess. Once bleaching has ceased, fresh Ph₃BnB⁻ can be added to the liposomes and bleaching will resume (Figure V-19).

![Graph showing bleaching of Cy3⁺ and Ph₃BnB⁻](image)

**Figure V-19.** Photosensitized (irradiation at 417 nm) bleaching of Cy3⁺; concentrations of liposomes, P4⁺, Cy3⁺, and Ph₃BnB⁻ (initially) are the same as in Figure V-12. After bleaching ceases, addition of fresh Ph₃BnB⁻ (800 per liposome) allows bleaching to resume.
For the experiment illustrated in Figures V-15 and 16, the initial ratio of the three components was 400 : 400 : 2000 (P⁴⁺ : Cy³⁺ : Ph₃BnB⁻) per liposome. However, after ten minutes irradiation, the bleaching of both pigments stops with only 25% of the porphyrin and 38% of the cyanine consumed while apparently the borate is completely destroyed. This corresponds to 100 P⁴⁺ and 150 Cy³⁺ consumed per liposome and eight borates consumed for every pigment bleached. This suggests that a chain reaction initiated by photoinduced electron transfer to either P⁴⁺ or Cy³⁺ consumes the borate. This is discussed further in Section V. E. 3.
V. E. DISCUSSION

V. E. 1. Assembly of the System

$P^{4+}$ binds to the surface of DOPC/DOPA (9:1) liposomes as evidenced by the resulting bathochromic shift of the Soret band and the enhanced fluorescence yield. The observation that the Soret band remains narrow upon binding indicates that the porphyrin is not aggregated on the membrane surface. The porphyrin is believed to lie flat on the surface of the liposome because of the symmetrical distribution of the four positive charges around the periphery of the macrocycle. $Cy^3+$ and $Ph_3BnB^-$ are known to bind to the surface and interior, respectively, of the lipid bilayer (Chapter IV). Thus addition of all three components to DOPC/DOPA liposomes produces the configuration depicted in Scheme V-1.

V. E. 2. $Cy^3+$ Emission Sensitized by Energy Transfer from $P^{4+}$

Liposome-bound $P^{4+}$ gives a doubly peaked emission profile characteristic of porphyrins whether excited in the Soret band or in the lower energy $Q$ bands. Addition of $Cy^3+$ to the liposome surface results in progressive quenching of the porphyrin fluorescence at 650 nm with simultaneous appearance of an emission band at 696 nm attributed to the cyanine. The ratio of the intensities at 696 nm and 650 nm is related to the energy transfer efficiency as they represent the emission maxima of the isolated pigments. As energy transfer becomes more efficient, this ratio increases. Figure V-7 shows that this ratio increases progressively with added cyanine. The relatively clean isoemissive point in Figure V-6 indicates that the quenching of the porphyrin
fluorescence occurs solely by energy transfer. Although photoinduced electron transfer between the two pigments is thermodynamically possible, it would not result in cyanine emission since ground state products would most likely result.

One point of concern regards the relative orientation of the transition moments for \textit{P}^{4+} and \textit{Cy}^{3+} when bound to the liposome. If the molecules were bound such that their respective transition moments were mutually perpendicular, energy transfer would not be possible. The porphyrin should bind to the liposome by lying flat on the surface. This would orient the transition moment parallel to the surface, although it would be isotropic in two dimensions. The cyanine, on the other hand, is more likely to bind in an intercalative fashion in which the chromophore penetrates in between the lipid head groups while the trimethylammoniopropyl tails extend into the aqueous phase. This type of binding would orient the transition moment (which lies along the long axis of the chromophore) parallel to the membrane surface as well. This permits the transition moments of the donor and acceptor to align, leading to energy transfer. This configuration would facilitate energy transfer between \textit{P}^{4+} and \textit{Cy}^{3+}, but it would likely hinder electron transfer since the \textit{pi}-systems of the two chromophores would be oriented perpendicular to one another.

The absorption spectra in Figure V-4 indicate that \textit{P}^{4+} can be selectively excited at 417 nm in the presence of \textit{Cy}^{3+}. This is verified by the observations that virtually no cyanine emission or bleaching occurs upon excitation at this wavelength in the absence of the porphyrin (Figures V-8 and 12, respectively). Thus the system is spectrally "clean" in the sense that for blue-light irradiation, any chemistry involving excited \textit{Cy}^{3+} must be sensitized by energy transfer from the porphyrin.
V. E. 3. PET from Ph3BnB\(^-\) to Cy\(^{3+}\) using Blue Light

Addition of the electron donor Ph3BnB\(^-\) to the system results in quenching of the emission at 696 nm (Figure V-9). This observation does not prove that the quenching is due to electron transfer from Ph3BnB\(^-\) to the cyanine. An equally plausible explanation is that rather than reducing the cyanine after energy transfer, the borate reduces the porphyrin before energy transfer. The quenching experiments summarized in Figure V-10 demonstrate that electron transfer appears to be occurring to both P\(^{4+}\) and Cy\(^{3+}\). If electron transfer completely quenched the excited porphyrin, then the Stern-Volmer kinetics for the mixed system should be identical to those of the porphyrin alone. Likewise, if the porphyrin completely transferred its energy before electron transfer could occur, the kinetics for the mixed system should be identical to those of the cyanine alone. Intermediate kinetics are indicative of competitive energy- and electron-transfer quenching of the excited porphyrin. Approximately two-thirds of the observed quenching appears to be the result of sensitized electron transfer to Cy\(^{3+}\).

The branching of the system after excitation of P\(^{4+}\) by electron- and energy transfer paths can also be observed by photobleaching experiments. The porphyrin undergoes similar chemistry upon direct irradiation in the presence of Ph3BnB\(^-\) as described for Cy\(^{3+}\) in Chapter IV: electron transfer quenching followed by irreversible bleaching. Thus irradiation of the P\(^{4+}\)-Cy\(^{3+}\)-Ph3BnB\(^-\) system with blue light leads to simultaneous bleaching of both the porphyrin and cyanine absorption bands (Figures V-15 and 17). The reaction sequences are illustrated in Scheme V-2.
Scheme V-2

For the bleaching of Cy$_{3}^{3+}$, Figures V-11 through 14 demonstrate that the chemistry sensitized by blue light has the same characteristics as that obtained by direct irradiation using red light: an electron transfer from the borate triggers the bleaching chemistry. While this could certainly result from the desired energy transfer-electron transfer sequence (see Scheme V-2, bold arrows; $E =$ energy transfer, $e^-$ = electron transfer), the alternative possibility is a two-step electron transfer sequence (Scheme V-2, dashed arrows). In this latter mechanism, the porphyrin is first reduced by the borate, then is reoxidized by a cyanine on the liposome surface. Cy$_{3}^{3+}$ should have a lower reduction potential than P$_{4}^{4+}$ by approximately 125 mV permitting the second step to occur.$^{164,165}$
As mentioned above, the relative orientations of the porphyrin and cyanine might hinder this reaction, but it cannot be ruled out at this point.

The observed bleaching of $P^{4+}$ only occurs when the porphyrin is directly irradiated. Moreover, this chemistry is independent of that involving the excited cyanine. Thus once $P^{4+}$ has transferred its energy to the cyanine, it returns to the ground state and is completely stable under the reaction conditions until it is excited again. Apparently the only entry point to the porphyrin bleaching sequence is by electron transfer from $\text{Ph}_3\text{SnS}^-$.

Finally, it was pointed out in Section V. D. 6. that $\text{Ph}_3\text{BnB}^-$ is consumed significantly faster than either of the pigments. One possible explanation for this effect is reoxidation of either $P^{4+}$ or $\text{Cy}^{3+}$ subsequent to reduction by $\text{Ph}_3\text{BnB}^-$, regenerating the even-electron dye molecule. The phenomenon of faster borate consumption than dye bleaching has been observed in a wide variety of systems possessing different lipid compositions, dyes, buffers, and oxygen contents. It seems unlikely that the unidentified electron acceptor would be present in each of these experiments.

An alternative explanation is a chain reaction involving the borate occurring within the bilayer. As described in Chapter IV, the oxidized borate rapidly decomposes to triphenylboron and a benzyl radical. If one of these fragments can act as an oxidizing agent, a chain reaction may be initiated. The redox potentials for the benzyl radical$^{166}$ and $\text{Ph}_3\text{BnB}^-$ $^{89,118}$ indicate that the former is incapable of oxidizing the latter. Thus $\text{Ph}_3\text{B}$ is the proposed oxidant (Scheme V-3). While its reduction potential is unknown, $\text{Ph}_3\text{B}$ is certainly an electrophile, its strong Lewis acidity being exploited in the synthesis of $\text{Ph}_3\text{BnB}^-$. 
Although this self-sacrificial feature diminishes the potential utility of Ph₃BnB⁻ as a membrane-bound electron donor, it may prove beneficial with respect to the generation of free radicals. Apparently multiple benzyl radicals are released for every dye molecule consumed, indicating that this chemistry may be exploited for the photochemical production of free radicals with fairly high quantum yields.
V. F. SUMMARY

The three-component system described in this chapter utilizes blue light, an antenna pigment and resonance energy transfer to sensitize the same photochemical reactions involving Cy3+ and Ph3BnB− triggered by red light irradiation in Chapter IV. The system is spectrally "clean" in that virtually all of the chemistry involving Cy3+ is sensitized via initial excitation of the porphyrin. Unfortunately, some of the excited porphyrin manages to make it past the two branch points on the left side of Scheme V-2, resulting in its own irreversible bleaching. Higher concentrations of Cy3+ may prevent this, particularly by enhancing electron transfer from the reduced porphyrin to the cyanine.

This system takes advantage of the unique ability of lipid bilayers to organize and concentrate small molecules both at the membrane surface as well as in its hydrophobic interior. Two reaction sequences may be operative after excitation of the porphyrin: 1) Energy transfer from the excited P4+ to Cy3+ occurs parallel to the liposome surface and triggers a vectorial electron transfer from Ph3BnB− to the excited cyanine, proceeding perpendicular to the surface. Thus the two steps occur orthogonally in space. 2) The other sequence considered also features orthogonal reactions but these are both electron transfers: first from Ph3BnB− to the excited porphyrin perpendicular to the liposome surface and then from the reduced porphyrin to Cy3+ parallel to the membrane surface. The fluorescence quenching data indicate that the first pathway is in fact an important pathway for the system.
CHAPTER VI

PET AND EET REACTIONS IN POLYMERIZED, PHASE-SEPARATED PHOSPHOLIPID MEMBRANES

VI. A. INTRODUCTION

The membrane-bound Cy$^{3+}$-Ph$_3$BnB$^-$ system described in Chapter IV features a vectorial photoinduced electron transfer reaction triggered by irradiation with red light. Addition of the antenna pigment P$^4+$ extends the sensitivity of the system to blue light (Chapter V). Further improvement of the system would result from enhancing the efficiency of the electron and energy transfer reactions. A number of approaches can be envisioned for achieving this:

1) Increasing the exergonicity of the electron transfer reaction would enhance the efficiency of the reaction (presuming the reaction rate is not in the Marcus inverted region$^{82-84}$). This could be achieved by adding electron donating groups such as methoxy to the phenyl rings of the borate. Of course the disadvantage to this strategy is that more of the excitation energy would be consumed by the electron transfer reaction (Section II. D.).

2) Increasing the absolute concentrations (i. e. the number per liposome) of the quenchers (Cy$^{3+}$ for energy transfer, Ph$_3$BnB$^-$ for electron transfer) should also lead to more efficient reactions.
3) An alternative to increasing the absolute concentrations is to increase the effective concentrations of the various components. Conceptually, the most direct way of accomplishing this is to covalently link the components via chemical synthesis. This strategy has been used in several cases to produce "molecular wires" which can be inserted into lipid bilayers. While this approach allows for considerable control over intercomponent distances and orientations, it often requires elaborate synthetic schemes. A further problem arises from the difficulty of inserting the wires into lipid bilayers with the same orientation. This is critical since a vectorial system requires that energy/electrons flow in the same direction. Two wires inserted into the bilayer in an antiparallel orientation could effectively short-circuit one another.

The strategy described below focuses on the membrane rather than on the energy- and electron-transfer components. Rendering a large percentage of the membrane volume inaccessible to the components via partial polymerization of the lipid bilayer effectively increases their concentration, leading to enhanced reactivities.
VI. B. EXPERIMENTAL

Materials. Syntheses of polymerizable lipids (see Table VI-1 for structures) were performed by Eric Oblinger and Todd D. Sells (BAPC) and by Henry Lamparski (Bis-C8-SorbPC).\textsuperscript{170,171} Bovine brain phosphatidylserine (PS) was obtained from Sigma Chemical Co. (St. Louis, MO) and used as received. Azobisisobutyronitrile (AIBN) obtained from Eastman Chemical Co. (Rochester, NY) was recrystallized from ethanol before use.

VI. B. 1. Energy Transfer Experiments

Vesicle Preparation. BAPC/DOPA (9:1) and Bis-C8-SorbPC/DOPA (9:1) vesicles were prepared in either Milli-Q water (pH adjusted to 8.3 using potassium hydroxide) or buffer (10 mM imidazole, pH=7.0). The freeze-thaw and extrusion protocols outlined in Chapter IV were used with the following parameters: 0.1 μm filters were used for the extrusion, which was performed at 37 °C for Bis-C8-SorbPC-containing vesicles and at 50 °C for BAPC-containing vesicles.

Determination of Vesicle Concentration. The concentrations of some of the vesicle preparations were determined by a combination of UV-vis absorption spectroscopy (for the polymerizable lipid concentration) and dynamic laser light scattering (for the number of lipids per vesicle).

BAPC has an absorption maximum which varies between 193-196 nm for different preparations ($\varepsilon_{\text{max}} = 12,000 \text{ M}^{-1} \text{ cm}^{-1}$) in water. This peak was used
to determine the BAPC content of BAPC/DOPA (9:1) vesicles prepared in Milli-Q water. Light scattering showed that these vesicles had an average diameter of 106 +/- 12 nm. Determination of (1) the number of lipids in a single vesicle by light scattering and (2) the vesicle concentration was done as described in Section V. B.

BAPC/DOPA (9:1) vesicles were also prepared in imidazole buffer using procedures identical to those described for vesicles prepared in Milli-Q water. In this case, the imidazole buffer masks the BAPC absorption band; therefore the BAPC concentration could not be determined by UV-vis spectroscopy. The two vesicle preparations were assumed to be of the same concentration.

Bis-C8-SorbPC/DOPA (9:1) vesicles prepared in either buffer or Milli-Q water were assayed for Bis-C8-SorbPC content by UV-vis absorption spectroscopy. This lipid has an absorption maximum which varies between 256-262 nm (ε_max = 35,000 M^-1 cm^-1 in aqueous media,^{172} 48,000 M^-1 cm^-1 in methanol^{62}) which is not masked by buffer. Light scattering was not done on these preparations; it was assumed that the average diameter was 100 nm, giving 80,000 lipids per vesicle.

**Vesicle Polymerization.** Two different methods were utilized to polymerize the vesicles: 1) direct irradiation of the polymerizable lipid with UV light and 2) AIBN photolysis in which AIBN is decomposed upon irradiation with UV light, generating two free radicals.

Direct Irradiation: This method was used for one experiment involving Bis-C8-SorbPC/DOPA vesicles. A quartz cuvette containing 3.0 mL of 5.0 nM vesicles (in imidazole buffer) was irradiated using filtered light from a 200W
Hg(Xe) lamp (Corning CS7-54, 240 nm < λ < 410 nm). The cuvette was 11.0 cm from the front of the lamp and the sample temperature was maintained at 37 °C. For the first 15 min, a 5% transmitting neutral density filter was also used but this was subsequently removed in order to accelerate the polymerization. The reaction was complete in 45 min (total) as judged by the depletion of the UV-absorption band of the polymerizable lipid.

AIBN Photolysis: This method was used for BAPC/DOPA (2.5 nM, in water and buffer) and Bis-C8-SorbPC/DOPA (5.0 nM, in water) vesicles. AIBN (10 mM in methanol) was added to 3.0 mL of vesicles by Hamilton syringe to give a ratio of polymerizable lipid/AIBN ([M]/[I]) = 10. The sample was bubbled with argon gas for 30 min and then irradiated using the Hg(Xe) lamp. A Corning CS0-52 filter (λ > 345 nm) was used to selectively irradiate the AIBN. Argon was continuously bubbled through the sample or over its surface for the duration of the polymerization, which was followed by monitoring the absorption band of the lipid (except for BAPC/DOPA in buffer). For BAPC/DOPA in water, 100% conversion was achieved after 14 hours of irradiation. BAPC/DOPA vesicles in imidazole buffer were also allowed to polymerize for 14 hours; complete polymerization was assumed. For Bis-C8-SorbPC/DOPA vesicles (in water), 90% conversion occurred after 6 hours polymerization. Some evaporation of water (ca. 20%) was inevitable in these experiments; after irradiation, the volume was returned to its initial value by adding water.

Energy Transfer. All experiments were performed at 37 °C. P4+ (10 per vesicle) was added to the vesicles either before or after polymerization. The fluorescence emission spectrum was recorded with excitation at 417 nm. Cy3+
was then added in increments of 10 or 20 dyes per vesicle, up to 100 or 200 Cy³⁺ per vesicle. The emission spectrum was recorded after each addition and corrected for dilution. In one experiment with 100 % polymerized BAPC/DOPA vesicles in water, fresh vesicles were used for samples containing 20, 50 and 100 Cy³⁺ per vesicle and compared with the case where the same set of vesicles was used for each measurement.

VI. B. 2. Electron Transfer Experiments

**Vesicle Preparation.** BAPC/DOPA (9:1) and BAPC/PS (9:1) vesicles were prepared in Milli-Q water and the vesicle concentrations were determined by UV absorption spectroscopy and dynamic laser light scattering as described in Section VI. B. 2. for BAPC/DOPA. Light scattering showed these vesicles to have an average diameter of only 61 +/- 5 and 69 +/- 4 nm, respectively. Since the methods of preparation were identical to those described above, the reason for this significant decrease in the vesicles' sizes is unknown.

**Vesicle Polymerization.** AIBN (10 mM in methanol) was added to 5.0 mL of 5.0 nM BAPC/DOPA or BAPC/PS vesicles to give [M]/[I] = 40. The samples were bubbled with ultrapure argon gas for 30 min and then placed in a 60 °C bath for 48 hours. The samples were kept under positive argon pressure through the reaction. UV absorption spectroscopy indicated 60-70 % conversion was achieved in both cases. Light scattering showed the polymerized BAPC/DOPA and BAPC/PS vesicles to have average diameters of 56 +/- 3 and 65 +/- 2 nm, respectively.
**Binding of Rhodamine-6-G to BAPC/DOPA (9:1) Vesicles.** The visible absorption spectrum of 1.08 μM Rhodamine-6-G (Rh⁺, see Table III-1 for structure) was recorded in water (λ_max = 526 nm) and in the presence of unpolymerized BAPC/DOPA (9:1) vesicles (70 nM in water, λ_max = 532 nm). The bathochromic shift is attributed to binding of the cationic dye to the anionic vesicle surface.

**Photoinduced Electron Transfer.** All experiments were performed at 37 °C. BAPC/DOPA vesicles were diluted to 1.0 nM with water. Rh⁺ was added to give 100 rhodamines per vesicle. The fluorescence emission spectrum was recorded with excitation at 532 nm. Then 5.0 molar equiv of either Ph₃BnB⁻ or Ph₄B⁻ were added to the vesicles and the spectrum recorded again. The spectra were integrated and the percent quenching determined as follows:

\[
\text{% Quenching} = 100 \left( \frac{l_0 - l}{l_0} \right)
\]

where \(l_0\) and \(l\) correspond to the integrated spectra without and with borate, respectively. Three trials were done for each sample.

BAPC/PS vesicles were diluted to 1.0 nM with water and 100 Rh⁺ per vesicle were added. Fluorescence emission spectra were recorded with from 0 to 2.0 molar equiv Ph₃BnB⁻. The ratio of the integrated emission spectra in the absence and presence of borate was plotted versus borate concentration for both polymerized and unpolymerized vesicles.
VI. C. DESIGN OF THE SYSTEM

Enhanced electron and energy transfer efficiencies may be attainable by decreasing the volume of the membrane which is accessible to the donor (D) and acceptor (A) molecules. The resulting increase in the effective concentrations of D and A should lead to faster reaction rates and higher efficiencies since both electron transfer\(^{173-181}\) and energy transfer\(^{93}\) rate constants depend on distance (r):

Electron transfer: \( k \propto e^{-r} \)

Energy transfer: \( k \propto r^{-6} \)

Figure VI-1 illustrates how this strategy would impact the membrane-bound Cy\(^{3+}\)-Ph\(_3\)BnB\(^-\) system described in Chapter IV.

\[ \text{Figure VI-1. Restricting the access of Cy}^{3+} \text{ and Ph}_3\text{BnB}^- \text{ to portions of the membrane increases their effective concentration. This increases the likelihood that Ph}_3\text{BnB}^- \text{ will reduce Cy}^{3+} \text{ within the excited state lifetime of the dye.} \]
Polymerization of the lipid bilayer is one way in which the two-component membrane illustrated in Figure VI-1 could be obtained. Polymerization of a binary lipid mixture, only one component of which is polymerizable, would necessarily lead to phase-separation of the membrane into polymerized and unpolymerized domains (Figure VI-2).\textsuperscript{182}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure_vi-2.png}
\caption{Polymerization of a binary lipid membrane in which the two components are randomly mixed before polymerization leads to phase separation of the membrane into polymerized and unpolymerized domains.}
\end{figure}
In order for substantial concentration enhancements to result from polymerization, D and A must exhibit considerably greater affinities for the unpolymerized versus the polymerized domains. This is likely to be true for hydrophobic molecules since covalent linkage of the lipid tails should render binding within the polymerized domains unfavorable relative to the more fluid unpolymerized domains. This physical differentiation between the two domains should be particularly pronounced if the polymerizable lipid is bis-substituted as this would produce cross-linked polymeric networks which should be even less penetrable to hydrophobic molecules. An additional concern regarding monofunctional lipids is that the linear polymers formed from such a lipid might not induce phase separation of the membrane. Cross-linking virtually guarantees domain formation.

While the polymerization-induced phase separation should be sufficient to permit molecules bound in the lipid interior to distinguish between the two types of domains, further differences between the domains are required in order to facilitate recognition of the unpolymerized domains by surface-associated molecules. Such differentiation can be achieved by using neutral polymerizable lipids and anionic nonpolymerizable lipids in the initial mixture. Polymerization would then lead to domains which could be distinguished electrostatically. In such a system the water-soluble cationic dyes described in Chapters III-V would bind selectively to the unpolymerized domains.

The polymerizable lipids used for the electron and energy transfer experiments are BAPC and Bis-C8-SorbPC (see Table VI-1 for structures). The anionic lipids DOPA and bovine brain phosphatidylyserine (PS) are used as nonpolymerizable components. P⁴⁺ and Cy³⁺ are used as donor and acceptor,
respectively, in the energy transfer experiments. For the electron transfer studies, the donor is again \( \text{Ph}_3\text{BnB}^- \) but Rhodamine-6-G (Rh\(^+\), structure in Table III-1) was used in place of \( \text{Cy}^3+ \) as the light absorber and electron acceptor in the system. The higher fluorescence quantum yield of Rh\(^+\) permits less polymerizable lipid to be used in a given experiment than required for \( \text{Cy}^3+ \).

The design of the membrane-based energy and electron transfer systems and the effect of membrane polymerization are depicted in Schemes VI-1 and 2. (Only the outer leaflets are shown for simplicity.)
Scheme VI-1

Polymerization

○ = Cy$^{3+}$

○ = P$^{4+}$

Scheme VI-2

Polymerization

○ = Rh$^+$

○ = Ph$_3$BnB$^-$
Table VI-1. Polymerizable lipids used in the energy and electron transfer experiments.

\[
\begin{align*}
\text{BAPC} & \quad \text{Bis-C8-SorbPC}
\end{align*}
\]
VI. D. RESULTS

VI. D. 1. EET on Unpolymerized BAPC/DOPA (9:1) Vesicles

Energy transfer between P⁴⁺ and Cy³⁺ occurs readily at the surface of unpolymerized BAPC/DOPA vesicles as shown by the quenching of the porphyrin emission and sensitization of the cyanine emission illustrated in Figure VI-3.

![Fluorescence spectra](image)

**Figure VI-3.** Fluorescence spectra (excitation at 417 nm) for P⁴⁺ (25.0 nM, 10 per vesicle) in the presence of 2.5 nM unpolymerized BAPC/DOPA (9:1) vesicles (in water) with addition of Cy³⁺ in increments of 20 cyanines per vesicle.
The efficiency of the energy transfer reaction is related to the ratio of the fluorescence intensities of the cyanine and porphyrin as described in Section V. D. 2. This ratio (R) is plotted versus the number of Cy\textsuperscript{3+} per vesicle in Figure VI-4 for vesicles prepared in imidazole buffer and in water. Interestingly, the energy transfer efficiency exhibits a stronger dependence on the number of cyanines bound per vesicle if the vesicles are prepared in the buffered system. This may be due to a difference in interfacial pH for the two systems, but further experimentation would be required to verify this.

![Graph showing variation of R with number of Cy\textsuperscript{3+} per vesicle for imidazole buffer and water.]

**Figure VI-4.** Variation of R with the number of Cy\textsuperscript{3+} bound per BAPC/DOPA (9:1) vesicle prepared in either water or imidazole buffer (pH = 7.0).
For vesicles prepared in imidazole buffer, the dependence of the energy transfer efficiency on the Cy\textsuperscript{3+} concentration is very similar to that observed for DOPC/DOPA (9:1) vesicles prepared in imidazole buffer (Figure VI-5).

**Figure VI-5.** Variation of $R$, the ratio of fluorescence intensity at 696 nm (Cy\textsuperscript{3+} maximum) to 650 nm (P\textsuperscript{4+} maximum), with the number of Cy\textsuperscript{3+} bound per vesicle for unpolym,erized BAPC/DOPA and DOPC/DOPA. Note that the DOPC/DOPA data utilized the intensity at 653 nm (rather than 650 nm), the porphyrin emission maximum in that case. [Vesicles] = 5.0 nM; [P\textsuperscript{4+}] = 50.0 nM.
VI. D. 2. EET on Polymerized BAPC/DOPA (9:1) Vesicles

All of the experiments described in this section were performed using vesicles prepared in Milli-Q water. BAPC/DOPA (9:1) vesicles were polymerized using the free-radical precursor AIBN which decomposes to give two isobutynitrile radicals and molecular nitrogen:

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
\text{C} & \quad \text{C} \\
\text{H}_3 & \quad \text{H}_3 \\
\text{N} & \quad \text{N} \\
\text{CN} & \quad \text{CN} \\
\rightarrow & \\
2 \quad \text{CH}_3 & + \quad \text{N}_2
\end{align*}
\]

The bond homolyses can be triggered either thermally \( (\tau_{1/2} = 10 \text{ hours at 64 } ^\circ\text{C})^{183} \) or photochemically \( (\lambda_{\text{max}} = 345 \text{ nm}, \varepsilon_{\text{max}} = 10 \text{ M}^{-1} \text{ cm}^{-1}) \).

AIBN was added to BAPC/DOPA vesicles to give a ratio of BAPC/AIBN = 10. Oxygen was purged from the vesicles using argon gas. The vesicles were then irradiated at 37 ºC with UV light \( (\lambda > 345 \text{ nm}) \). The polymerization reaction can be followed by UV absorption spectroscopy, provided the vesicles are prepared in water. (Buffers mask the BAPC absorption band.) Approximately 50 % conversion of monomer to polymer was achieved in 6 hours of irradiation while 14 hours gave complete polymerization.
Dynamic laser light scattering showed that within experimental error, the vesicles retained their size after 100 % polymerization (Table VI-2).

**Table VI-2.** Diameters of BAPC/DOPA (9:1) vesicles before and after AIBN-initiated polymerization.

<table>
<thead>
<tr>
<th>BAPC/DOPA (9:1) Vesicles</th>
<th>Average Diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Polymerization</td>
<td>106 +/- 12</td>
</tr>
<tr>
<td>After Polymerization</td>
<td>98 +/- 15</td>
</tr>
</tbody>
</table>

In the single experiment performed, the fluorescence yield of P₄⁺ was attenuated by 20 % on the polymerized vesicles, relative to unpolymerized vesicles. Since only 10 porphyrins were bound to each vesicle, self-quenching seems unlikely in either case. The explanation for this observation is unknown.
In spite of the reduced fluorescence yield, energy transfer from $P^{4+}$ to $Cy^{3+}$ is more efficient after polymerization than before (Figure VI-6). Moreover, vesicles which are completely polymerized afford greater enhancement than do the 50% polymerized vesicles.

![Graph showing variation of R with the number of Cy3+ bound per BAPC/DOPA (9:1) vesicle polymerized to different extents.](image)

**Figure VI-6.** Variation of $R$ with the number of $Cy^{3+}$ bound per BAPC/DOPA (9:1) vesicle polymerized to different extents. 2.5 nM vesicles and 25.0 nM $P^{4+}$ in each case. The data for 0% polymerization represent the average of two separate trials; the other data are for single experiments.
The clean isoemissive point observed at 661 nm in the unpolymerized vesicles is not preserved at higher Cy\textsuperscript{3+} concentrations for the polymerized vesicles. Figure VI-7 shows how the normalized fluorescence intensity at 661 nm varies with cyanine concentration for 0, 50 and 100 % polymerized vesicles.

**Figure VI-7.** Plot of the normalized fluorescence intensity at 661 nm as a function of added Cy\textsuperscript{3+} for P\textsuperscript{4+} (25.0 nM) on BAPC/DOPA (9:1) vesicles (2.5 nM) at various extents of polymerization. The data for 0 % polymerization represent the average of two separate trials; the other data are for single experiments. The intensities are normalized to 1.0 at zero added Cy\textsuperscript{3+}. 
The loss of the isoemissive point is correlated to a decrease in the relative fluorescence yield, defined as the ratio of the integrated fluorescence spectra in the presence and absence of Cy\textsuperscript{3+} (I/I\textsubscript{o}). This ratio is plotted versus the number of Cy\textsuperscript{3+} per vesicle for 0, 50 and 100 % polymerization in Figure VI-8. I/I\textsubscript{o} rises most quickly for the completely polymerized sample but it reaches a maximum after 40 Cy\textsuperscript{3+} (per vesicle) have been added. In the case of 50 % polymerization, the relative yield does not turn over, although it does level off.

Figure VI-8. Relative fluorescence yield for P\textsuperscript{4+}-Cy\textsuperscript{3+} system on BAPC/DOPA (9:1) vesicles as a function of Cy\textsuperscript{3+} concentration. 2.5 nM vesicles and 25.0 nM P\textsuperscript{4+} in each case. The data for 0 % polymerization represent the average of two separate trials; the other data are for single experiments.
The turnover of the fluorescence yield observed for the polymerized vesicles cannot be attributed to quenching of Cy$^3+$ alone, since this would not result in loss of the isoemissive point. It is the porphyrin which must be undergoing additional chemistry in the polymerized vesicles. This quenching reaction could be reversible or irreversible; to investigate this, an experiment was performed in which the relative fluorescence yield was measured as Cy$^3+$ was 1) added successively to the same set of vesicles (as in the experiments just described) and 2) added to a fresh set of vesicles for each sample. Figure VI-9 shows that using fresh vesicles for a number of data points prevents the relative yield from turning over, although it still levels off. This establishes that the porphyrin is consumed by an irreversible reaction.

Figure VI-9. Relative fluorescence yield for P$^4+$-Cy$^3+$ system on BAPC/DOPA (9:1) vesicles (100 % polymerized) as a function of Cy$^3+$ concentration. 2.5 nM vesicles and 25.0 nM P$^4+$ in each case. In A) fresh vesicles were used for each data point whereas in B) Cy$^3+$ was added sequentially to the same set of vesicles.
VI. D. 3. EET on Unpolymerized Bis-C8-SorbPC/DOPA (9:1) Vesicles

The efficiency of energy transfer between P4+ and Cy3+ shows the same cyanine concentration dependence on unpolymerized Bis-C8-SorbPC/DOPA (9:1) vesicles as on unpolymerized BAPC/DOPA (9:1) and DOPC/DOPA (9:1), provided the vesicles are buffered (Figure VI-10).

![Graph showing variation of R with the number of Cy3+ bound per vesicle](image)

**Figure VI-10.** Variation of R with the number of Cy3+ bound per vesicle. for DOPC/DOPA, unpolymerized BAPC/DOPA, and unpolymerized Bis-C8-SorbPC/DOPA, [Vesicles] = 2.5 nM for Bis-C8-SorbPC/DOPA, 5.0 nM otherwise. 10 P4+ bound per vesicle in each case. Vesicles were prepared in imidazole buffer (pH = 7.0).
Energy transfer from $P^{4+}$ to $Cy^{3+}$ is more efficient at a given $Cy^{3+}$ concentration when the vesicles were prepared in imidazole buffer rather than in water. This is similar to the observation for unpolymerized BAPC/DOPA vesicles (Section VI. D. 1). Figure VI-11 shows that the results are similar for the two types of polymerizable lipid in both water and imidazole buffer.

![Graph](image)

**Figure VI-11.** Variation of $R$ with the number of $Cy^{3+}$ bound per (unpolymerized) vesicle for A) BAPC/DOPA in buffer ($pH = 7.0$); B) Bis-C8-SorbPC/DOPA in buffer ($pH = 7.0$); C) BAPC/DOPA in water; D) Bis-C8-SorbPC/DOPA in water. 10 $P^{4+}$ bound per vesicle in each case.
VI. D. 4. EET on Polymerized Bis-C8-SorbPC/DOPA (9:1) Vesicles

Bis-C8-SorbPC/DOPA (9:1) vesicles (in water) were polymerized using AIBN photolysis under the same irradiation conditions and [M]/[I] = 10 as was used for BAPC/DOPA; irradiation for 6 hours resulted in 90 % polymerization. Energy transfer from P$^4+$ to Cy$^3+$ is considerably enhanced on the polymerized vesicles (Figure VI-12).

![Graph](image)

**Figure VI-12.** Variation of R with the number of Cy$^3+$ bound per Bis-C8-SorbPC/DOPA (9:1) vesicle (in water) before and after 90 % polymerization. 5.0 nM vesicles and 50.0 nM P$^4+$ in each case.
The fluorescence spectra for the unpolymerized vesicles exhibit an isoemissive point at 662 nm which is preserved throughout the range of Cy\textsuperscript{3+} concentration studied. After polymerization, this point moves to 658 nm and is lost after 30 Cy\textsuperscript{3+} have been added (Figure VI-13). Similar results were obtained for completely polymerized BAPC/DOPA vesicles (Figure VI-7).

**Figure VI-13.** Plot of normalized fluorescence intensity at the isoemissive points for unpolymerized (662 nm) and polymerized (658 nm) Bis-C8-SorbPC/DOPA vesicles as a function of added Cy\textsuperscript{3+}; [P\textsuperscript{4+}] = (50.0 nM) and [vesicles] = 5.0 nM. The intensities are normalized to 1.0 at zero added Cy\textsuperscript{3+}. 
Unlike the polymerized BAPC/DOPA vesicles, the relative fluorescence yield on polymerized Bis-C8-SorbPC/DOPA does not turn over at higher Cy\textsuperscript{3+} concentrations, although it does level off (Figure VI-14). Again, reasonable agreement is found between the concentrations of Cy\textsuperscript{3+} at which the isoemissive point is lost and the relative fluorescence yield begins to level off: 30 Cy\textsuperscript{3+} per vesicle. This experiment was not repeated with fresh vesicles for each data point.

![Graph](image)

**Figure VI-14.** Relative fluorescence yield for P\textsuperscript{4+}-Cy\textsuperscript{3+} system on Bis-C8-SorbPC/DOPA (9:1) vesicles before and after polymerization as a function of Cy\textsuperscript{3+} concentration. 5.0 nM vesicles and 50.0 nM P\textsuperscript{4+} in each case.
VI. D. 5. EET on Bis-C8-SorbPC/DOPA (9:1) Vesicles Polymerized by Direct Irradiation

The enhancement of energy transfer observed after polymerization should be sensitive to the lengths of the polymer chains since this determines the number and size of DOPA domains which are formed. It has been found that direct irradiation of pure Mono-C10-SorbPC (see Table VII-1 for structure) bilayers generally produces very short polymers (degree of polymerization \([X_n] = 10\)), while longer polymers are formed for AIBN-initiated systems \((X_n = 10^2\) for \([M]/[I] = 10)\). Direct irradiation of mixed Bis-C8-SorbPC/DOPA should produce considerably smaller polymers and thus more domains than the AIBN-initiated process. Polymerized Bis-C8-SorbPC/DOPA vesicles (in imidazole buffer) prepared by direct irradiation exhibited virtually no enhancement of energy transfer efficiency (Figure VI-15).

![Graph showing variation of R with the number of Cy³⁺ bound per Bis-C8-SorbPC/DOPA (9:1) vesicle before and after polymerization by direct irradiation. 5.0 nM vesicles and 50.0 nM P⁴⁺ in each case.](image)

**Figure VI-15.** Variation of R with the number of Cy³⁺ bound per Bis-C8-SorbPC/DOPA (9:1) vesicle before and after polymerization by direct irradiation. 5.0 nM vesicles and 50.0 nM P⁴⁺ in each case.
The enhancement of energy transfer was determined by comparing the initial increase of R (from 0 to 40 $\text{Cy}^{3+}$ per liposome) for polymerized with unpolymerized vesicles; the results are given in Table IV-3. For BAPC/DOPA and Bis-C8-SorbPC/DOPA, similar enhancements are observed after extensive polymerization initiated by AIBN.

**Table VI-3.** Enhancement of energy transfer (EET) on polymerized, phase-separated vesicles.

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Relative EET efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAPC/DOPA (AIBN)</strong></td>
<td></td>
</tr>
<tr>
<td>0 % Polymerized</td>
<td>1.00</td>
</tr>
<tr>
<td>50 % Polymerized</td>
<td>2.90</td>
</tr>
<tr>
<td>100 % Polymerized</td>
<td>7.80</td>
</tr>
<tr>
<td><strong>Bis-C8-SorbPC/DOPA (AIBN)</strong></td>
<td></td>
</tr>
<tr>
<td>0 % Polymerized</td>
<td>1.00</td>
</tr>
<tr>
<td>90 % Polymerized</td>
<td>8.06</td>
</tr>
<tr>
<td><strong>Bis-C8-SorbPC/DOPA (Direct)</strong></td>
<td></td>
</tr>
<tr>
<td>0 % Polymerized</td>
<td>1.00</td>
</tr>
<tr>
<td>100 % Polymerized</td>
<td>0.88</td>
</tr>
</tbody>
</table>
VI. D. 6. PET in Polymerized, Phase-Separated Phospholipid Membranes

The water-soluble, cationic dye Rhodamine-6-G (Rh⁺) binds to BAPC/DOPA (9:1) as indicated by the bathochromic shift observed in the absorption spectrum of Rh⁺ in the presence of the anionic vesicles (Figure VI-16).

**Figure VI-16.** Visible absorption spectra of 1.08 μM Rh⁺ A) in water (λ_max = 526 nm) and B) on BAPC/DOPA (9:1) vesicles (λ_max = 532 nm). [Vesicles] = 70 nM.
Addition of 5.0 molar equiv Ph$_3$BnB$^-$ to BAPC/DOPA-bound Rh$^+$ results in 66% quenching of the rhodamine fluorescence while an equivalent amount of Ph$_4$B$^-$ quenches only 19% of the fluorescence. The weaker quenching by the weaker electron donor ($E_{red}$ [Ph$_3$BnB$^-$] = 1.09 V, $E_{red}$ [Ph$_4$B$^-$] = 1.44 V)$^{89}$ indicates that the quenching is due to electron transfer from the borate to the excited rhodamine. After polymerization of the vesicles using AIBN thermolysis (70% conversion), quenching by both borates is enhanced (Table VI-4).

Table VI-4. Percent Quenching of BAPC/DOPA-bound Rh$^+$ by Borates

<table>
<thead>
<tr>
<th>Vesicles</th>
<th>Ph$_3$BnB$^-$</th>
<th>Ph$_4$B$^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unpolymerized</td>
<td>66 +/- 2</td>
<td>19 +/- 2</td>
</tr>
<tr>
<td>70% Polymerized</td>
<td>80 +/- 2</td>
<td>30 +/- 1</td>
</tr>
</tbody>
</table>
Quenching of Rh$^+$ fluorescence by Ph$_3$BnB$^-$ was also investigated in BAPC/PS (9:1) liposomes. After 70% polymerization, quenching was more efficient than before polymerization, particularly at higher borate concentrations (Figure VI-17).

Figure VI-17. Stern-Volmer analysis for fluorescence quenching of BAPC/PS-bound Rh$^+$ by Ph$_3$BnB$^-$. [Vesicles] = 1.0 nM; [Rh$^+$] = 100 nM.
VI. E. DISCUSSION

VI. E. 1. EET on Polymerized, Phase-Separated Vesicles

The results for BAPC/DOPA (Figure VI-6) and Bis-C8-SorbPC/DOPA (Figure VI-12) vesicles show that the transfer of energy from P$^{4+}$ to Cy$^{3+}$ at the vesicle surface is clearly enhanced after the vesicles are polymerized by AIBN photolysis. This is attributed to a polymerization-induced phase separation of the lipid bilayer, producing domains enriched in DOPA (pure DOPA at complete polymerization) surrounded by cross-linked polymeric domains, as depicted in Scheme VI-1. Binding of P$^{4+}$ and Cy$^{3+}$ to the DOPA domains should give a smaller average distance of separation versus unpolymerized vesicles, resulting in a more efficient energy transfer reaction. This interpretation assumes that the two lipids are randomly mixed before polymerization. While phase separation has been observed in many unpolymerized, binary lipid mixtures$^{185-190}$, it usually occurs when the temperature is below one or both of the lipids' main phase transition temperatures. For example in mixtures of neutral DPPC ($T_m = 41.4$ °C) with anionic DPPS ($T_m = 51$ °C), a single phase ($L_\alpha$) was observed at all compositions when the temperature was above 51 °C.$^{188}$ In the energy transfer experiments, the temperature was maintained at 37 °C which is above the $T_m$ of all of the individual components. ($T_m$ was not measured for the lipid mixtures utilized but it is 30 °C for pure BAPC$^{171}$ and 11 °C for pure Bis-C8-SorbPC.$^{170}$) The similar dependence of energy transfer efficiency on Cy$^{3+}$ concentration observed for DOPC/DOPA (9:1), unpolymerized BAPC/DOPA (9:1) and unpolymerized Bis-C8-SorbPC/DOPA (9:1) vesicles (Figure VI-10)
lends further support to the contention that the bilayers are not phase-separated prior to polymerization.

Energy transfer is enhanced on polymerized Bis-C8-SorbPC/DOPA vesicles when AIBN photolysis is used to initiate the polymerization but not when direct irradiation of the polymerizable lipid is employed, even though complete polymerization was observed in both cases. The probable explanation for this effect is that direct irradiation produces significantly shorter polymers than AIBN-photolysis, as described in Section VI. D. 5. Assuming the vesicles have the same number of lipids, polymerization by AIBN should yield vesicles with fewer domains since the domains would be bigger. The fewer domains, the greater the likelihood that a porphyrin and cyanine will be bound to the same domain at a given time, a significant concern when only ten porphyrins are bound per vesicle. For a 100 nm diameter vesicle, there are approximately 40,000 lipids in the outer leaflet-the site for energy transfer; 36,000 of these are polymerizable lipids. The degrees of polymerization cited in Section V. D. 5 were determined for linear polymers and thus cannot be used to quantitatively determine the number of domains in the cross-linked systems presumably produced in these experiments. However, they do indicate that the systems polymerized by AIBN and direct irradiation yield tens and hundreds of domains per vesicle, respectively. The increased probability that a cyanine will be close enough to an excited porphyrin to accept energy leads to the enhanced energy transfer efficiency for the AIBN-polymerized vesicles when virtually no enhancement is evident for the vesicles polymerized by direct irradiation.

The loss of the isoemissive point (Figure VI-7) and corresponding turnover (Figure VI-8) in the relative fluorescence yield which are evident on
polymerized BAPC/DOPA and, to a lesser extent, on polymerized (AIBN) Bis-C8-SorbPC/DOPA (Figures VI-13 and 14) are attributed to an irreversible reaction consuming the porphyrin. (While consumption of the cyanine is most likely occurring as well, this would not affect the isoemissive point, only the efficiency.) This is supported by the results in Figure VI-9, where fresh samples of vesicles significantly enhanced the relative fluorescence yield. One possible explanation for these observations is the attack of singlet oxygen on vesicle-bound porphyrins by the sequence outlined below:

\[
\begin{align*}
P^{4+} & \rightarrow (P^{4+})^* \quad (1) \\
(P^{4+})^* & \rightarrow (P^{4+})^* \quad (2) \\
(P^{4+})^* + O_2 & \rightarrow P^{4+} + O_2 \quad (3) \\
P^{4+} + O_2 & \rightarrow \text{Bleached Porphyrin} \quad (4)
\end{align*}
\]

The high extinction coefficient favoring step (1) combined with the large intersystem crossing yield for the porphyrin (0.80 in water\textsuperscript{163}) should lead to efficient production of triplet $P^{4+}$ (2). Generation of singlet oxygen would result from triplet-triplet energy transfer (3). While singlet oxygen could attack the porphyrin from which it was generated, it would also be free to attack other porphyrins. The larger the effective concentration of $P^{4+}$, the more important step (4) becomes. This is why larger effects are observed on the isoemissive point and the relative fluorescence yield at greater conversion to polymer (Figures VI-9 and 10) for BAPC/DOPA. The less drastic effects observed with polymerized Bis-C8-SorbPC/DOPA vesicles may be due to shorter polymers and thus, more domains being formed. Size exclusion chromatography analysis
indicates that mono-substituted acryloyl lipids form longer polymers at a given [M]/[I], than do mono-sorbyl lipids for AIBN-initiated polymerizations. For example, at [M]/[I] = 10, Xₙ (MAPC, see p. 54, for structure) = 400 while Xₙ (Mono-C₁₀-SorbPC) = 100.

VI. E. 2. PET in Polymerized, Phase-Separated Phospholipid Membranes

Binding of Rhodamine-6-G (Rh⁺) to the surface of unpolymerized BAPC/DOPA vesicles is accompanied by a bathochromic shift of its visible absorption maximum from 526 nm to 532 nm (Figure VI-18). The fluorescence of vesicle-bound Rh⁺ is quenched by Ph₃BnB⁻ and, to a lesser extent, Ph₄B⁻ (Table VI-3), apparently by electron transfer from the borate to the rhodamine. The fact that Ph₄B⁻ quenches Rh⁺ but not Cy³⁺ (Chapter IV) indicates that Rh⁺ is a stronger oxidant in the excited state than is the cyanine.

The quenching efficiency of both borates increases after the BAPC/DOPA vesicles were 70% polymerized. As with the energy transfer experiments, this enhancement is attributed to polymerization-induced phase separation of the membrane, leading to higher effective concentrations of the borates in the unpolymerized domains. Ph₃BnB⁻ also quenches the fluorescence of Rh⁺ in BAPC/PS membranes (Figure VI-19). The quenching efficiency increases after 70% polymerization, particularly at higher borate concentrations. This may be due to the greater probability that Rh⁺ and Ph₃BnB⁻ will bind in the same domains at high borate concentrations.

The quenching results indicate that the photoinduced electron transfer between Rh⁺ and Ph₃BnB⁻ is enhanced in the polymerized, phase-separated vesicles. One point of concern regarding these experiments involves the
unexpectedly small vesicles produced by extrusion. Whereas the BAPC/DOPA vesicles used in the energy transfer experiments were approximately 100 nm, those used for the electron transfer experiments were 30-40 % smaller although they were prepared in the same manner. This observation is inexplicable at this time.
VI. F. SUMMARY AND FUTURE WORK

The experimental results described in this chapter indicate the goal of enhancing the efficiencies of membrane-based photochemical reactions via polymer chemistry is realistic. Polymerization-induced phase separation of a two-component lipid bilayer was used to increase the effective concentrations of donor and acceptor molecules employed in the types of energy and electron transfer reactions discussed in Chapters IV and V, leading to greater reactivity upon illumination with visible light.

These systems are by no means optimized. The results from the energy transfer experiments suggest several directions for future study. As discussed previously, the enhancements are going to be sensitive to the size and, therefore, number of domains formed in the membrane by polymerization. A simple way of varying this is by changing the ratio [M]/[I] in the vesicles. Using a lower concentration AIBN will yield longer polymers and fewer domains in the membrane. This is important with regard to the question of "occupancy": if a donor and acceptor are bound to remote domains after polymerization, they might actually be further away from one another than before polymerization. With fewer domains, this potential problem would be minimized.

Another factor to consider is the ratio of polymerizable and nonpolymerizable lipid used in the initial mixture. All experiments reported above used 90% polymerizable lipid. Membranes with a larger percentage of polymerizable lipid should lead to even greater reactivity enhancements since polymerization will render more of the membrane inaccessible to the donors and acceptors. An important point to consider as the proportion of
polymerizable lipid in the mixture is increased regards the degree of polymerization which is attainable. Significant phase separation will occur only when the polymer chain length is considerably larger than the ratio of polymerizable to nonpolymerizable lipid. If the polymers are too short, the enhancements will be negligible because phase separation of DOPA will not occur. The results for Bis-C8-SorbPC/DOPA vesicles polymerized by AIBN and by direct irradiation support this hypothesis. In the latter case, the polymer chain length was most likely comparable to the ratio of Bis-C8-SorbPC to DOPA and thus there was no enhancement in the energy transfer efficiency. The AIBN-polymerized vesicles exhibited far greater enhancements, presumably due to formation of longer polymers. Clearly, variation of the lipid ratio as well as of the monomer to initiator ratio should result in quite different levels of enhancement of these types of reactions.

With regard to the actual reactions studied in these polymerized, phase-separated systems, a logical next step would be to repeat the experiments with the three-component system described in Chapter V. Fluorescence quenching experiments (see Section V. D. 3 and Figure V-10) would provide a convenient means of studying enhanced, sensitized electron transfer from Ph3BnB+ to Cy3+. 
CHAPTER VII

VISIBLE-LIGHT SENSITIZED POLYMERIZATION OF VESICLES

VII. A. INTRODUCTION

Many in vivo uses for lipid vesicles can be envisioned, including applications as diagnostic and imaging agents. However, most efforts have been directed toward the use of vesicles as drug delivery agents. In the ideal case, a vesicle would transport a drug through the bloodstream, arrive at the desired location and then release the drug. Such a system would permit selective treatment of infected or damaged tissue without affecting healthy tissue, thus permitting the use of drugs which would otherwise be too toxic for in vivo use.

Vesicles are in fact good transportation vehicles for drug molecules. They are generally nontoxic, being constructed from naturally occurring and/or synthetic lipids. Thus a drug molecule which is extremely toxic when introduced into the body alone may be rendered far less so when encapsulated within a vesicle prior to introduction. Considerable expertise has been developed in the design of vesicles which effectively evade the immune system, thus permitting prolonged circulation time in the body.

Arrival of the vesicles at the appropriate place in the body is the most problematic aspect of the drug delivery system. Simple strategies take advantage of the fact that tumor tissues often take up vesicles (i.e. the vesicles will leave the bloodstream, wedging their way between the tissue cells by a
process called extravasation) more efficiently than healthy tissues.\textsuperscript{195} This is generally not exclusive as there may still be significant uptake by the healthy tissues. An unacceptably large amount of the drug may still get to the wrong place in this approach.

Greater selectivity might be obtained by the use of immunoliposomes, vesicles with surface-associated antibodies, which target the vesicles to a specific cell type.\textsuperscript{196} The antibodies are covalently attached to the head groups of lipids and then incorporated into the lipid bilayer. As the vesicles pass through the bloodstream, they preferentially associate with the desired cells. This approach requires that the appropriate antibodies can be produced and collected for the targeted cells. Note that both of the strategies discussed benefit from the prolonged circulation times mentioned above.

Once the vesicle has arrived, it must release its contents in order to complete the delivery. Most systems operate passively, that is, the release step is controlled \textit{in vivo}. Upon partitioning out of the bloodstream into the tissue, vesicles may be taken up into actual cells via endocytosis.\textsuperscript{197} Once inside the cell, the vesicle will ultimately be broken down by the lysosome resulting in the release of the drug. Alternatively, the vesicle may deliver its contents to the cellular interior by fusing with the outer membrane of the cell. This is the method by which most viruses inject their genomic material into host cells, but in the viral systems, specific mechanisms exist which facilitate the fusion event.\textsuperscript{198} Simple lipid vesicles which are not fusogenic will generally not adopt this approach. Finally, particularly troublesome is the finding that those vesicles which are not taken up into cells will often simply remain in the tissue without releasing their contents.\textsuperscript{199-201}
Clearly the release of the vesicular contents is the process over which one has the least control. A controlled release mechanism would certainly improve the efficacy of the treatment. Vesicles which release their contents upon increased temperature\textsuperscript{202} or acidity\textsuperscript{203-208} have been reported. Both approaches would take advantage of the fact that tumor tissues exhibit lower pH and higher temperature than healthy tissues. An alternative strategy is to make the vesicles sensitive to visible light for the release of entrapped contents. (Fiber optics would be used for nontopical, \textit{in vivo} applications.) Early attempts focused on the use of photoisomerization to increase the permeability of the membrane. Lipids containing all-trans retinyl\textsuperscript{26,209} or azobenzene units\textsuperscript{137} were incorporated into lipid bilayers. Irradiation with visible light resulted in trans-cis isomerization of the lipid tails. The kinked tails presumably could not pack as well as the trans isomers, resulting in the increased permeability. In another approach, irradiation of the protein rhodopsin reconstituted in egg PC membranes was found to increase the permeability of the membranes to various transition metal ions.\textsuperscript{210}

More recently, Thompson and coworkers reported a system in which photochemically generated singlet oxygen cleaves a vinyl ether linkage between one tail of a lipid and the glycerol backbone.\textsuperscript{211} The buildup of single chain molecules (which do not favor bilayer morphologies) in the membrane ultimately leads to a destabilization of the bilayer. Glucose was released from the photodestabilized vesicles approximately twice as fast as from unirradiated vesicles.

O'Brien and coworkers have recently described an alternative method for photoinduced destabilization of vesicles.\textsuperscript{62,212} In that approach, vesicles were
constructed from a mixture of a polymerizable PC and DOPE. (DOPE will not form vesicles by itself, favoring nonbilayer morphologies such as the inverted hexagonal phase; see Chapter I. Mixing DOPE with PCs, including polymerizable PCs, permits formation of stable bilayer vesicles.) Polymerization of the bilayer necessarily results in a lateral redistribution or phase separation of the lipid components into polymerized domains and pure DOPE domains (Scheme VII-1). The DOPE domains, preferring to adopt nonbilayer morphologies, are destabilized. This instability is manifested as a tendency to undergo fusion events. If multilamellar vesicles are used, intravesicular fusion occurs, resulting in the release of entrapped contents. Unilamellar vesicles must undergo intervesicular fusion, resulting in delivery of the entrapped contents of one vesicle interior to the other. In principle, the multilamellar vesicles could be used to release drugs on the outside of cells whereas the unilamellar vesicles could deliver therapeutic agents to the cellular interior via fusion with the lysosomal membrane, analogous to viral infection.

The major obstacle to in vivo use of this strategy is the method of initiation of polymerization. This has been achieved heretofore by direct irradiation of the polymerizable lipid, which absorbs in the UV. Light of this wavelength could have adverse effects on cellular proteins and nucleic acids, precluding its use in any nontopical application. A variety of initiating systems have been used to polymerize vesicles. These initiators typically generate free radicals upon thermolysis, UV photolysis, γ-ray irradiation or via redox chemistry. Unfortunately, none of these approaches would be useful for in vivo systems. Extending the sensitivity of the system depicted in Scheme VII-1 to visible light would alleviate this problem. An initiating system is
described in this chapter which upon irradiation in the yellow polymerizes the types of lipids used in the earlier experiments under physiological conditions.

1) **Multilamellar Liposomes: Leakage**

![Diagram of leakage](image)

Before Polymerization | After Polymerization | Leakage

2) **Unilamellar Liposomes: Fusion**

![Diagram of fusion](image)

Before Polymerization | After Polymerization | Fusion

Scheme VII-1. Photoinduced destabilization of lipid vesicles occurs via polymerization-induced phase separation of a two-component membrane. 1) Multilamellar systems undergo intravesicular fusion while 2) unilamellar systems undergo intervesicular fusion.
VII. B. EXPERIMENTAL

Materials. *N,N'-Dioctadecyldimethylindocarbocyanine perchlorate (DiIC18(3)), N,N'-Dioctadecyldimethylindodicarbocyanine perchlorate (DiIC18(5)), and N,N'-Dimethyldimethylindocarbocyanine iodide (DiIC1(3)) were purchased from Molecular Probes (Eugene, OR) and used as received. N,N'-Dioctadecyloxacarbocyanine p-toluenesulfonate (DiOC18(3)) was purchased from Eastman (Rochester, NY) and used as received. Hydrogen peroxide (30%, J. T. Baker Inc., Phillipsburg, NJ) was used as received.

Polymerizable lipids were synthesized by the following coworkers (see Table VII-2 for structures): H. Lamparski (Bis-C8-SorbPC and Mono-C10-SorbPC) and T. Sells and E. Oblinger (BAPC). DenPC was a generous gift from the Nippon Oil and Fat Co. Stock benzene solutions of polymerizable lipids (ca. 20 mg/mL) were stored at -40 °C. (At this temperature, the solutions form an amorphous ice.) The lipids were checked periodically by TLC and found to give a single spot (CHCl₃:Methanol:H₂O, 65:25:4 by volume as eluent).

Preparation of Vesicles with Cyanine Dye in Both Leaflets. For preparation of vesicles, an aliquot of the appropriate lipid stock solution was removed by pipet and combined with an appropriate amount of methanolic cyanine dye stock solution in a 10 mL pear-shaped flask. The organic solvent was removed by passing a gentle stream of argon gas over the sample. Immersion of the flask in a warm water bath during this step appeared to aid in the removal of the organic solvent. The remaining dye/lipid film was then dried in vacuo overnight to remove trace organic solvent. In early experiments, the
film was hydrated with 3.0 mL PBS (phosphate-buffered saline: 10 mM Na$_2$HPO$_4$, 100 mM NaCl, pH=7.4), placed in a 50 °C bath for 1 min, briefly sonicated and vortexed. In later experiments, the hydrated sample was incubated above the phase transition of the lipid for at least one hour and subsequently vortexed. This procedure completely removed the film from the wall of the flask and dispersed it homogeneously in the buffer. (Note: BAPC vesicles were prepared in Milli-Q water, pH adjusted to 7.4 using NaOH, in order to observe the absorption peak at 193 nm. All buffers mask this peak.) The sample was then subjected to ten freeze-thaw cycles, accomplished by immersion for three min in a dry ice-isopropanol bath followed by ten min in a water bath. The sample was placed in a 50 °C bath for 30 seconds prior to each freeze-thaw cycle. Vesicles were formed by extrusion ten times through two 0.1 μm polycarbonate filters (Nuclepore Co.; Pleasanton, CA) at a pressure of 250 psi. The extruder (Upex Biomembranes, Inc.; Vancouver, B. C.) was warmed to 37 °C.

**Preparation of Vesicles with Cyanine Dye in the Outer Leaflet.** Pure polymerizable lipid vesicles were prepared by extrusion as described above. After determining the lipid concentration (see below), the desired amount of cyanine dye was added from a concentrated (1.0 mM) methanol solution to the vesicle suspension (1.3 mL) by syringe. The volume of added organic solvent did not exceed 1 % of the total volume.
**Determination of the Lipid and Dye Concentrations after Extrusion.**

Aliquots of extruded vesicles were added either to buffer or methanol. The concentrations were then determined using UV-vis absorption spectroscopy and the following extinction coefficients. Lipids: BAPC: 12,000 M\(^{-1}\) cm\(^{-1}\), Monoc10-SorbPC: 17,500 M\(^{-1}\) cm\(^{-1}\) (in buffer), Bis-C8-SorbPC: 35,000 M\(^{-1}\) cm\(^{-1}\) (in buffer) and 48,000 M\(^{-1}\) cm\(^{-1}\) (in methanol), and DenPC: 35,000 M\(^{-1}\) cm\(^{-1}\) (in buffer); Dyes: DilC18(3): 140,000 M\(^{-1}\) cm\(^{-1}\), DiOC18(3): 150,000 M\(^{-1}\) cm\(^{-1}\), and DilC18(5): 170,000 M\(^{-1}\) cm\(^{-1}\).

**Steady-State Irradiations.** Irradiations were performed using visible light obtained from the filtered output of a 200W Hg(Xe) arc lamp (Oriel Corp.; Stratford, CT). A CS3-72 (Corning Glass Works, \(\lambda > 440\) nm) filter was used, giving a light flux of ca. 10\(^{16}\) photons/s\(^{-1}\). The samples (1.3 mL) were irradiated in a quartz cuvette at a distance of 11.0 cm from the lamp. Sample temperature was controlled by using a thermostatted cell holder connected to a water circulator. Irradiations were performed at 37 °C unless otherwise indicated. The samples were incubated at the desired temperature for 15 min prior to irradiation. UV-vis absorption spectra were recorded at regular intervals. (The cell holders in the instrument were also thermostatted at the desired temperature to prevent temperature change during data acquisition.) For samples irradiated under anaerobic conditions, argon gas was bubbled through the sample for 15 min prior to irradiation.
Calculation of Lipid and Dye Consumption. Irradiation results in bleaching of the UV and visible absorption bands for the lipid and the dye, respectively. Complete polymerization of the vesicles by direct irradiation typically leads to approximately 85% loss in optical density at the lipid maximum; thus, the % monomer remaining at a given time (t) in the sensitized polymerizations was estimated as follows:

\[
\% \text{ Monomer Remaining (at time } t) = 100 \times \left\{ 1 - \frac{OD_{\text{max}(t=0)} - OD_{\text{max}(t)}}{OD_{\text{max}(t=0)} \times 0.85} \right\}
\]

The cyanine dyes bleach completely, thus the % dye remaining at a given time (t) was calculated from the loss in optical density in the visible absorption band:

\[
\% \text{ Dye Remaining (at time } t) = 100 \times \left\{ 1 - \frac{OD_{\text{max}(t=0)} - OD_{\text{max}(t)}}{OD_{\text{max}(t=0)}} \right\}
\]

Chromatographic and \(^1\)H-NMR Spectroscopic Characterization of Lipid Photoproduct. The products of irradiation of Mono-C10-SorbPC/DilC18(3) (12.9:1) vesicles was also investigated by thin-layer chromatography and \(^1\)H-NMR spectroscopy. The vesicles were prepared in Milli-Q water (pH adjusted to 7.1 by addition of NaOH). Concentrated samples of vesicles (OD\(_{\text{max(Dye)}} = 1.0\)) were irradiated for one hour. The samples were then poured into excess methanol followed by evaporation of the solvent. A thin, red film was left behind which was mostly soluble in chloroform. The chloroform-soluble material was subjected to TLC analysis (CHCl\(_3\):Methanol:H\(_2\)O, 65:25:4 by volume as eluent) and \(^1\)H-NMR spectroscopy.
VII. C. RESULTS

The cyanine dyes and polymerizable lipids used in these studies are shown in Tables VII-1 and VII-2, respectively. Three configurations have been investigated for the polymerization of vesicles triggered by visible light. The dye may be incorporated into both leaflets, the outer leaflet or neither leaflet of the polymerizable bilayer, depending on the preparation sequence.

\[
\text{Table VII-1. Cyanine dye structures.}
\]

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<thead>
<tr>
<th>n</th>
<th>m</th>
<th>Dye</th>
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<tr>
<td>1</td>
<td>0</td>
<td>DilC1(3)</td>
</tr>
<tr>
<td>1</td>
<td>17</td>
<td>DilC18(3)</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>DilC18(5)</td>
</tr>
</tbody>
</table>

DiOC18(3)
Table VII-2. Polymerizable lipid structures.

<table>
<thead>
<tr>
<th>DenPC</th>
<th>Bis-C8-SorbPC</th>
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<tr>
<td>(CH₃)₃N⁺</td>
<td>(CH₃)₃N⁺</td>
</tr>
<tr>
<td>O</td>
<td>O</td>
</tr>
<tr>
<td>O</td>
<td>O</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Mono-C10-SorbPC</td>
<td>BAPC</td>
</tr>
<tr>
<td>(CH₃)₃N⁺</td>
<td>(CH₃)₃N⁺</td>
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<tr>
<td>O</td>
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VII. C. 1. Polymerizable Membranes with Dye in Both Leaflets

Polymerizable lipid-cyanine dye vesicles can be prepared by standard extrusion techniques. It is assumed that vesicles generated in this manner have cyanine dye molecules distributed throughout both the inner and outer leaflets of the polymerizable bilayer. The absorption spectrum of DenPC/DiIC18(3) (15:1) vesicles prepared in this way exhibits maxima in the ultraviolet ($\lambda_{\text{max}} = 256$ nm) and the visible ($\lambda_{\text{max}} = 553$ nm) for the polymerizable lipid and the cyanine dye, respectively (Figure VII-1).

![Absorption Spectrum](image)

**Figure VII-1.** UV-vis absorption spectrum of DenPC/DiIC18(3) (15:1) vesicles. Maxima at 256 nm for DenPC and at 552 nm for DiIC18(3).
The vesicles are sensitive to visible light: irradiation with yellow light causes irreversible bleaching of both the lipid and cyanine absorption bands (Figure VII-2), with the lipid being consumed at a significantly faster rate than the dye (Figure VII-3).

**Figure VII-2.** Effect of visible-light irradiation at 37 °C on absorption spectrum of DenPC/DilC18(3) (15:1) vesicles. (A)-(G) correspond to t=0 to t=60 min in 10 min increments.
Figure VII-3. Plot of loss of lipid and dye as a function of irradiation time at 37 °C for DenPC/DilC18(3) (15:1) vesicles.

Both the lipid and the dye are stable in the absence of light (Figure VII-4, A and B, respectively). Additionally, purging the vesicle suspension with argon gas for 15 min prior to irradiation leads to slower consumption of both the lipid and the dye (Figure VII-5, A and B, respectively), implicating molecular oxygen as a reactant in the system. The slow residual bleaching may be due to trace oxygen left in the sample.
Figure VII-4. Plots of lipid (A) and dye (B) consumption at 37 °C with and without visible-light irradiation of DenPC/DilC18(3) (15:1) vesicles.

Figure VII-5. Plots of photoinduced lipid (A) and dye (B) consumption at 37 °C with and without argon purging of DenPC/DilC18(3) (15:1) vesicles.
Vesicles composed of Bis-C8-SorbPC/DiIC18(3) (21.6:1) are also sensitive to visible light. Irradiation leads to depletion of the lipid and the dye with the former reacting faster (Figure VII-6). The spectral changes occurring in the UV region in Figure VII-6 are similar to those which occur upon irradiation of pure Bis-C8-SorbPC vesicles with UV light, a reaction which has been identified as polymerization of the lipids within the vesicle by linkage of the sorbyl moieties in the tails of adjacent lipids.\textsuperscript{62}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure_vii_6.png}
\caption{Effect of visible-light irradiation at 37 °C on absorption spectrum of Bis-C8-SorbPC/DiIC18(3) (21.6:1) vesicles. (A)-(G) correspond to t=0 to t=60 min in 10 min increments.}
\end{figure}
Irradiation of Mono-C10-SorbPC/DiIC18(3) (12.9:1) vesicles leads to greater than 90% consumption of lipid in 60 min (Figure VII-7). After irradiation, the vesicle suspension was poured into excess methanol which was then evaporated. The resulting thin, red film was mostly soluble in chloroform and methanol although some material was insoluble. Thin-layer chromatography of the soluble fraction is illustrated in Figure VII-8. Spots are evident for unreacted dye and lipid as well as a streak extending from the unreacted lipid down to an immobile spot at the origin.

![Absorption Spectrum](image)

**Figure VII-7.** Effect of visible-light irradiation at 37 °C on absorption spectrum of Mono-C10-SorbPC/DiIC18(3) (12.9:1) vesicles. (A) t=0, (B) 60 min irradiation.
Figure VII-8. TLC of polymerized sample of Mono-C10-SorbPC/DiIC18(3) (12.9:1) along with unpolymerized lipid and dye controls.

$R_f = 0.88$

$R_f = 0.36$

$^1$H-NMR of the soluble material indicates loss of the vinyl resonances of the sorbyl moiety in the lipid tail (Figure VII-9). The remaining resonances match those observed for a sample of Mono-C8-SorbPC which was polymerized by the thermal initiator AIBN, the lone exception being the broad resonance at $\delta_{2.4-2.7}$ which is likely due to the octadecyl tails of the cyanine dye.$^{62,184}$ On the basis of the TLC and $^1$H-NMR evidence, the bleaching of the UV absorption band for the polymerizable lipids in Figures VII-2, 6 and 7 is attributed to a photoinduced polymerization of the lipid bilayer.
Figure VII-9. $^1$H-NMR spectrum of polymerized sample of Mono-C10-SorbPC/DilC18(3) (12:9:1) (in CDCl$_3$).
The rates at which the lipid and the dye are consumed depend on the ratio of the two components in the vesicle. Figure VII-10 shows that the half-life for bleaching of both components in Bis-C8-SorbPC/DilC18(3) vesicles decreases with decreasing dye content in the membranes. Consumption of the dye is more sensitive to the lipid/dye ratio than is lipid consumption.

Figure VII-10. Plot of half-life for photoinduced bleaching versus lipid/dye ratio in Bis-C8-SorbPC/DilC18(3) vesicles at 37 °C.
The lipid and dye bleaching rates in Bis-C8-SorbPC/DilC18(3) (9.2:1) vesicles show an interesting dependence on vesicle concentration (Figures VII-11 and 12). The dye bleaching is more sensitive to vesicle concentration, with its reaction rate decreasing with increasing concentration.

**Figure VII-11.** Plot of photoinduced lipid consumption versus irradiation time at 37 °C for Bis-C8-SorbPC/DilC18(3) (9.2:1) vesicles. (A) [Dye] = 0.71 µM, [vesicles] = 0.15 nM; (B) [Dye] = 1.42 µM, [vesicles] = 0.31 nM; (C) [Dye] = 2.83 µM, [vesicles] = 0.61 nM; (D) [Dye] = 4.25 µM, [vesicles] = 0.92 nM.
Figure VII-12. Plot of cyanine photobleaching versus irradiation time at 37 °C for Bis-C8-SorbPC/DiiC18(3) (9.2:1) vesicles. The legend is the same as in Figure VII-11.
The quantity \( E(t) \) defined below is a measure of the polymerization efficiency with regard to the dye. When \( E(t) \) is greater than one, more lipid has been consumed than dye and the polymerization can proceed to high conversion.

\[
E(t) = \frac{\% \text{ Dye Remaining (at time}=t)}{\% \text{ Lipid Remaining (at time}=t)}
\]

Plotting \( E(t) \) as a function of time for the four concentrations of vesicles studied reveals that at low concentrations, the dye bleaches faster than the lipid whereas the relationship is inverted at higher vesicle concentrations (Figure VII-13). Thus the efficiency of the polymerization increases with increasing vesicle concentration.

![Figure VII-13. Plot of \( E(t) \) versus irradiation time at 37 °C for Bis-C8-SorbPC/DiIC18(3) (9.2:1) vesicles. The legend is the same as in Figure VII-11.](image)
Temperature also has a significant effect on the efficiency $E(t)$ of the photoinduced polymerization of Bis-C8-SorbPC/DiIC18(3) (12.2:1). Although the rate at which the lipid reacts does not appear to depend on temperature (Figure VII-14), dye consumption accelerates noticeably with increasing temperature (Figure VII-15). This leads to a progressive decrease in the efficiency of the polymerization as the temperature increases (Figure VII-16).

**Figure VII-14.** Plot of lipid consumption as a function of temperature for irradiation of Bis-C8-SorbPC/DiIC18(3) (12.2:1) vesicles. [Dye] = 0.89 $\mu$M, [vesicles] = 0.26 nM.
Figure VII-15. Plot of dye consumption as a function of temperature for irradiation of Bis-C8-SorbPC/DiIC18(3) (12.2:1) vesicles. [Dye] = 0.89 μM, [vesicles] = 0.26 nM.

Figure VII-16. Plot of E(t) as a function of temperature for irradiation of Bis-C8-SorbPC/DiIC18(3) (12.2:1) vesicles. [Dye] = 0.89 μM, [vesicles] = 0.26 nM.
Other amphiphilic cyanine dyes were utilized in an attempt to extend the sensitivity of the system to other wavelengths of visible light. Extending the methine bridge of DiIC18(3) by two carbons yields the commercially available DiIC18(5) which absorbs at 649 nm. In Bis-C8-SorbPC/DiIC18(5) (13.8:1) vesicles, the lipid was found to bleach slower than the dye (Figure VII-17) with the half-lives being $t_{1/2} > 30$ min for the lipid and $t_{1/2} = 23$ min for the dye. For DiIC18(3) under similar conditions, Figure VII-9 indicates that the half-lives for lipid and dye consumption are 14 and 37 min, respectively. Other experiments involving this dye are presented in Section VII. C. 2.

![Figure VII-17](image)

**Figure VII-17.** Plot of loss of lipid and dye as a function of irradiation time at 37 °C for Bis-C8-SorbPC/DiIC18(5) (13.8:1) vesicles. [Dye] = 2.0 μM, [vesicles] = 0.65 nM.
Experiments with DiOC18(3) ($\lambda_{\text{max}} = 490$ nm) show a similar diminuion in polymerization efficiency relative to DilC18(3). Blue light irradiation of Bis-C8-SorbPC/DiOC18(3) (16.2:1) vesicles resulted in faster bleaching of the dye than the lipid at all times (Figure VII-18).

![Graph showing loss of lipid and dye as a function of irradiation time](image)

**Figure VII-18.** Plot of loss of lipid and dye as a function of irradiation time at 37 °C for Bis-C8-SorbPC/DiOC18(3) (16.2:1) vesicles. [Dye] = 1.77 μM, [vesicles] = 0.67 nM.
Increasing the vesicle concentration by a factor of 2.2 led to a slightly slower consumption of dye (Figure VII-19) but also to a significantly retarded polymerization (Figure VII-20). Overall, polymerization of Bis-C8-SorbPC/DiOC18(3) vesicles is less efficient at the higher vesicle concentration (Figure VII-21), contrary to the observation for Bis-C8-SorbPC/DiIC18(3) vesicles.

Figure VII-19. Plot of dye consumption versus irradiation time at 37 °C for Bis-C8-SorbPC/DiOC18(3) (16.2:1) vesicles. (A) [Dye] = 1.77 µM, [vesicles] = 0.67 nM (B) [Dye] = 3.86 µM, [vesicles] = 1.46 nM.
Figure VII-20. Plot of lipid consumption versus irradiation time at 37 °C for Bis-C8-SorbPC/DiOC18(3) (16.2:1) vesicles. Dye and vesicle concentrations are the same as in Figure VII-19.

Figure VII-21. Plot of E(t) versus irradiation time at 37 °C for Bis-C8-SorbPC/DiOC18(3) (16.2:1) vesicles. Dye and vesicle concentrations are the same as in Figure VII-19.
VII. C. 2. Polymerizable Vesicles with Dye in the Outer Leaflet

Addition of an amphiphilic cyanine dye such as DiIC18(3) to polymerizable vesicles should lead to its insertion into the outer leaflet of the closed lipid bilayer. Moreover, the dye should remain localized since experiments using vesicles composed of the naturally occurring egg-PC have shown that cyanine dyes inserted into membranes in this manner do not "flip-flop" into the inner leaflet. Such an approach would afford greater control over the ratio of lipid-to-dye than the method in which dye and lipid are extruded together.

DiIC18(3) was added to pure polymerizable Bis-C8-SorbPC vesicles at a ratio of 19 lipids per dye molecule. (Although this gives a total content of 5 mol % dye, the effective content is approximately 10 mol % in the outer leaflet, assuming the dye does not flip to the inner leaflet.) The vesicles were then irradiated with yellow light. As observed for the system featuring DiIC18(3) in both leaflets, bleaching of both the lipid and the dye occurs with the lipid reacting faster (Figure VII-22).
Figure VII-22. Plot of loss of lipid and dye as a function of irradiation time at 37 °C for Bis-C8-SorbPC vesicles with 5 mol % DilC18(3) added. [Vesicles] = 1.0 nM, [Dye] = 2.30 µM.
Similar experiments were attempted with DenPC. The absorption spectrum of the dye (10 mol % total) inserted into the vesicles was considerably broadened and diminished in intensity relative to that measured in Bis-C8-SorbPC vesicles (Figure VII-23). Irradiation led to greater than 80% loss of dye in 30 min but virtually no polymerization. This is drastically different from the configuration where the dye was present in both leaflets. In that case, the dye was not aggregated and efficient photoinduced polymerization occurred (Figure VII-2).

Figure VII-23. Absorption spectra for DilC18(3) added to (A) Bis-C8-SorbPC and (B) DenPC vesicles.
DilC18(3) inserted into BAPC vesicles monomerically at 5 mol % dye. The sample was irradiated under conditions identical to those used for Bis-C8-SorbPC (Figure VII-22). Although the dye bleached at similar rates in the two vesicles (Figure VII-24A), only the Bis-C8-SorbPC polymerized (Figure VII-24B).

Figure VII-24. Photoinduced consumption of lipid and dye at 37 °C for BAPC and Bis-C8-SorbPC vesicles with 5 mole % DilC18(3) added to the outer leaflet. [Vesicles] = 0.44 nM, [Dye] = 2.31 μM in each case. (A) DilC18(3) photobleaching; (B) lipid consumption.
Experiments were also performed in which DilC18(5) was inserted into the outer leaflet of Bis-C8-SorbPC vesicles. Although the dye bleaching followed similar kinetics as found for DilC18(3) (Figure VII-25A), lipid consumption was found to be significantly retarded (Figure VII-25B).

Figure VII-25. Photoinduced consumption of lipid and dye at 37 °C for Bis-C8-SorbPC vesicles with either DilC18(5) or DilC18(3) added to the outer leaflet. [Vesicles] = 0.78 nM in each case. [DilC18(3)] = 4.77 μM, 13 mol %, while [DilC18(5)] = 3.46 μM, 10 mol %. This gave the same total optical density for each sample. (A) Dye photobleaching; (B) lipid consumption.
VII. C. 3. Comparison of Membrane-Bound vs Water-Soluble Sensitizers

As shown in the previous section, addition of DilC18(3) to Bis-C8-SorbPC vesicles and subsequent visible-light irradiation leads to efficient polymerization of the vesicle. If the water-soluble analog DilC1(3) is used instead, both lipid and dye consumption are dramatically retarded (Figures VII-26 and 27). Although the lipid/dye ratio is the same for both samples, the effective concentration of DilC18(3) is much greater since it is restricted to the volume of the vesicles' outer leaflets while DilC1(3) is free to diffuse throughout the bulk aqueous medium. The higher effective concentration and closer proximity to the polymerizable lipid lead to the efficient polymerization triggered by the membrane-bound dye.

![Graph](image)

**Figure VII-26.** Plot of photoinduced consumption at 37 °C of Bis-C8-SorbPC with 5 mol % added DilC18(3) or DilC1(3).
Figure VII-27. Plot of photobleaching at 37 °C of 5 mol % DilC18(3) or DilC1(3) added to Bis-C8-SorbPC vesicles.
VII. C. 3. Effect of Hydrogen Peroxide on Sensitized Polymerization

The results shown in Figures VII-5A and 5B indicate that molecular oxygen is required in order to initiate polymerization. It is likely that oxygen reacts with the excited state of the cyanine dye to produce a reactive oxo species, either singlet oxygen by triplet energy transfer or superoxide anion ($O_2^-$) by single electron transfer. Generation of singlet oxygen, although documented in some dye-sensitized photopolymerizations \cite{219,220}, is unlikely in this case since simple carbocyanines typically undergo intersystem crossing with very low efficiencies ($< 1\%$).\cite{121} Without formation of the triplet state of the dye, singlet oxygen cannot be produced in appreciable amounts.

Photoinduced electron transfer from the cyanine to oxygen yields the oxidized dye and superoxide anion; the latter is an intermediate in the production of hydrogen peroxide:\cite{221}

$$\text{O}_2^- + 2\text{H}^+ + \text{e}^- \longrightarrow \text{H}_2\text{O}_2$$

(The second electron could be supplied by a second excited cyanine dye.) Hydrogen peroxide is a well-known redox initiator of radical polymerizations.\cite{68} One electron reduction of hydrogen peroxide results in cleavage of the oxygen-oxygen bond, giving hydroxide anion and hydroxy radical:

$$\text{H}_2\text{O}_2 + \text{e}^- \longrightarrow \text{HO}^- + \text{HO}^+$$

This electron could again be supplied by an excited cyanine dye. The hydroxy radical would then be the initiating species.
Such a mechanism suggests that in the absence of oxygen, polymerization can still proceed if hydrogen peroxide is present. To test this prediction, Bis-C8-SorbPC vesicles were prepared with 5 mol % DilC1(3) added to the outer leaflet. Irradiation of the vesicles results in approximately 90 % loss of monomer in 30 min (Figure VII-28, A). Purging the sample with commercial argon significantly reduces the rate of polymerization, with less than 30 % conversion attained in 30 min (B). (The lack of complete inhibition is probably due to trace oxygen remaining in the sample.) However irradiation of a purged sample to which hydrogen peroxide was added results in a partial recovery of the polymerization, with 65 % conversion occurring in 30 min (C).

Figure VII-28. Plot of photoinduced consumption at 37 °C of Bis-C8-SorbPC (16.7 μM) with 5 mol % added DilC18(3): (A) ambient conditions, (B) argon-purged for 15 min, (C) H₂O₂ (23 mM), argon-purged for 15 min.
An alternative possibility is that the hydrogen peroxide generated by photoinduced electron transfer simply decomposes on its own via oxygen–oxygen bond homolysis, generating two hydroxy radicals:

\[ \text{H}_2\text{O}_2 \rightarrow 2 \text{HO}^- \]

This possibility was investigated by adding commercially available aqueous hydrogen peroxide to a suspension of Bis-C8-SorbPC vesicles. As shown in Figure VII-29, the lipid is consumed without any cyanine dye.

![Figure VII-29. Effect of H\textsubscript{2}O\textsubscript{2} (55 mM) at 37 °C on absorption spectrum of Bis-C8-SorbPC vesicles. [Lipid] = 21.7 μM. H\textsubscript{2}O\textsubscript{2} added to both the sample and reference cuvettes in order to subtract its absorbance in the UV. Spectra taken every ten min.](image)
The consumption of monomer exhibits a significant temperature dependence (Figure VII-30). Moreover, addition of the hydroxyl radical scavenger D-mannitol effectively inhibits lipid consumption (Figure VII-31).

**Figure VII-30.** Effect of temperature on the H$_2$O$_2$-induced consumption of Bis-C8-SorbPC. [Lipid] = 21.7 μM, [H$_2$O$_2$] = 55 mM.

**Figure VII-31.** Effect of D-mannitol (50 mM) on the H$_2$O$_2$-induced consumption at 37 °C of Bis-C8-SorbPC. [Lipid] = 21.7 μM, [H$_2$O$_2$] = 55 mM.
Significantly, H$_2$O$_2$ also bleaches the absorption band of Bis-C8-SorbPC-bound DilC18(3) in the dark (Figure VII-32). Simultaneously, consumption of the lipid is inhibited (Figure VII-33).

**Figure VII-32.** H$_2$O$_2$-induced bleaching at 37 °C of Bis-C8-SorbPC-bound DilC18(3). [Lipid] = 21.7 μM, [DilC18(3)] = 2.15 μM, [H$_2$O$_2$] = 55 mM.

**Figure VII-33.** Effect of DilC18(3) on the H$_2$O$_2$-induced bleaching at 37 °C of Bis-C8-SorbPC. [Lipid] = 21.7 μM, [DilC18(3)] = 2.15 μM, [H$_2$O$_2$] = 55 mM.
Finally, while D-mannitol effectively inhibits the consumption of Bis-C8-SorbPC, it has virtually no effect on the bleaching of DilC18(3) (Figure VII-34).

Figure VII-34. Effect of D-mannitol (50 mM) on the $\text{H}_2\text{O}_2$-induced bleaching at $37 \, ^\circ\text{C}$ of membrane-bound DilC18(3). [Lipid] = 21.7 $\mu$M, [Dye] = 2.15 $\mu$M $[\text{H}_2\text{O}_2]$ = 55 mM.
VII. D. DISCUSSION

VII. D. 1. Evidence for Polymer Formation

The results presented in Section VII-C indicate that irradiation of membrane-bound cyanine dyes leads to vesicle polymerization for the lipids DenPC, Bis-C8-SorbPC, and Mono-C10-SorbPC. The irreversible bleaching of the UV absorption band of the lipid provides clear evidence of disruption of the conjugation in the polymerizable groups. It has been shown that similar spectral results (i.e. decreased optical density at the maximum but increased optical density at shorter wavelengths) for direct irradiation of the SorbPC lipids corresponds to polymerization of the lipid tails. Moreover, the faster rate of reaction of the lipid compared to the dye in many of the figures is suggestive of the monomer being consumed by a chain process.

The experiment performed with Mono-C10-SorbPC (Figures VII-7-9) provided further evidence of polymerization. After irradiation and removal of water, some of the material was insoluble in chloroform. Although the proposed polymers would be linear rather than cross-linked with this monomer, the amphiphilic nature of lipid polymers often renders even linear polymers insoluble in most organic solvents. (Since the vesicles were prepared in pure water, the insoluble material cannot be buffer salts. However it may be the dye photoproduct, which was not observed by TLC.) TLC of the soluble material showed spots for unreacted dye and lipid as well as a streak extending from just below the unreacted lipid down to the origin. This is characteristic of polymer samples with the longer polymer chains exhibiting the lower Rf values.
The $^1$H-NMR spectrum of the soluble material is also consistent with polymer formation. The monomer has characteristic resonances at $\delta 1.85$ (doublet) for the terminal methyl on the polymerizable chain and at $\delta 5.72-5.80$ (doublet) for the vinyl proton adjacent to the carbonyl. In Figure VII-9, the latter of these resonances has disappeared completely while the former is barely discernible. A broad resonance has appeared at $\delta 5.2$ which is likely due to vinyl protons in the polymer. Overall the spectrum is very similar to that obtained from a sample of the same lipid which was polymerized thermally using AIBN. These results indicate that the visible-light irradiation of the membrane-associated cyanine dye triggers polymerization of the lipids in the bilayer.

VII. D. 2. Mechanism of Initiation

The initiating system for these polymerizations appears to be quite simple. Of the cyanine dyes investigated, the dimethylindocarbocyanine DilC18(3) was clearly the most effective based on its slower rate of consumption as compared to the lipid. Moreover, polymerization of Bis-C8-SorbPC was efficient even if the dye were added only to the outer leaflet. This is most likely due to the fact that since the polymerizable groups are at the tail termini, they are at roughly the same depth in the membrane whether the lipid is in the outer or inner leaflet. The initiating species which is presumably generated at the vesicle surface has equal access to the polymerizable groups of both leaflets, regardless of the surface at which it is generated. Addition of the dye to preformed vesicles is beneficial for two reasons: 1) If the dye is to be incorporated into both leaflets, the freeze-thaw and extrusion procedures must
be done in the dark. Adding the dye only to the outer leaflet permits vesicle preparation to be done under yellow lights. 2) A considerable amount of the dye (as much as 25%) adsorbs to the filters during extrusion. This results in a different and unpredictable lipid/dye ratio for each experiment. Preparing the vesicles before addition of dye allows the lipid/dye ratio to be controlled and varied within a single vesicle preparation as well as in separate preparations. The results in Figure VII-22, where the dye was in the outer leaflet, can be compared with those of Figures VII-11 and 12, where the dye was presumably in both leaflets. The data in Figure VII-22 was acquired for [Dye] = 2.30 μM, the closest data sets in Figures VII-11 and 12 had 1.42 and 2.83 μM dye. Interpolating between those data sets, one would expect half-lives of 3.2 and 4.6 min for lipid and dye consumption, respectively. The results in Figure VII-22 give 4 and 6 min for these same quantities. Thus, the reaction appears to be slowed only slightly by having the dye present in just one leaflet, again assuming it does not flip to the inner leaflet. In the case where the dye was in both leaflets, 10 mole % of the dye was present. In the other case, only 5 mole % was added. However, the effective concentration would be 10 mole % if the dye were restricted to the outer leaflet. This could account for the similarity in the kinetics for the two experiments.

Unfortunately, this approach could not be extended to DenPC since the dye aggregated in that case (Figure VII-20). Two possible explanations are given for the failure of the aggregated dye to initiate polymerization: 1) Alternative photochemical pathways might be open to the aggregate leading to rapid bleaching of the dye but no reaction of the lipid. 2) The same chemistry outlined in Scheme VII-1 is initiated by irradiation but the initiating species are
merely consumed by the dye which is present at higher effective concentration than for monomeric dye. The data shown in Figure VII-34 support the latter hypothesis as the cyanine dye actually inhibits the hydrogen peroxide-initiated consumption of Bis-C8-SorbPC. The observation that DilC18(3) is monomeric when bound to both leaflets of DenPC but aggregated when added to the outer leaflet is intriguing but inexplicable at this point. Interestingly, completely removing the dye from the bilayer by using the water-soluble analog DilC1(3) drastically retarded the polymerization. Apparently, a high effective concentration of the dye is required for polymerization, but not so high that the dye aggregates.

Figures VII-4 and 5 establish the need for visible-light irradiation and molecular oxygen in the system. Oxygen can interact with photoexcited dyes either by energy transfer to form singlet oxygen or by electron transfer to form the superoxide radical anion. Quenching by energy transfer to produce singlet oxygen seems unlikely since the simple cyanines generally have low intersystem crossing yields and this process would regenerate ground state dye, a process which would not lead to its photobleaching. Although bleaching may result from attack by singlet oxygen on ground state dye molecules, the temperature dependence data shown in Figure VII-15 do not support this. At lower temperature, intersystem crossing should be enhanced since nonradiative decay will be less efficient. (The cyanines decay primarily by twisting about the methine bridge.) This should lead to greater singlet oxygen production and thus more extensive photobleaching of the dye, but the opposite relation is found.
It is more likely that oxygen is an electron acceptor in the system. Photoinduced electron transfer from a cyanine to oxygen is the entry point to the following sequence:

**Scheme VII-2**

\[
\begin{align*}
{^\cdot}Cy^+ + O_2 & \rightarrow Cy^{2+} + O_2^- \quad (1) \\
O_2^- + H^+ & \rightarrow HO_2^- \quad (2) \\
{^\cdot}Cy^+ + HO_2^- & \rightarrow Cy^{2+} + HO_2^- \quad (3) \\
HO_2^- + H^+ & \rightarrow H_2O_2 \quad (4) \\
{^\cdot}Cy^+ + H_2O_2 & \rightarrow Cy^{2+} + H_2O_2^- \quad (5) \\
H_2O_2^- & \rightarrow HO^- + HO^- \quad (6)
\end{align*}
\]

This reaction sequence (often referred to as Fenton chemistry\textsuperscript{222}) is commonly encountered in biochemical processes such as lipid oxidation\textsuperscript{183} and DNA cleavage.\textsuperscript{223} The principal variable among the different systems is the electron donor.

The hydroperoxy and hydroxy radicals are emphasized as potential initiating species. Initiation of polymerization by the hydroperoxy radical may occur, but it is believed to be a minor pathway at best, since a high effective concentration of the dye is required for polymerization to occur. The hydroperoxy radical could be formed in the aqueous phase using DilC\textsubscript{1}(3) and would certainly be free to diffuse into vesicles and initiate polymerizations. The
fact that polymerization does not occur in such an experiment suggests that at least two electron transfers are required for initiation (steps 1-4).

The dependence of polymerization and dye photobleaching rates on vesicle concentration (Figures VII-11 and 12, respectively) provide evidence that while the initiating species is likely produced at the surface of one vesicle, it is free to diffuse to other vesicles. As the vesicle concentration increases, the rate of dye photobleaching decreases, presumably due to inner filtering within the (unstirred) sample. If the initiating species were restricted to the vesicle where it was formed, the polymerization should concomitantly decrease. However, the opposite result is found: higher vesicle concentrations favor faster polymerization rates, particularly at longer reaction times. Thus while light may not be able to penetrate throughout the cuvette, the initiating species can. A possible explanation for these effects is that higher vesicle concentrations push more oxygen through the sequence (1)-(4). While hydroperoxy radical may not be the initiating species, it is probably a key intermediate. It is a very reactive molecule and as such is the most likely candidate to leave the sequence by side reaction. As a diffusible species, the likelihood that it will react with another excited cyanine increases with both the number of cyanines per vesicle and with the total number of vesicles. This keeps the sequence going, ultimately resulting in more efficient polymerization. In fact both of these results are observed: Figure VII-10 for the number of cyanines per vesicle and Figure VII-11 for the total number of vesicles.

The observation that while the polymerization is significantly retarded under anaerobic conditions, addition of hydrogen peroxide and irradiation in the absence of oxygen enhances the polymerization (Figure VII-28) indicates that
step 5 is important in the photosensitized polymerization. However, the results given in Figures VII-29-31 suggest that simply generating hydrogen peroxide is sufficient as the spontaneous decomposition of $H_2O_2$ to hydroxy radicals should lead to polymerization. Overall, the evidence indicates that polymerization results from photoinduced generation of hydrogen peroxide at the vesicle surface. The peroxide then decomposes to give hydroxy radicals, which initiate polymerization of the bilayer.

VII. D. 3. Mechanism of Dye Photobleaching

While the dye photoproducts have been neither isolated nor identified, some evidence is available regarding the bleaching chemistry. 1) Only the membrane-bound cyanine dyes bleach; the water-soluble DilC1(3) was very stable to irradiation. 2) As the number of cyanines per vesicle increases, the rate of photobleaching increases. 3) DiOC18(3) and DilC18(5) are consumed faster than the polymerizable lipid upon irradiation. 4) Addition of hydrogen peroxide to vesicle-bound DiIC18(3) causes bleaching of the dye without irradiation.

The first two observations demonstrate that bleaching is concentration dependent. It is tempting to ascribe this reaction to a bimolecular process. In studying the fate of electrochemically oxidized cyanine dyes, Parton and Lenhard found that cyanine radical dications often dimerize, giving irreversible bleaching.154 The reaction results in coupling at odd-numbered positions along the methine bridge, as the odd electron density resides primarily at those positions.224 However, this reaction was important only for dicarbocyanine and sterically unencumbered carbocyanines. Furthermore, those experiments
involved the \(N,N'\text{-diethyl}\)substituted cyanines as opposed to the dioctadecyl analogs used in the vesicles. It is difficult to imagine covalent bond formation between the methine bridges of two oxidized DilC18(3) molecules in solution, let alone in the geometrically restricted environment of a lipid bilayer.

Another possibility is that the hydroxy radicals produced in (1)-(6) bleach the membrane-bound cyanine dyes in addition to initiating polymerization. The polymethine bridge would be the most susceptible region to attack. As the number of dye molecules per vesicle increases, the number of radicals produced increases, leading to enhancement of both polymerization and photobleaching. Importantly, the hydroxy radicals would most likely be capable of attacking not only oxidized cyanines but also ground-state, even-electron cyanines. Key evidence for this hypothesis comes from the experiments in which hydrogen peroxide was found to bleach the membrane-bound dye in the dark (Figure VII-32). Interestingly, D-mannitol effectively inhibited lipid consumption but not dye consumption. A potential explanation for these apparently conflicting observations is given in Scheme VII-3.

Attack of the hydroxy radical on a dye (D) or on D-mannitol inhibits polymerization, as observed in Figures VII-31 and 33. However, the most likely mode of attack on the sugar is via hydrogen abstraction. The resulting radical should be sufficiently hydrophilic to remain in the aqueous phase. Thus polymerization is inhibited. However, this radical should retain access to the dye chromophore at the vesicle surface, permitting bleaching to proceed. Further experiments are required to verify this.
The attack by hydroxy radicals on dye molecules could also explain why
the lipid is consumed faster than the dye for DilC18(3) but slower for DiOC18(3)
and DilC18(5). The latter two have lower oxidation potentials than the former
($E_{ox} = 1.088$ V, 1.020 V and 0.775 V for the $N, N'$-diethyl analogs of DilC18(3),
DiOC18(3) and DilC18(5), respectively),$^{154,165}$ meaning more radicals would be
produced upon irradiation. In addition, the polymethine bridges of these two
dyes are more exposed to attack than in DilC18(3) where the gem dimethyl
groups afford steric protection to the bridge. This feature is not present for
DiOC18(3). In DilC18(5) not only are the gem dimethyl groups further apart but there are more potential sites of attack for the radical along the bridge. Thus the oxacarbocyanine and the indodicarbocyanine should produce more hydroxy radicals and be more susceptible to attack of those radicals leading to faster bleaching and less efficient initiation of polymerization.

VII. D. 4. Factors Controlling the Efficiency of Initiation

Table VII-3 summarizes the results presented in Section VII. C. Entries 1-4 correspond to the data utilized to construct Figure VII-10. There is a clear dependence for reaction of both the lipid and the dye on the lipid-to-dye ratio ([L]/[D]). Difficulty arises in attempting to draw further comparisons from experiments involving dye in both leaflets due to the limited control over the [L]/[D] resulting from loss of the dye during extrusion. However, for experiments where the [L]/[D] was held constant, some conclusions can be drawn. 1) Increased vesicle concentration leads to slower dye bleaching for both DilC18(3) (entries 5-8) and DiOC18(3) (entries 12-13) in Bis-C8-SorbPC vesicles, presumably because of inner filtering. 2) Entries 10 and 11 compare DilC18(3) and DilC18(5) added to the outer leaflet of Bis-C8-SorbPC vesicles. The concentrations of the two dyes are different but the total optical densities are equivalent for the two samples. The dyes bleach at comparable rates but polymerization initiated by DilC18(3) proceeds approximately 10 times faster than that triggered by DilC18(5). Moreover, the dye bleaches considerably faster than the lipid for the dicarbocyanine. Qualitatively, similar behavior is found for DiOC18(3) (entries 12 and 13): the dye bleaches faster than the lipid. In order to quantitatively compare this dye with DilC18(3), the experiment
should be done by adding DiOC18(3) to the outer leaflet such that its total optical density matches that of DiIC18(3), as was done for DiIC18(5).

The lack of polymerization observed for BAPC under conditions where Bis-C8-SorbPC is efficiently polymerized (entries 17 and 18, Figure VII-24B) could be due to the effect of oxygen on the growing polymer chains. Oxygen is known to strongly inhibit polymerization of acrylate monomers in solution by rapidly reacting with the propagating polymer chain terminus. The propagating SorbPC radical should be more stable than the acryloyl radical due to more extensive resonance stabilization. Thus, oxygen should have a more deleterious effect on the latter. These observations are a manifestation of oxygen's dual capabilities in the system as coinitiator and inhibitor.
Table VII-3. Half-lives for consumption of lipid and dye in photosensitized polymerizations.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Lipid (L)</th>
<th>Dye (D)</th>
<th>[L]/[D]</th>
<th>[D] (μM)</th>
<th>t$_{1/2}$ (L, min)</th>
<th>t$_{1/2}$ (D, min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bis-C8-SorbPC</td>
<td>DilC18(3)</td>
<td>21.6</td>
<td>2.5</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>&quot;</td>
<td>16.6</td>
<td>2.5</td>
<td>16</td>
<td>46</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>&quot;</td>
<td>11.8</td>
<td>2.5</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>&quot;</td>
<td>7.0</td>
<td>2.5</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>&quot;</td>
<td>9.2</td>
<td>0.7</td>
<td>&gt;5</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>&quot;</td>
<td>9.2</td>
<td>1.4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>&quot;</td>
<td>9.2</td>
<td>2.8</td>
<td>3</td>
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<td>8</td>
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<td>&quot;</td>
<td>9.2</td>
<td>4.2</td>
<td>3</td>
<td>&gt;5</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
<td>DilC18(5)</td>
<td>13.8</td>
<td>2.0</td>
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<td>23</td>
</tr>
<tr>
<td>10$^a$</td>
<td>&quot;</td>
<td>&quot;</td>
<td>10.0</td>
<td>3.1</td>
<td>&gt;30</td>
<td>20</td>
</tr>
<tr>
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<td>&quot;</td>
<td>DilC18(3)</td>
<td>10.0</td>
<td>4.4</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>12$^a$</td>
<td>&quot;</td>
<td>DiOC18(3)</td>
<td>16.2</td>
<td>1.8</td>
<td>26</td>
<td>20</td>
</tr>
<tr>
<td>13$^a$</td>
<td>Mono-C10-SorbPC</td>
<td>&quot;</td>
<td>16.2</td>
<td>3.6</td>
<td>&gt;30</td>
<td>30</td>
</tr>
<tr>
<td>14$^a$</td>
<td>&quot;</td>
<td>DilC18(3)</td>
<td>16.6</td>
<td>5.6</td>
<td>14</td>
<td>&gt;30</td>
</tr>
<tr>
<td>15$^a$</td>
<td>&quot;</td>
<td>&quot;</td>
<td>16.6</td>
<td>2.7</td>
<td>9</td>
<td>&gt;30</td>
</tr>
<tr>
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<td>DenPC</td>
<td>&quot;</td>
<td>15.0</td>
<td>3.2</td>
<td>43</td>
<td>&gt;60</td>
</tr>
<tr>
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<td>2.3</td>
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<td>6</td>
</tr>
<tr>
<td>18$^a$</td>
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<td>&quot;</td>
<td>20.0</td>
<td>2.3</td>
<td>&gt;30</td>
<td>9</td>
</tr>
</tbody>
</table>

$^a$ Dye added to the outer leaflet only.
This chapter describes an initiator for vesicle polymerizations which is sensitive to yellow light. Extension of the sensitivity of the initiating system to red light seems to require two properties of the sensitizer: 1) the sensitizer should be able to reduce oxygen but 2) should not be readily attacked by hydroxy radicals. This is believed to be the problem with DilC18(5). Based on its oxidation potential, the dye should be readily oxidized by molecular oxygen. Its failure to efficiently sensitize the polymerization is probably due to the relatively easy access that hydroxy radicals would have to the exposed pentamethine bridge.

Because of the potential drug delivery applications, it was important to develop a system which was operative under physiological conditions: 37 °C, pH = 7.4 and ambient oxygen conditions. The final requirement presents the greatest challenge to a strategy based on polymerization chemistry since oxygen is an effective inhibitor of radical polymerizations. Interestingly, the system described above requires oxygen in order to initiate polymerization. It should be pointed out that since the polymerization is likely a chain reaction occurring in the presence of oxygen the polymers formed in this system are probably short. It remains to be seen whether or not the polymers will be long enough to induce phase separation in mixed DOPE/SorbPC vesicles, triggering membrane fusion events.
CHAPTER VIII

USE OF BORATE COINITIATORS FOR PHOTOSENSITIZED VESICLE POLYMERIZATIONS

VIII. A. INTRODUCTION

In addition to the simple cyanine dye-sensitized polymerizations described in Chapter VII, experiments were undertaken to investigate the potential utility of hydrophobic borate anions (such as Ph₃BnB⁻) as coinitiators for vesicle polymerizations. In fact, cyanine dye-triphenyl-n-butylborate ion pairs have been reported to initiate bulk polymerization of butyl acrylate upon visible light irradiation.¹⁸⁸,¹⁸⁹,¹¹⁸ That system relied on the instability of the oxidized borate to produce free radicals (Scheme VIII-1). The butyl radicals then initiate polymerization of the acrylate monomer.

Scheme VIII-1

```
\[
\begin{align*}
\text{Cy}^+ + \text{Ph}_3\text{BuB}^- & \quad k_p \\
\rightarrow & \quad \text{Cy}^- + \text{Ph}_3\text{BuB}^+ \\
& \downarrow \text{k} \\
\text{Cy}^+ + \text{Ph}_3\text{BuB}^- & \quad k_{nr} \\
& \downarrow \text{hv} \\
& \quad \text{Cy}^- + \text{Ph}_3\text{BuB}^+ \\
& \quad \downarrow \text{k}_{\text{bet}} \\
& \quad \text{Cy}^- + \text{Ph}_3\text{B}^- + \text{Bu}^- \\
& \quad \downarrow \text{k}_c \\
& \quad \text{Bu} - (\text{M})_{n-1} - \text{M}^+ \\
\end{align*}
\]
```
Extension of this chemistry to lipid bilayers requires simultaneous binding of both a dye and a borate to the membrane. Two strategies were devised for achieving this: 1) An amphiphilic dye was used which anchored itself to the bilayer. The borate was bound to the hydrophobic interior, as in Chapters IV-VI. (Scheme VIII-2) 2) A hydrophilic cationic dye was bound to the membrane surface via either an anionic lipid or an excess of the borate. (Scheme VIII-3)
VIII. B. EXPERIMENTAL

Materials. t-Butyllithium (1.7 M in Pentane) and 1-bromododecane were purchased from the Aldrich Chemical Co. and used as received. Pentane and THF were freshly distilled before use. N, N'-Ditetradecyloxacarbocyanine perchlorate (DiOC14(3)) and acridine orange-C12 (AOC12) were purchased from Molecular Probes, Inc (Eugene, OR) and used as received. Mono-C8-SorbPC was synthesized by H. Lamparski. All other solvents and chemicals were used as described in previous chapters.

Synthesis of Tetramethylammonium Triphenyl-n-dodecylborate (Ph₃C₁₂B⁻). All manipulations were performed under argon atmosphere. A solution of 2.4 mL (4.1 mmoles) t-Butyllithium in 10 mL pentane was added dropwise to a solution of 0.5 mL (2.1 mmoles) 1-Bromododecane in 10 mL THF at -78 °C with stirring. Each drop of the colorless organolithium produced a yellow color in the reaction mixture. Initially this color faded before the next drop was added, but ultimately it persisted. After the addition was complete (15 min), the bright yellow solution was stirred at -78 °C for 1.5 hours. At that point the reaction mixture had become cloudy but was still yellow.

A solution of triphenylboron (0.5 g, 2.1 mmoles) in 10 mL benzene was prepared and cooled to 0 °C. The dodecyllithium solution prepared above was transferred to this solution by canula over a 15-20 min period. The final solution was clear and light yellow. The stir bar left in the original flask had turned dark blue but this color disappeared upon exposure to air. The solvent (pentane, THF, benzene) was removed from the final reaction mixture under inert
atmosphere leaving a light brown solid. The solid was washed with 5x10 mL portions of pentane and dried by blowing argon gas through the flask.

The solid was then dissolved in 10 mL water. A solution of tetramethylammonium bromide (0.5 g, 3.0 mmoles) in 15 mL water was added dropwise, giving a precipitate. The reaction mixture was centrifuged at 10,000 rpm for 20 min and the supernatant poured off. A yellow-brown solid was obtained which upon recrystallization from methanol gave 13.5 mg white crystals (1.4 % yield, relative to t-butyllithium). $^1$H-NMR (DMSO-d$_6$): δ 7.2 (d, 6H), 6.9 (t, 6H), 6.7 (t, 3H), 3.1 (s, 12H), 1.2 (br s, 20H), 0.85 (br, 5H). Full spectrum is given in Appendix A-1.

**Ion Exchange Procedure for Preparation of Cyanine-Borate Salts.**

All manipulations were performed in a darkroom equipped with red safelights. Separate solutions of DiOC14(3) perchlorate (4.0 mg, 5.2 μmoles) and tetramethylammonium triphenyl-n-butylborate (1.9 mg, 5.2 μmoles), each in 2.0 mL acetonitrile, were mixed. The solvent was evaporated by passing a stream of argon gas over the solution. After drying, 10 mL dichloromethane was added. The solution was extracted with 4x10 mL water and 4x10 mL saturated sodium chloride. The organic layer was then dried over anhydrous sodium sulfate and filtered. Evaporation of the solvent gave the dye-borate salt as determined by an increased absorbance in the UV relative to the perchlorate salt as well as photosensitivity (see Section VIII-C).
Vesicle Preparation. The standard freeze-thaw and extrusion procedures described in Chapters IV and VII were used to prepare vesicles. Extrusion was done through 0.1 μm pore size polycarbonate filters. For samples containing extra borate, the desired amount of tetramethylammonium borate was dried from an acetonitrile stock solution along with the lipid and the dye.

Determination of Lipid and Dye Concentrations after Extrusion. The concentrations were determined by UV-vis absorption spectroscopy as described in Chapter VII.

Steady-State Irradiations. Samples were irradiated using the filtered output of a 200W Hg(Xe) lamp. Oxygen was removed by bubbling argon gas through the vesicle suspension for at least 10 min prior to irradiation. Irradiations were performed at room temperature unless otherwise indicated. Calculation of the percent loss of monomer was complicated by the fact that the triphenylalkylborate salts absorb light in the same region as the polymerizable lipids. The percent loss was calculated simply from the loss of optical density at the lipid absorption maximum. This gives an upper limit on the loss of monomer.
VIII. C. RESULTS

VIII. C. 1. Amphiphilic Dye Sensitizers

Bis-C8-SorbPC vesicles were prepared in the presence of 5 mole % of either the \( \text{Ph}_3\text{BuB}^- \) or the \( \text{ClO}_4^- \) salt of DiOC14(3). Visible-light irradiation of the borate-containing vesicles in the absence of oxygen results in approximately 10 \% bleaching of the lipid absorption band and 15-20 \% loss of dye (Figure VIII-1). The perchlorate-containing vesicles exhibited no loss of dye or lipid upon irradiation. The bleaching of the dye with the borate counterion is likely the result of the same PET chemistry presented in Chapters III-VI.

![Figure VIII-1. Effect of visible-light irradiation on the lipid and dye absorption bands for Bis-C8-SorbPC/DiOC14(3)/Ph\textsubscript{3}BuB\textsuperscript{-} vesicles. [L]/[D] = 20, [Dye] = 3.0 \mu\text{M.}](image)

Figure VIII-1. Effect of visible-light irradiation on the lipid and dye absorption bands for Bis-C8-SorbPC/DiOC14(3)/Ph\textsubscript{3}BuB\textsuperscript{-} vesicles. [L]/[D] = 20, [Dye] = 3.0 \mu\text{M.}
In order to obtain greater conversion of monomer, a five-fold excess of Ph₃BuB⁻ (relative to the dye) was included during the preparation of vesicles. Irradiation resulted in 26 % loss of the lipid absorption band in 30 min. However, greater than 90 % bleaching of the dye occurred during this period. Better results were obtained when vesicles were prepared with a large excess of the borate (equimolar relative to lipid). Figure VIII-2 shows the effect of visible-light irradiation on the absorption spectrum of Bis-C8-SorbPC/DiOC14(3)/Ph₃BuB⁻ vesicles prepared in this manner. Approximately 44 % conversion of monomer is obtained in 60 min irradiation. However, the dye bleaches considerably faster than the lipid with greater than 90 % bleaching occurring over the same period. In the presence of oxygen, the dye bleaches at the same rate but lipid consumption is slowed considerably (Figures VIII-3 and 4).

Figure VIII-2. Effect of visible-light irradiation on the absorption spectrum of Bis-C8-SorbPC/DiOC14(3)/Ph₃BuB⁻ (1:0.2:1, before extrusion). [L]/[D] = 16.7; [dye] = 1.8 µM
Figure VIII-3. Effect of oxygen on dye bleaching in Bis-C8-SorbPC/DiOC14(3)/Ph₃BuB⁻ vesicles. [L]/[D] = 16.7, [dye] = 1.9 μM.

Figure VIII-4. Effect of oxygen on lipid bleaching in Bis-C8-SorbPC/DiOC14(3)/Ph₃BuB⁻ vesicles. [L]/[D] = 16.7, [Dye] = 1.9 μM.
A number of experiments were performed at various [L]/[D] values (Table VIII-1). Since the initial optical densities varied considerably, the data are not directly comparable. However, in all cases the dye bleached faster than the lipid, indicating that high conversions will be difficult to obtain in these systems.

**Table VIII-1.** Percent conversion at 5 and 15 min irradiation for Bis-C8-SorbPC/DiOC14(3)/Ph3BuB- vesicles.

<table>
<thead>
<tr>
<th>[L]/[D]</th>
<th>OD&lt;sub&gt;dye&lt;/sub&gt;</th>
<th>% Conversion (5 min)</th>
<th>% Conversion (15 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33.6</td>
<td>0.078</td>
<td>16</td>
<td>25</td>
</tr>
<tr>
<td>18.8</td>
<td>0.293</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>18.0</td>
<td>0.412</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>16.7</td>
<td>0.264</td>
<td>25</td>
<td>33</td>
</tr>
<tr>
<td>12.6</td>
<td>0.412</td>
<td>27</td>
<td>38</td>
</tr>
</tbody>
</table>

Similar experiments were attempted with the single-chain dye AOC12 and the chromophore-containing lipid N-NBD-PE. In both cases, visible-light irradiation led to bleaching of the dyes but less than 50% conversion of monomer in 30 min irradiation (UV-vis spectra are in Appendix A-4).
The dodecyl analog of Ph₃BuB⁻ was synthesized in the hope that a more hydrophobic initiating radical would be more effective at triggering vesicle polymerizations. However, as Figure VIII-5 shows, this system did not improve on the efficiency of the Ph₃BuB⁻ system.

**Figure VIII-5.** Photoinduced consumption of lipid for Bis-C₈-SorbPC/DiOC14(3)/Ph₃RB⁻ vesicles where R = n-butyl or n-dodecyl. [L]/[D] = 35.0 for R = n-butyl and 32.5 for R = n-dodecyl. [Dye] = 0.86 μM for R = n-butyl and 0.98 μM for R = n-dodecyl.
An interesting feature of these experiments is that the absorption spectrum of the cyanine dye DiOC14(3) was invariably broadened in the presence of Ph₃BuB⁻ (Figure VIII-6). This is evidence of dye aggregation within the membrane, driven by association with the borate. This might be the same phenomenon that drives aqueous dye-borate complex formation (Chapter III).

**Figure VIII-6.** Effect of Ph₃BuB⁻ on the UV-vis absorption spectra of DiOC14(3) in Bis-C8-SorbPC vesicles.
As described in Chapter VII, the adsorption of the cyanine dye to the filters during extrusion results in the loss of control over the [L]/[D] for a given preparation. Experiments were performed in which DiIC18(3) was added to the outer leaflet of preformed Bis-C8-SorbPC vesicles. The dye was added at a level of 10 mole % relative to the lipid (20 mole % effectively, assuming the dye does not flip to the inner leaflet). In the absence of Ph3BnB⁻, the dye absorption spectrum was monomeric but addition of 10 mole % (relative to lipid) of the borate caused extensive aggregation of the dye (Figure VIII-7). The same spectrum was obtained if 20 or 30 mole % Ph3BnB⁻ were added indicating that 10 mole % was sufficient to completely aggregate the dye.

Figure VIII-7. Absorption spectrum of 10 mol % DiIC18(3) added to the outer leaflet of Bis-C8-SorbPC vesicles before and after addition of 10 mol % Ph3BnB⁻. [Bis-C8-SorbPC] = 25 μM.
Visible-light irradiation of the vesicles leads to rapid bleaching of the dye and much slower consumption of monomer (Figure VIII-8). Interestingly, the rate of lipid bleaching increases in the presence of oxygen (Figure VIII-9).

**Figure VIII-8.** Effect of visible-light irradiation at 37 °C on Bis-C8-SorbPC vesicles with 10 mole % DiIC18(3) and Ph₃Bn⁺ added. [Dye] = 2.6 μM.

**Figure VIII-9.** Effect of oxygen on the photosensitized consumption at 37 °C of Bis-C8-SorbPC vesicles with 10 mole % DiIC18(3) and Ph₃Bn⁺ added. [Dye] = 2.6 μM.
The rate of lipid consumption in unpurged samples is insensitive to the borate concentration, although the dye bleaching is more extensive at higher borate concentrations (Figures VIII-10 and 11).

**Figure VIII-10.** Effect of borate concentration on the photosensitized consumption at 37 °C of Bis-C8-SorbPC vesicles with 10 mole % DilC18(3). [Dye] = 2.6 μM.

**Figure VIII-11.** Effect of borate concentration on the photobleaching at 37 °C of Bis-C8-SorbPC bound DilC18(3). [L]/[D] = 10.0, [dye] = 2.6 μM.
If Ph$_3$SnS$^-$ is not included in the system and the sample is not purged with argon, one is left with the cyanine dye-oxygen initiator which polymerizes Bis-C8-SorbPC effectively (Chapter VII). Two samples, one with Ph$_3$SnS$^-$ and one without, were irradiated under identical conditions. As seen in Figures VIII-12 and 13, the borate accelerates dye photobleaching but simultaneously retards lipid consumption. Rather than acting as coinitiators in these systems, the borates seem to be functioning primarily as inhibitors.

![Graph](image)

**Figure VIII-12.** Effect of Ph$_3$SnS$^-$ on photobleaching of DiiC18(3) at 37 °C in Bis-C8-SorbPC/DiiC18(3) (7.8:1) vesicles. [dye] = 5.1 μM; [borate] = 15.3 μM
Figure VIII-13. Effect of Ph₃BnB⁺ on photosensitized consumption of lipid at 37 °C in Bis-C8-SorbPC/DilC18(3) (7.8:1) vesicles. [dye] = 5.1 μM; [borate] = 15.3 μM
VIII. C. 2. Surface-Associated Sensitizers

The aggregated state of the amphiphilic dyes seen in the last section could seriously undermine the efficiency of initiation. Since the aggregation occurs only in the presence of the borate, it is reasonable to assume that the borate molecules are bound among the hydrophobic tails of the aggregated dyes. This would give a high effective concentration of \( \text{Ph}_3\text{RB}^- \) ultimately leading to production of a large number of radicals within a small region of the membrane. Termination of the radicals by coupling should be favored over escape of the radicals from the aggregate and subsequent initiation. Moreover, the chain reaction proposed in Section V. E. would lead to generation of even more free radicals. In light of these considerations, experiments were designed to bind dyes to polymerizable vesicles without aggregation. The highly charged \( \text{Cy}^{3+} \) and \( \text{P}^{4+} \) introduced in Chapter III were selected for these experiments.

Bis-C8-SorbPC/DOPA (10:1) vesicles were prepared in water. \( \text{Cy}^{3+} \) and \( \text{Ph}_3\text{BuB}^- \) (5 and 15 mole %, respectively) were bound to the vesicles. No evidence for aggregation of the dye was observed. Visible-light irradiation in the absence of oxygen for ten minutes resulted in 80 % bleaching of the dye but only 5 % bleaching of the lipid. The rapid bleaching of \( \text{Cy}^{3+} \) might be due to attack by butyl radicals on the dye.

Further experiments were done with both \( \text{Cy}^{3+} \) and \( \text{P}^{4+} \). In separate experiments, \( \text{Ph}_3\text{BnB}^- \) (7.5 mole %) was used to bind either \( \text{Cy}^{3+} \) or \( \text{P}^{4+} \) (2.5 mole %) to Mono-C8-SorbPC vesicles. In the presence of the borate, the absorption bands of the dyes was red-shifted and broadened (Figure VIII-14 and Table VIII-2). This could be due either to incomplete binding of the dyes or to complex formation between the dyes and the borate.
Table VIII-2. Spectral parameters of Cy$^{3+}$ and P$^{4+}$ (2.5 mole %) bound to Mono-C8-SorbPC by Ph$_3$BnB$^-$.

<table>
<thead>
<tr>
<th>Dye</th>
<th>Mole % Ph$_3$BnB$^-$</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>FWHM (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy$^{3+}$</td>
<td>0</td>
<td>650</td>
<td>42</td>
</tr>
<tr>
<td>Cy$^{3+}$</td>
<td>7.5</td>
<td>667</td>
<td>58</td>
</tr>
<tr>
<td>P$^{4+}$</td>
<td>0</td>
<td>411</td>
<td>14</td>
</tr>
<tr>
<td>P$^{4+}$</td>
<td>7.5</td>
<td>419</td>
<td>24</td>
</tr>
</tbody>
</table>

Figure VIII-14. UV-vis absorption spectra of Cy$^{3+}$ and P$^{4+}$ (2.5 mole % relative to lipid) with Mono-C8-SorbPC (A) before and (B) after addition of 7.5 mole % Ph$_3$BnB$^-$. [Dye] = 1.4 μM.
Irradiation of the vesicle suspensions in the absence of oxygen results in bleaching of both the dyes and to a lesser extent the lipids (Figure VIII-15). For Cy\textsuperscript{3+}, approximately 80 % dye photobleaching occurs with less than 5 % of the lipid consumed in ten minutes irradiation. For the porphyrin, 60 minutes irradiation leads to approximately 50 % P\textsuperscript{4+} photobleaching and 23 % loss of monomer.

Figure VIII-15. Effect of irradiation at 37 °C on absorption spectra of Bis-C8-SorbPC/Dye/Ph\textsubscript{3}BnB\textsuperscript{−} vesicles. [Dye] = 1.4 µM. A) Dye = Cy\textsuperscript{3+}, total irradiation time = 10 min. B) Dye = P\textsuperscript{4+}, total irradiation time = 60 min.
The best results using P$_4^+$ were obtained from Bis-C8-SorbPC vesicles with 5 mole % P$_4^+$ and 15 mole % Ph$_3$BnB$^-$. Unlike previous experiments, the sample was irradiated at 37 °C with oxygen present. After 30 minutes of irradiation, 67 % monomer was consumed (Figure VIII-16).

![Absorption Spectra](image.png)

**Figure VIII-16.** Effect of irradiation at 37 °C on absorption spectra of Bis-C8-SorbPC vesicles with 5 mole % P$_4^+$ and 15 mole % Ph$_3$BnB$^-$. [P$_4^+$] = 15.3 µM.
Finally, polymerization of DenPC and Bis-C8-SorbPC under identical conditions revealed the sorbyl lipid to be more reactive (Figure VIII-17). This may be due to greater access of benzyl radicals to the polymerizable groups in both leaflets for Bis-C8-SorbPC than for DenPC, where the radical produced in the outer leaflet is required to diffuse up to the level of the glycerol backbone in the inner leaflet to initiate polymerization.

![Graph showing the effect of irradiation time on polymerization of DenPC and Bis-C8-SorbPC vesicles.](image)

**Figure VIII-17.** Effect of irradiation at 37 °C on polymerization of Bis-C8-SorbPC and DenPC vesicles with 5 mole % P4+ and 15 mole % Ph3BnB⁺: [P4+] = 15.3 μM.
VIII. D. DISCUSSION

The experiments described above indicate that initiation of vesicle polymerizations via the PET-bond cleavage chemistry of borate anions proceeds with modest success. Approximately 60-70% conversion of monomer was obtained in 30 minutes of visible-light irradiation for the two types of systems investigated. The simpler cyanine dye-oxygen initiator described in Chapter VII is considerably more effective, as illustrated in Figure VIII-13.

Oxygen inhibits the polymerization of Bis-C8-SorbPC when the vesicles are prepared in the presence of a large excess of Ph$_3$BuB$^-$ whereas it enhances the polymerization when Ph$_3$BnB$^-$ is present in equimolar amounts relative to the dye. Although both the cyanine dye and borate are different in the two cases, this result could also be due to the competition between borate- and oxygen-quenching of the excited cyanine dyes. In the first case, it is likely that the cyanine is quenched primarily by the borate, meaning that the initiating species would be benzyl radicals. Oxygen would scavenge these radicals leading to a net decrease in the polymerization efficiency. However, if the dye is not completely quenched by the borate, oxygen could also quench and initiate the reaction sequence leading to production of hydroxy radicals proposed in Chapter VII. Under such circumstances, the presence of oxygen could actually enhance the rate of polymerization.

The polymerizations sensitized by surface-associated dyes also show limited success. Both Cy$^{3+}$ and P$^{4+}$ appeared to bind to Mono-C8-SorbPC vesicles based on the observed bathochromic shift in the absorption spectra although the broadened profiles and depressed extinction coefficients suggest
that either incomplete binding occurs or that aqueous dye-borate complexes are being formed as well. The best results were obtained with the porphyrin in which 30 minutes of irradiation led to over 60% consumption of monomer at 37 °C and in the presence of oxygen. Further experiments were not conducted with these systems because of the greater success achieved with cyanine dye-oxygen initiator described in Chapter VII.

At least two potential sources of inefficiency can be identified for the borate-containing systems:

1) The aggregated/complexed state of the dyes used in these experiments favors release of radicals in high local concentration, perhaps promoting radical coupling. The chain reaction involving Ph₃RB⁻ proposed in Chapter V also leads to enhanced release of radicals. While this should favor initiation at low concentration, the high concentration could bias the system toward coupling.

2) Radicals may directly attack dye molecules in the membrane, rather than polymerizable groups.

Both of these pathways reduce the efficiency of initiation by diverting the radicals from attacking a polymerizable lipid. Further experimentation involving the borates should focus on elucidation of the fate of the alkyl radicals. For Ph₃BnB⁻, coupling should lead to production of 1,2-diphenylethane while attack on a cyanine dye should yield a bleached dye with a benzyl substituent bound most likely on the polymethine bridge.

A final concern involves the potential utility of the borate-containing initiating systems. While these might find use in the laboratory, use in vivo is most unlikely. The large amount of borate (> 10 mole %) required to attain
significant loss of monomer could have a deleterious effect in the body. Hydrophobic anions such as \( \text{Ph}_4\text{B}^- \) are known to uncouple the transmembrane pH gradient which is produced by respiration and is required for active transport and ATP synthesis.\(^2\) \( \text{Ph}_3\text{BnB}^- \) and \( \text{Ph}_3\text{BuB}^- \) could have a similar effect. In addition, the triphenylboron byproduct of the PET-bond cleavage chemistry would most likely be hydrolyzed to boric acid and benzene.

In conclusion, the hydrophobic borate anions do not appear to be effective cointiators for visible-light sensitized vesicle polymerizations despite their reported utility in bulk polymerizations.\(^{88,89,118}\) In virtually all cases studied, the dye sensitizer was consumed considerably faster than the lipid monomer, indicative of poor initiating efficiencies. This is in contrast with the simpler system described in Chapter VII, lacking the borate, in which the lipid was generally consumed faster than the dye, allowing high conversion to polymer to be attained.
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$^1$H-NMR SPECTRA

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UV-VIS SPECTRA OF AQUEOUS DYE-BORATE COMPLEXES

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Figure A-2-c. Absorption spectra for titration of 10.0 μM Rh⁺ in water with Ph₃BnB⁻. Each spectrum corresponds to successive addition of 0.2 molar equiv of borate.
Figure A-2-d. Absorption spectra for titration of 3.85 μM P⁴⁺ in water with Ph₄B⁺. Each spectrum corresponds to successive addition of 0.4 molar equiv of borate.
APPENDIX A-3

JOB PLOTS FOR AQUEOUS DYE-BORATE COMPLEXES

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Figure A-3-c. Job plot for formation of aqueous Cy\(^{+}\)-Ph\(_4\)B\(^{-}\) complex.

Figure A-3-d. Job plot for formation of aqueous Rh\(^{+}\)-Ph\(_3\)BnB\(^{-}\) complex.
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UV-VIS SPECTRA FOR BORATE-INITIATED POLYMERIZATIONS

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**Figure A-4-b.** Effect of visible-light irradiation at room temperature on absorption spectrum of Bis-C8-SorbPC/N-NBD-PE/Ph₃BuB⁺ vesicles. [L]/[D] = 9.1. Before extrusion, 50 mole % borate (relative to lipid) present. Sample was purged for 30 min with argon gas prior to irradiation.
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(164) While the reduction potential for Cy$^{2+}$ has not been measured, $E_{\text{red}}$ for the $N$, $N'$-diethyl analog is -0.885 V (Ref. 154). $E_{\text{red}}$ for $P^{4+}$ is -1.10 V (Ref. 163). Thus the reduced porphyrin can transfer an electron to the cyanine.


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