Determination of Functional Group Contributions to Transport through Lipid Bilayers: A Study Using a Hippuric Acid Series

by

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Abstract

Functional group contributions were determined for six α-methylene substituted p-hippuric acid compounds across egg lecithin lipid bilayers. These data were compared to results from the analogous series of p-toluic acid compounds that were carried out using egg Lecithin planar lipid bilayers. Through a vesicular efflux method the rate at which each compound permeated through a large unilamellar vesicle (LUV) was measured. An analysis of this rate versus the pH profile for each compound yielded membrane permeability coefficients (PRX) that varied from $4.5 \times 10^{-4}$ cm/s for α-methyl p-hippuric acid to $1.06 \times 10^{-8}$ cm/s for α-carbamoyl p-hippuric acid. These values are greater than three orders of magnitude smaller than their counter parts in the p-toluic acid series, necessitating the use of more stable LUV's rather than planar lipid. Functional group contributions to the free energy of transfer, ΔΔG, from water to the barrier domain derived from the permeability data were determined for the functional groups Cl, OCH₃, CN, OH, and CONH₂ and were found to be 180, 860, 2290, 4020, and 6310 (cal/mol), respectively. These data were compared to those from the α-methyl-substituted p-toluic acid series and exhibited similar behavior. This similarity indicates that the functional group contributions are independent of the molecule to which they are attached. Using the bulk organic solvent/water partition coefficient (KRX) from the toluic acid experiment, the log PRX was plotted against the log KRX to identify which bulk solvent most closely resembles the chemical nature of the bilayer barrier for these permeants. The slopes of the plots were .87, .93, 1.05, and 2.24 for hexadecane/water, hexadecene/water, decadiene/water, and octanol/water, respectively, with the best model solvent being that which yielded a slope closest to unity. These results suggest that the more non-polar solvents are a better model for the barrier domain region of the lipid bilayer, indicating that the barrier domain is more hydrocarbon-like and is probably located in the hydrocarbon-chain region and away from the polar-head region.
Acknowledgements

Throughout my project there were many people that gave their support and time to helping me learn and grow in and outside of the lab. I am very grateful for all the help that Peter Mayer gave to me throughout the summer. He not only answered all my questions, but helped me to learn the ways of the lab and the specifics of the project. I would like to thank Dr. Brad Anderson and the Department of Pharmaceutics and Pharmaceutical Chemistry at the University of Utah for allowing me the opportunity to work in their graduate labs. I also appreciated all the support and help that Anderson’s lab gave me. I not only learned about chemistry and biology, but about different aspects of life. Finally I would like to thank my parents for the support they have given me throughout my life. They have permitted me to learn from experiences and allowed me to make my own decisions.
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**Introduction**

The investigation of drug transport through a biological membrane will enable a better understanding of the cell and how the human body works. The most widely accepted description of biological membranes is the “fluid mosaic” model, which implies that the two major components in the membrane, lipid bilayers and proteins, exist individually without forming an intermediate substance. The basic structure of the complex membrane is that of the lipid with the proteins embedded in the bilayer structure as shown in Figure 1. Determining the membrane function requires knowledge of how the protein and lipid components interact and contribute to the properties of the membrane.¹

![Image of the lipid and protein components of the cell membrane.](image)

**Figure 1:** The Lipid and Protein Components of the Cell Membrane.¹

One of the primary functions of the cell membrane is to serve as a means of controlling the transport of molecules into and out of the cell² and this can involve the proteins associated with the membrane as well as the lipid bilayer.¹ This semipermeable barrier ultimately controls the passive transport of drugs as well as the transport of other nonelectrolyte solutes. To begin to comprehend the drug transport through a complex biomembrane one must use a simpler model, a phospholipid bilayer. Lipid bilayers are
commonly used as models for biological membranes because they are easier to work with in the laboratory and have properties similar to the biological membrane - such as a hydrophobic interior and ability to control transport of small molecules and ions.¹

There are two theories used to characterize the lipid bilayer. The first results from observations dating back to Overton’s rules. These rules state that the diffusion through the lipid region of the membrane resembles the partitioning that would occur in a homogeneous organic solvent, such as olive oil, octanol, or hydrocarbon. Therefore the lipid bilayer is believed to be homogeneous. This model is not complete since it does not account for many observations regarding molecular permeability in lipid bilayer membranes, such as interfacial constraints.³

The second theory describes the lipid bilayer as a heterogeneous membrane that accounts for the interfacial constraint. This theory generates a model of the barrier domain from a correlation with an organic solvent. Since the lipid bilayer is both oil and water in nature, the permeabilities of nonelectrolytes through lipid bilayers, or the biomembrane, could correlate with a bulk solvent/water partition coefficient. From this correlation the establishment of functional group contributions to the free energy of permeant transfer from water to the barrier domain is used to investigate the chemical nature of the barrier environment. The following linear equation (from reference 4) shows the fit between the partitioning to bulk solvent and the transport of permeant across a lipid bilayer.

\[
\log P_{RX} = s \log K_{RX} + i \tag{1}
\]

\(P_{RX}\) is the permeability of a solute RX of the series and \(K_{RX}\) is the bulk solvent/water partition coefficient of the same compound. The slope, \(s\), calculated by linear regression
analysis determines the best model solvent for the barrier domain. An s value of ~1 implies that the microenvironment of the barrier domain is similar to that of the reference solvent. An s value < 1 suggests a less lipophilic barrier domain and therefore a barrier which is less selective, or the barrier permits more of the compound to flow through the bilayer than the reference solvent permits. The opposite is true for s > 1.  

Using the heterogeneous membrane model, the lipid bilayer is described as having three regions and an outside water phase, as depicted in Figure 2. The bilayer contains a polar head group in contact with water, an ordered acyl hydrocarbon chain region, and a relatively disordered acyl chain region. The chain regions are nonpolar tails within the interior of the lipid bilayer held together by noncovalent interactions such as van der Waals interactions. Each region has its own chemical and diffusional properties leaving the contribution of each region to the overall barrier properties as an unresolved issue central to the complete understanding of molecular permeability through the lipid bilayer.

![Figure 2: Illustration of the Three Distinctive Regions of the Lipid Bilayer.](image)

Permeability can be broken down into two categories, passive and active transport. Active transport is the movement of a substance by a specific mechanism that
many not be in the same direction as the concentration gradient. This situation is similar to pumping water uphill. The energy required to move a substance against the gradient comes from an exergonic reaction such as hydrolysis of ATP to ADP and Pi. Through two conformational changes in the protein the pumping process occurs. The first change opens a protein pore or channel and allows a substance to move into extracellular fluid. The second change regenerates the original form of the enzyme allowing another substance to enter the cell.

In comparison, passive transport is the permeation of a substance from a region of higher concentration to one of a lower concentration, or flowing with the gradient. Passive transport can be subdivided into two smaller categories, simple diffusion and facilitated diffusion. Facilitated diffusion is when the molecule binds to a carrier protein to transport through the membrane. Simple diffusion is when the molecule passes through the lipid bilayer or an opening in the bilayer such as a pore or an opening caused by a protein.¹

Experiments have been conducted in this laboratory using a series of toluic acid compounds, Figure 3, to generate chemical selectivity data, such as the change in Gibbs free energy for transport of compounds containing different functional groups.

![Figure 3: α- Methyl Substituted -p-Toluic Acid](image)

\[ X = -\text{H}, -\text{Cl}, -\text{OCH}_3, -\text{OH}, -\text{CN}, -\text{COOH}, -\text{CONH}_2 \]
By determining functional group contributions to transport, the chemical selectivities of the barrier domain in lipid bilayers were investigated.\(^5\) The chemical environment of the bilayer barrier domain was studied using the toluic acid series. Toluic acid was chosen due to the isolation of its polar substituents which removes the possibility of intramolecular hydrogen bonding.\(^5\) The various compounds of the toluic acid series are similar in molecular size and shape and are likely to exhibit similar diffusional characteristics and volume displacement within the lipid bilayer.\(^5\) From the transport studies of the toluic acid series it appeared that the primary aliphatic group contributions were independent properties of the substituents themselves, but this needed to be further studied and verified with another series. It was also determined that the barrier region was located in the ordered hydrocarbon chain region of the lipid bilayer. This was determined by finding a bulk solvent that best modeled the chemical properties of the bilayer. Comparing the thermodynamic group contributions calculated from the transport data for the toluic acid series, using eq. 1, to those from partitioning into various bulk/solvent water systems, such as hexadecane, hexadecene, decadiene, and octanol, the best model was found to be decadiene. This implies that the barrier domain is hydrocarbon-like, which is characteristically similar to the ordered hydrocarbon chain region of the lipid bilayer.

We chose a hippuric acid series, as shown in Figure 4, to further study the passive transport process and verify the independence of the functional group contributions as well as the nature of the barrier domain within the lipid bilayer. Hippuric acid has similar characteristics to those of the toluic acid series. It is a small molecule, but larger than the
toluic acid where the polar substituents are isolated removing the possibility of intramolecular hydrogen bonding.

\[
\begin{array}{c}
\text{X= -H, -Cl, -OCH}_3, -\text{OH, -CN, -COOH, -CONH}_2
\end{array}
\]

Figure 4: α- Methyl Substituted p- Methyl Hippuric Acid Series

Due to the greater stability required for the monitoring of the more slowly diffusing hippuric acid series large unilamellar vesicles (LUV’s), shown in Figure 5, are used rather than planar “black” lipid membranes.²

Figure 5: A Large Unilamellar Vesicle and the Transport of a Weak Acid.²

A vesicular efflux method will be used whereby LUV’s are formed with a permeant solution inside and outside of the vesicle. A concentration gradient is then formed by retaining the permeant solution on the outside of the vesicles allowing the inside permeant, weak acid, to begin to diffuse through the vesicle with the assumption that only
the neutral species is allowed to transport. Through ultrafiltration, using filters and High Performance Liquid Chromatography (HPLC), we will be able to study the transport process and calculate the rate at which each permeant solution diffuses through the vesicle. From these rates the permeability coefficients and changes in Gibbs free energies can be calculated and compared to those of the toluic acid series. This comparison will verify the independence of the functional group contributions to transport across a lipid bilayer if the functional group contributions are similar for the two series. From these functional group contributions we can also determine the nature of the vesicle barrier domain because the heterogeneous model breaks the lipid bilayer into component parts. Through a comparison of group contributions of the hippuric acid transport and toluic acid partition coefficients we can determine whether the barrier domain is in the hydrocarbon chain region or the polar head region due to their similarities or differences to various bulk solvents.
Materials and Methods

Chemicals—The various α-methylene substituted p-methyl hippuric acids used as permeants in these transport experiments are shown in figure 4. Methyl-hippuric acid (98%) was purchased from Aldrich Chemical Co. (Milwaukee, WI) and the remaining compounds were synthesized earlier in this laboratory. Final purities of synthesized compounds were >95% by HPLC analysis. These compounds were stored at 5°C after synthesis.

pKa Determinations—Through pH titration, ionization constants for the various α-methylene substituted p-methyl hippuric acids were determined at room temperature with solute concentrations ranging from 0.01 to 0.009 M. A solution (3mg/ml) of each compound prepared in deionized water was stirred with nitrogen gas. A known normality of NaOH was slowly added while the pH was recorded (PHM 82 Standard pH meter). Plots of pH versus volume of titrant added were fitted using Micromath Scientist and a theoretical model, correcting for ionic strength of 0.1M, Appendix 1.

The pKa of p-chloromethyl hippuric acid was determined by partition coefficients due to impurities in the compound. A known amount of p-chloromethyl hippuric acid was dissolved in enough ethyl acetate to make eight 1.5 ml samples in 4ml vials. To each sample 1.5 ml of .01M formic acid buffer, adjusted to the desired pH, was added. The eight pH values ranged from 3 to 4.4 in intervals of 0.2. The samples were then tumbled slowly while in a 25°C oven for 30 minutes and allowed to stand outside the oven to equilibrate. The aqueous phase was then separated from the ethyl acetate by pipetting.
Figure 6: Illustration of Method for Producing LUV's and the Transport Process.
nitrogen gas in a Meyer N-EVAP Analytical Evaporator (Berlin, MA), then held under vacuum for fifteen minutes at \( \approx 50^\circ C \). They were then covered and stored at -20\(^\circ\)C until used.

**LUV Preparation**—Aqueous solutions containing .04M buffer (morphiloethan sulfonic acid (MES), phosphoric acid, tris(hydroxy methyl)amino methane, (TRIS) carbonic acid, or boric acid, depending on the pH value being buffered) with an ionic strength of 0.1 (adjusted with NaCl) were prepared with 3 x 10\(^{-3}\)M permeant at various pH values ranging from 5.5 to 10.5. This range was used due to stability concerns of the vesicles outside of this range. The aqueous permeant solution was then added to the lipid vials and vortexed. Large multilamellar vesicles, which spontaneously form, were subjected to extrusions through 0.2\(\mu\)m Nuclepore polycarbonate membrane filters (Nuclepore Inc., Pleasanton, CA) resulting in the desired large unilamellar vesicles. After 7 extrusions, the pH was checked and adjusted, if necessary, and again checked after 17 extrusions. The size of the LUV, (140-180 angstroms), was determined by Dynamic Light Scattering as explained below. The sample was then placed in a 25 \(^\circ\)C oven overnight. The final pH was measured and the respective buffer adjusted to this pH, the following morning. A size exclusion PD-10 Sephadex G-25Medium (15 x 50mm, polypropylene column, Supelco Inc., Bellefonte, PA) gel column was equilibrated to desired pH by running buffer though the gel column until eluted buffer pH was constant at the pH adjusted value of the sample. An aliquot (600\(\mu\)l) of the LUVs prepared above was added to the gel column to separate the extravesicular permeant from the vesicle with entrapped permeant and centrifuged, using an IEC Clinical Centrifuge (Needham HTS).
MA) at setting 1 for 2 minutes and setting 3 for 1 minute. The filtrate placed in 25°C bath immediately.

**Ultrafiltration**—Aliquots of this solution were taken at various intervals to determine the rate of diffusion. Each sample was collected by centrifuging an aliquot through an Amicon Centricon-100 centrifugal concentrator (Beverly, MA), at setting 7 until 150μL of permeant filtered through, approximately 8 minutes. Before use, the filters were preconditioned by washing with methanol and deionized water. Diffused permeant filtered through the Centricon-100 and was collected in a vial to store in the refrigerator until analysis. Total permeant concentrations in the filtrate was determined by collecting 350μL directly from the 12 ml vial and lysing the sample with a small amount of surfactant (Triton-100 10% v/v (Sigma)) prior to analysis. Appropriate amounts of 10N HCl were added to each sample to lower the pH to < 3 and then the samples were analyzed by High Performance Liquid Chromatography (HPLC), to detect permeant concentration at each time point.

**HPLC Analysis**—To analyze samples taken during the transport process a modular HPLC system was used, consisting of a syringe-loaded sample injector (Rheodyne Model 7125, Rainin Instrument Co., Woburn, MA) with a 200μL loop, a solvent delivery module (Model 110B, Beckman Instruments, Inc., Fullerton, CA) operated at a flow rate of 1.0 ml/min, a dual wavelength absorbance detector (Model 441, Water Associates, Milford, MA) operated at 254nm, an integrator (Model 3392A, Hewlett-Packard Co., Avondale, PA), and a reversed-phase column packed with 5μm C-18-300Å° (Phenomenex Jupiter, 4.66 mm i.d. x 25 cm, C-18). An acetonitrile:water mobile phase was used, varying from 7% to 33% acetonitrile, depending on lipophilicity
where \( V_i \) and \( V_o \) are total internal and external permeant volumes, respectively.

Combining eqs. 1 and 2 and taking into account that \( V_i \ll V_o \), eq. 1 becomes

\[
\frac{d C_0}{dt} = k_{\text{obs}} (C_0^\infty - C_0^1) 
\]

Solving this differential equation with known initial concentration, \( C_0^0 \), and noting that \( C_0^\infty \) is when all the concentration is outside the vesicle, the equation becomes

\[
\ln \left( \frac{C_0^\infty - C_0^0}{C_0^\infty - C_0^1} \right) = k_{\text{obs}} t 
\]

Plotting \( \ln \left( \frac{C_0^\infty - C_0^0}{C_0^\infty - C_0^1} \right) \) vs. time produces \( k_{\text{obs}} \), \( \text{(time}^{-1}\text{)} \), the rate constant for permeant diffusion through the lipid bilayer. The apparent permeability coefficient, \( P_{\text{app}} \), was determined from the first-order rate constant, \( k_{\text{obs}} \), (per second) and the ratio between entrapped volume and surface area of the LUV's, \( V/A \), with the following equation

\[
P_{\text{app}} = k_{\text{obs}} \frac{V}{A_t} 
\]

The values for \( V/A \) were determined from the vesicle thickness found using Dynamic Light Scattering and the geometry of a sphere, the volume and area. Taking the ratio of the volume over the area and canceling, the final result is \( d/6 \). Therefore the vesicles in these experiments had a \( V/A \) value of about 150nm/6.
Results

pKa Determinations

Ionization constants for α-methylene substituted p-methyl hippuric acid are needed to determine molar fractions of neutral species HA, the weak acid diffusing through the vesicle, Table 1.

Table 1: pKa Values Determined for Hippuric Acid Series Through pH Titration.

<table>
<thead>
<tr>
<th>X</th>
<th>pKa Values'</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>3.85 ± .01</td>
</tr>
<tr>
<td>Cl</td>
<td>3.80 ± .04</td>
</tr>
<tr>
<td>OCH₃</td>
<td>3.66 ± .01</td>
</tr>
<tr>
<td>CN</td>
<td>3.66 ± .00</td>
</tr>
<tr>
<td>OH</td>
<td>3.69 ± .01</td>
</tr>
<tr>
<td>COOH</td>
<td>ND</td>
</tr>
<tr>
<td>CONH₂</td>
<td>3.70 ± .02</td>
</tr>
</tbody>
</table>

'Confidence intervals are 95% range (S-Plane)

As desired the pKa values are within the same range and the confidence interval of 95% permits the conclusion that the values are accurate. The more polar compounds have a lower value than expected, however their values are close in value to that of the p-methyl hippuric acid.

Determination of Permeability Coefficients

Measured concentration of permeant increases with time as seen in Figure 7. Samples were analyzed by HPLC throughout the transport. The concentration of each sample was determined using peak areas and known standard concentrations. Using eq. 4, the linear form, and plotting the model values versus time, the estimated rate constants, \( k_{obs}(\text{hour}^{-1}) \), for each of the seven compounds at various pH values are calculated. As shown in Figure 8, the slope of the line is the estimated rate.
These rates were used to calculate the apparent permeabilities, $P_{app}$, at each pH using eq. 5. The plots of log ($P_{app}$) versus pH for the hippuric acid series are shown in Figure 9. The slope of approximately −1 shows that the transport is membrane controlled and that the transport rate of the ionized species is negligible.

The pKa values, Table 1, are then used in eq. 6 (from reference 5) to calculate the fraction of unionized species at each pH,

$$f_{HA} = 1 / (1 + 10^{pH-pKa}) \quad (6)$$

Using the apparent permeabilities, $P_{app}$, and the fraction of unionized species, $f_{HA}$, the intrinsic permeability coefficient, $P_{RX}$, for each compound is calculated using

$$P_{RX} = P_{app} / f_{HA} \quad (7)$$

The calculated permeability coefficients are shown in Table 2 along with those from the toluic acid series for comparison. As expected the values for the hippuric acid series are smaller due to the more polar nature of the hippuric acid series, so they take more time to diffuse through the bilayer.

**Table 2**: Permeability Coefficients(cm/sec) for Hippuric Acid and Toluic Acid Series’ Transport.

<table>
<thead>
<tr>
<th>-X</th>
<th>Hippuric Acid Series</th>
<th>Toluic Acid Series</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>4.5 E -4 (± 6.3 E -5)</td>
<td>1.1±0.2</td>
</tr>
<tr>
<td>Cl</td>
<td>3.3 E -4 (± 6.4 E -5)</td>
<td>6.4 E -1 (±0.1 E -1)</td>
</tr>
<tr>
<td>OCH3</td>
<td>1.0 E -4 (± 2.4 E -5)</td>
<td>3.5 E -1 (±0.1 E -1)</td>
</tr>
<tr>
<td>CN</td>
<td>9.3 E -6 (± 1.4 E -6)</td>
<td>2.7 E -2 (±0.5 E -2)</td>
</tr>
<tr>
<td>OH</td>
<td>5.05 E -7 (± 1.2 E -7)</td>
<td>1.6 E -3 (±0.4 E -3)</td>
</tr>
<tr>
<td>COOH</td>
<td>ND</td>
<td>1.8 E -4 (±0.3 E -4)</td>
</tr>
<tr>
<td>CONH2</td>
<td>1.06 E -8 (± 3.59 E -9)</td>
<td>4.1 E -5 (±0.4 E -5)</td>
</tr>
</tbody>
</table>

1 Standard Deviations
Figure 7: A Representative Plot of the Permeant Concentration versus Time

\( (p\text{-Methoxy Hippuric Acid at pH 9.18}) \)

Figure 8: A Representative Plot of the Determination of the First Order Rate Constant

\( (p\text{-Methoxy Hippuric Acid at pH 9.18}) \)
Calculations of Group Contributions to the Free Energy of Transfer

The intrinsic permeability coefficients are then used to calculate the changes in free energies of transfer into the barrier, $\Delta(G^0)$, attributable to the substituents. This can be calculated in two ways using the data one has (from reference 4), for transport with permeability coefficients, as we have,

$$\Delta(G^0) = -RT \ln \left( \frac{P_X}{P_{RH}} \right)$$  \hspace{1cm} (8)

and for partitioning with partition coefficients

$$\Delta(G^0) = -RT \ln \left( \frac{K_X}{K_{RH}} \right).$$  \hspace{1cm} (9)

**Figure 9**: Log ($P_{app}$) of the Hippuric Acid Series versus pH
The functional group contributions for the hippuric acid series are shown in Table 3 with the data from the toluic acid series for a comparison. This comparison will determine the independence of the functional groups.

**Table 3:** Functional Group Contributions (cal/mol) to Transport of the Hippuric Acid and Toluic Series.

<table>
<thead>
<tr>
<th>-X</th>
<th>Hippuric Acid Series</th>
<th>Toluic Acid Series*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cl</td>
<td>180</td>
<td>325</td>
</tr>
<tr>
<td>OCH₃</td>
<td>860</td>
<td>687</td>
</tr>
<tr>
<td>CN</td>
<td>2290</td>
<td>2170</td>
</tr>
<tr>
<td>OH</td>
<td>4020</td>
<td>3860</td>
</tr>
<tr>
<td>COOH</td>
<td>ND</td>
<td>5170</td>
</tr>
<tr>
<td>CONH₂</td>
<td>6310</td>
<td>6060</td>
</tr>
</tbody>
</table>

**Determination of the Nature of the Barrier Domain**

The thermodynamic functional group contribution values for the hippuric acid series can then be compared with the changes in free energies of transfer into bulk solvents from water for the toluic acid series calculated using eq. 9. Four different bulk solvents are used demonstrating characteristics that are either hydrocarbon like or more polar. The functional group contributions of the four bulk solvents are listed in Table 4.

**Table 4:** The Functional Group Contribution (cal/mol) for a Transfer Process for Partitioning of Various Functional Groups from Water into Bulk Solvents, for the Toluic Acid Series*.

<table>
<thead>
<tr>
<th>X</th>
<th>Hexadecane</th>
<th>Hexadecene</th>
<th>Decadiene</th>
<th>Octanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cl</td>
<td>409</td>
<td>126</td>
<td>314</td>
<td>350</td>
</tr>
<tr>
<td>OCH₃</td>
<td>1240</td>
<td>1260</td>
<td>1240</td>
<td>840</td>
</tr>
<tr>
<td>CN</td>
<td>2820</td>
<td>2400</td>
<td>2350</td>
<td>1640</td>
</tr>
<tr>
<td>OH</td>
<td>5100</td>
<td>4590</td>
<td>4210</td>
<td>2000</td>
</tr>
<tr>
<td>COOH</td>
<td>6140</td>
<td>5680</td>
<td>5270</td>
<td>1560</td>
</tr>
<tr>
<td>CONH₂</td>
<td>7250</td>
<td>6730</td>
<td>6010</td>
<td>2870</td>
</tr>
</tbody>
</table>
These data are plotted against the functional group contributions for the hippuric acid series listed in Table 3, as seen in Figure 10 (a,b,c,d).

**Figure 10:** Linear Free Energy Relationships Partition versus Permeability. Key: (a), Hexadecane/water; (b), Hexadecene/water; (c), decadiene/water; and (d), octanol/water.

The slope, s, determined by eq. 1, measures the similarity of the chemical properties between the bulk solvent and membrane barrier. These slope values are compared to the slopes determined in the toluic acid experiment to find the best model solvent to characterize the barrier in the lipid bilayer, listed in Table 5.
Table 5: Slope Values Determined to Characterize the Barrier Domain for Hippuric Acid and Toluic Acid Series

<table>
<thead>
<tr>
<th>Model Solvent</th>
<th>Hippuric Acid s values</th>
<th>Toluic Acid s values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecane</td>
<td>0.86 ± 0.01</td>
<td>0.85 ± 0.03</td>
</tr>
<tr>
<td>Hexadecene</td>
<td>0.93 ± 0.01</td>
<td>0.91 ± 0.04</td>
</tr>
<tr>
<td>Decadiene</td>
<td>1.0 ± 0.01</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>Octanol</td>
<td>2.2 ± 0.5</td>
<td>2.4 ± 0.5</td>
</tr>
</tbody>
</table>

When the slope, $s = 1$ the two solvents are nearly identical in chemical selectivity. As one can see, the two experiments show that the slope is near one for the hydrocarbon-like solvents, as opposed to octanol.
Discussion

Permeabilities

The rate at which a permeant diffuses through a lipid bilayer depends on the lipophilicity and size of the permeant. It has previously been shown that chain packing also plays an important role in determining the diffusion. Egg PC, a sixteen or eighteen carbon long chain, makes up a majority of our bilayer, in this experiment, with 72% of the chain being the highly ordered chain region, Figure 2. Due to these variables it is necessary that the permeant solutions span a wide range of hydrogen-bonding and differing lipophilicity abilities. The permeant concentration was not varied, though, due to previous observations that a systematic change is not noticeable in the permeability coefficient. Also, the permeant state, liquid crystalline, was not varied due to the lack of time. Many variables, such as pH and thickness of the bilayer, are controlled and varied to achieve accurate results.

The necessity to find a pH region for each permeant where the flux is lipid bilayer controlled, rather than water layer controlled is portrayed in Figure 9 illustrating the “pH window” where each permeant is membrane controlled. The selection of a pH region depends on when the net flux of a weak acid, HA, becomes membrane controlled. As illustrated in Figure 5, there exists both the ionized and unionized species inside and outside the LUV. During our transport experiments we are observing the rate at which the unionized species diffuses through the vesicle. As the fraction of unionized species decreases with the increase of the solution pH, the permeability decreases and the transport of the series becomes membrane controlled. This establishes that it is the neutral species, HA, that accounts for the bilayer flux. An insignificant amount of the
ionized species, A⁻, is diffusing through the vesicle, but in the determined membrane controlled pH region it is negligible, yielding the linear relationship between the apparent permeability and the concentration of unionized species.

The ionized species and the water layer, though, can become a significant part of the transport, as seen in Figure 11, when transport occurs outside the membrane-controlled region. The upper plateau in region 1 is due to the transport being aqueous boundary layer controlled, where the apparent permeability coefficient is independent of the pH and the permeant structure. The rate at which the permeant is diffusing through the vesicle in this region is altered due to the rate at which it diffuses through the aqueous boundary layer, causing it to be difficult to detect the membrane permeability coefficient. It is probably where the pKa is being approached and the linear relationship is no longer valid. Under the buffer conditions chosen for this experiment, though, the proton concentration remains constant across the aqueous boundary layers within the correct pH region. In region 3 the permeability of the ionized species, A⁻, and/or the rate of the vesicle leakage controls the rate of diffusion. Region 2 is the linear, membrane-controlled region that is used to determine the functional group contributions. The apparent permeability decreases linearly as long as the A⁻ anion contribution to transport is insignificant.

As seen in Figure 9, the slopes are not all the ideal slope of −1. This indicates that not all of the data were taken in the membrane controlled pH region, altering the slope slightly, but the majority of the transport studies were completed in the linear pH region for each permeant. The apparent permeabilities vary with pH which may be caused by the differing permeabilities of the neutral and ionic species and the changes in the relative
fractions of the weak acid to the anion in the pH solution. The determination of accurate permeability coefficients depends on the effects of the permeant ionization.

![Figure 11: Theoretical log (P_{app}) versus pH.](image)

Key: dotted lines are theoretical and solid line is determined by calculations with a slope of -1, where transport is membrane controlled

The comparison between the permeability coefficients for the hippuric acid and toluic acid Series, in Table 2, shows the differences in the magnitude of these values between the two series. As expected the values vary by several orders of magnitude because both polar and non-polar substituents are used. This difference in rates of transport indicates the need for the use of LUV’s rather than the planar “black” lipid membranes. Planar "black" lipid membranes can be used for more permeable solutes, but due to the instability of the planar bilayers the alternate method of LUV’s was used. Reasonable agreement has been observed for permeabilities using both methods, but
planar films seem to be better for chemical selectivity involving permeants varying in permeant coefficients over many orders of magnitude, and LUV's appear to be the favored method for examining lipid composition and other structural features.² In addition it has been previously suggested that the effects of the unstirred water layer may not be as important in transport studies involving LUV’s compared to those of planar lipid bilayers.² The use of LUV's, though, limits the transport measurements because rapid transport cannot be measured, which are grounds for using hydrophilic permeants only. It is also difficult to discern the leakiness and breakage of vesicles.

Functional Group Contributions

In the comparison of the data calculated for the functional group contributions for the hippuric acid series and toluic acid series, Table 3, it can be seen that the values are reasonably similar. These results lead to the conclusion that the effect of a given substituent on the free energy transfer is an independent contribution of the substituent itself, regardless of the molecule it is attached to. This is not expected, though, to hold for all molecules due to intramolecular interactions and steric effects. It also illustrates that the transport studies using large unilamellar vesicles appear to be equivalent to experiments completed with planar lipid bilayers. This functional group independence may seem to be a reasonable assumption for macroscopic systems such as bulk solvents, yet it is less obvious that this would hold for the barrier domain, a region with a thickness of only a few angstroms.
Bulk Solvent Model of the Barrier Domain

Linear free energies are calculated for the hippuric series transfer from water to the barrier domain and corresponding values for the transfer from water into four bulk solvents due to the similar diffusional behavior, molecular size, and shape between lipid bilayers and the relative ability to partition from water into the barrier domain. In the lipid bilayer there exists a distinct region that governs the permeant transport and is the same for all the permeants in this series. To characterize the barrier domain the best model solvent for bilayer chemical selectivity is determined using eq. 1 and the slopes, seen in Figure 10 (a,b,c,d). Potential model bulk solvents for characterizing the barrier domain of the lipid bilayer must vary in polarity, polarizability, and hydrogen bonding capacity. Octanol represents the polar, hydrogen-bonding solvents, whereas hexadecane, hexadecene, and decadiene all represent the non-polar, non hydrogen-bonding hydrocarbon solvents. The latter three solvents have different degrees of chain unsaturation, possibly describing the effects of double bonds in egg PC on barrier properties. Egg PC has an average of one double bond per acyl chain, which is hydrocarbon-like, but may be altered by the degree of chain unsaturation causing the chemical selectivity to vary.

The barrier region does not closely resemble the hydrogen-bonding region found in octanol, as seen in Figure 10 (d) and Table 5, thereby establishing the location of the barrier to be in the hydrocarbon chain region. Thus, the contributions of the interfacial region as the barrier domain seem negligible. Previous studies have broken the bilayer interior into a disordered region and highly ordered chain region. The disordered chain
region represents a significant barrier to diffusion if an inverse dependence of permeability on thickness is evident, however this is not observed. In the ordered hydrocarbon chain region the effective local polarity may be slightly higher than in bulk alkane solvents, indicating the reason hexadecane is not the chosen solvent. The polar nature of the bilayers can be affected by the closeness to the polar head region, by partial penetration of water molecules into the bilayer, or by the presence of double bonds in the chain. It can also be attributed to the dipole-dipole interactions between polar permeants and the methylene groups in lipid acyl chains. In both the toluic acid study and this study it was observed that the bulk solvent decadiene has a slope of approximately one, leading to the conclusion that it is the best model for the barrier domain. Previous studies have suggested that the more polarizable hydrocarbon solvents more closely resemble that of the lipid bilayer as is shown in this study.
Conclusion

An investigation of the principal function of a biological membrane, to control the permeation of solutes, is fundamental in fully understanding the cell function and biological behavior of drug delivery. The lipid bilayer, one of the primary aspects of the cell membrane, attributes to the rate at which the permeant diffuses through the cell. Because it was observed that the free energies calculated for the hippuric acid series was similar to those of the previous study using a toluic acid series it is determined that the contribution of the functional groups are independent. This is true provided that the functional groups are attached to sites offering similar steric and electronic environments and are well isolated from other polar residues in the parent compound. These similarities also demonstrate that the studies using large unilamellar vesicles (LUV’s) are equivalent to those using planar lipid bilayers.

Through the study of the transport of varying permeants across lipid bilayers, the unresolved issue of the chemical nature of the barrier domain controlling the diffusion of nonelectrolytes across biological membranes can be further investigated. In this study it is believed that the barrier domain appears to be hydrocarbon like due to the correlation of bulk solvent partition coefficients to permeabilities. This correlation was calculated using a linear fit to determine the rate of diffusion for each functional group, yet this has been verified further by using the non-linear fit, resulting in the same conclusions. (P. Mayer, unpublished observations)

The permeabilities are affected by the limitation of the LUV’s and instrumentation, which can only be improved upon through further studies. A larger pH range without the time restraint can be studied by using new hydraulic filters for the
transport. As well it is important to investigate the effect of vesicle breakage on the apparent permeability in the slower diffusing pH range.
Works Cited


Works not Cited

Appendix 1

// MicroMath Scientist Model File
InciVars: V
DepVars: pH
Params: pKa, Cl, Vl, W
RI=V/(V+Vl)
lsqr1=(W*R)/0.5
FA=10^(-0.509*lsqr1/(1+1.968*lsqr1))
FH=10^(-0.509*lsqr1/(1+2.952*lsqr1))
CT=Cl*Vl/(V+Vl)
BW=RI*W
KA=10^(pKa)
B=KA/FA+B*FH
pH=-log10(0.5*sqrt(B^2-4*FH*(BW-CT)*KA/FA)))
***