Independence of Substituent Contributions to the Transport of Small-Molecule Permeants in Lipid Bilayer

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ABSTRACT Purpose: To explore the independence of functional group contributions to permeability of nonelectrolytes across egg lecithin bilayers. **Methods.** The transport rates were measured of a series of α -substituted *p*-methylhippuric acids (-H, -Cl, -OCH₃, -CN, -OH, -COOH, and -CONH₂) across egg lecithin lipid bilayers, in the form of large unilamellar vesicles (LUVs) at 25EC. Intrinsic permeability coefficients (P_{HA}) were calculated from apparent permeability coefficients (P_{app}) measured as a function of pH. Group contributions to the free energy of transfer from water into the barrier domain, $\Delta(\Delta G)_{P,X}$, were calculated for the substituents and compared to the contributions of these groups when attached to *p*-toluic acid measured earlier. The $\Delta(\Delta G)_{P,X}$ values from permeability data were also correlated with $\Delta(\Delta G)_{PC,X}$ values of partitioning from water into organic solvents to determine the physicochemical selectivity of the barrier domain. **Results.** P_{app} values in LUVs were found to vary approximately linearly with the fraction of neutral permeant over a pH range of 5.5 to 10.5, suggesting that the transport of the ionized species is negligible over this pH range. The $\Delta(\Delta G)_{P,X}$ values from the 2 series of compounds appear to be the same, indicating that the functional group contributions are independent. 1,9-Decadiene was found to be the most similar to the chemical environment of the barrier domain. **Conclusions.** Functional group contributions to transport across egg lecithin bilayers appear to be independent of the compound to which they are attached, even though the thickness of the barrier domain in lipid bilayers is approximately the same as the extended length of the permeant.

INTRODUCTION

The ability to predict drug distribution into or transport across any given biomembrane based solely on the structure of the permeant and the composition of the membrane is the ultimate objective of much of the current research into these phenomena. At present, structure-distribution coefficients or structure-transport relationships rely heavily on the assumptions of independence and additivity of either functional group contributions or free energy component contributions to the overall transfer process (1). The thermodynamic additivity principle as applied to functional group contributions was articulated by Irving Langmuir nearly 75 years ago. Noting that the addition of each successive CH₂- to a hydrocarbon chain has about the same effect on volume, boiling point, and solubility, Langmuir suggested that it is reasonable to assume, therefore, that the field of force about any particular group or radical in a large organic molecule is characteristic of that group and, as a first approximation, is independent of the nature of the rest of the molecule. He referred to this as the principle of independent surface action (2). Of course, if the contributions of the constituent groups within a molecule to the free energy of a transfer process are independent, they are also additive. Because the assumptions of independence and additivity lie at the foundation of computational chemistry and biology, they have recently been referred to as the 4th law of thermodynamics (1,3).

There is abundant evidence that the independence and additivity of functional group contributions can generally be assumed for bulk phase transfer free energies as determined from organic solvent/water partition coefficients (4,5), provided that appropriate nonadditivity corrections are made to account for chain branching or other steric factors, electronic effects, intramolecular hydrogen-bonding, etc. (6).

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This success may be attributable to the likelihood that a given well-isolated functional group is surrounded by the same, uniform environment in a given bulk solvent, regardless of the size or complexity of the molecule to which it is attached. This independence of functional group contributions seems less likely, however, when one is considering the effect of structural changes on biomembrane transport. The two barrier domain regions within a solvent-free egg phosphatidylcholine bilayer have been estimated previously to account for about 72% of the chain segments in the bilayer, yielding a thickness for the barrier domain on each half of the bilayer of only 9 Å (7). One might expect nonadditivities to become increasingly apparent as the length of the permeant in its optimal orientation within the bilayer extends beyond the barrier domain thickness. Nonadditivity or the lack of independence in functional group contributions to membrane transport might also occur if the location of the barrier domain changes with the polarity of the permeant, as suggested by Diamond and Katz (8).

This paper examines the question of independence (and therefore additivity) of functional group contributions to the free energy of transfer of solute from water to the membrane barrier domain ($\Delta[\Delta G]_{P,X}$) by comparing the lipid bilayer membrane permeability coefficients of 2 series of compounds similar in molecular size and comparable in their fully extended length to the barrier domain thickness but differing by more than 3 orders of magnitude in their partition coefficients between the same members of each series. This was accomplished by measuring the transport rates of a series of α -substituted (-H, -Cl, -OCH₃, -CN, -OH, -COOH, and -CONH₂) *p*-methylhippuric acids shown in Figure 1 out of large unilamellar egg lecithin vesicles at 25°C employed as the model membrane system. These results were compared to those of the same functional groups attached to *p*-toluic acid (9) determined in planar lipid bilayer experiments using egg lecithin bilayers. The addition of a glycine residue to the *p*-toluic acids to form the *p*-methylhippuric acids greatly increases the polarity of the compounds with only a modest change in size, resulting in $\Delta(\Delta G)_{P,X}$ values determined over a wide range of permeability coefficients.

<i>p</i> -Toluic Acids			<i>p</i> -Methylhippuric Acids		
	X	Y		X	Y
1a	-H	-OH	2a	-H	-NHCH ₂ CO ₂ H
1b	-Cl	-OH	2b	-Cl	-NHCH ₂ CO ₂ H
1c	-OCH ₃	-OH	2c	-OCH ₃	-NHCH ₂ CO ₂ H
1d	-CN	-OH	2d	-CN	-NHCH ₂ CO ₂ H
1e	-OH	-OH	2e	-OH	-NHCH ₂ CO ₂ H
1f	-CO ₂ H	-OH	2f	-CO ₂ H	-NHCH ₂ CO ₂ H
1g	-CONH ₂	-OH	2g	-CONH ₂	-NHCH ₂ CO ₂ H

Figure 1. Structures of α -Substituted *p*-Toluic Acids and α -Methyl Substituted *p*-Methylhippuric Acids.

MATERIALS AND METHODS

Egg lecithin-phosphatidylcholine (egg-PC, >99%) and phosphatidic acid (egg-PA, >99%), were obtained from Avanti Polar-Lipids, Inc. (Pelham, AL). α -Chloro-*p*-toluic acid (1a) (95%) and *p*-methylhippuric acid (2a) (98%) were from Aldrich Chemical Co. (Milwaukee, WI). D-mannitol-[1-³H(N)] was purchased from Sigma Chemical Co. (St. Louis, MO). All commercially obtained reagents were used as received. The other compounds used as permeants in these experiments were synthesized as described below. Preparative scale high-performance liquid chromatography (HPLC) was performed using a C-18 column (Partisil 10, ODS-3, 25.4 i.d. $\times$ 300 mm, Whatman, Fairfield, NJ) with acetonitrile:water varying from 10% to 20% organic at a flow rate of 5.0 mL per minute. The aqueous phase for the preparative scale HPLC was pure water, with the pH adjusted to 3.0 using HCl.

*α -Chloro-*p*-methylhippuric Acid (Figure 1 and Figure 2)*

To a 60-mL volume of dimethyl formamide (DMF) was added 4.5 g of α -chloro-*p*-toluic acid in a vessel sealed to prevent exposure to water vapor. The

solution was cooled to <-10EC in an acetone/liquid nitrogen bath, and a 15% molar excess each of diisopropylethylamine and isobutyl chloroformate were added. The solution was allowed to react for 5 minutes with agitation. The reaction mixture was then removed to an ice water bath, and 3 equivalents of glycine were added via a 2.3 M aqueous solution at pH 11 and allowed to react for 10 minutes. The white solid that precipitated (glycine) was filtered off, and the desired product was isolated by extraction into ethyl acetate. The product was dried in vacuo and recrystallized from warm acetonitrile to yield 2.0 g of rod-shaped crystals (mp 174-175EC); ^1H NMR (CD_3CN): δ 7.80 (d, 2H, *o*), 7.51 (d, 2H, *m*), 4.70 (s, 2H, ClCH_2), and 4.03 (d, 2H, NCH_2). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 228$. Purity was >98% by HPLC.

Figure 2. Image of a Single Molecule of *p*-Methylhippuric Acid (see structure in Fig. 1) Inserted into a DPPC Bilayer Consisting of 98 DPPC Molecules and 2800 Water Molecules Prepared using the Insight II/Discover software (Molecular Simulations Inc., San Diego, CA) and the CVFF Forcefield (T.-X. Xiang and B.D. Anderson, unpublished results).

*α -Iodo-*p*-methylhippuric Acid*

To 250 mL of acetone was added 2 g of α -chloro-*p*-methylhippuric acid. To this solution 10 $\times$ molar equivalents (13.3 grams) of sodium iodide (J. T. Baker, Phillipsburg, NJ) were added. The solution was allowed to stir at room temperature for 1 to 2 hours, and the white solid formed (NaCl) was filtered off. The stirring was continued for another 1 to 2 hours and filtered again then stirred overnight. The acetone was evaporated off and the resulting yellow-

brown solid was then dissolved in 450 mL of water and the solution was adjusted to pH 1.8 with dilute H_2SO_4 . This aqueous suspension was stirred for 1 hour and the undissolved product was isolated via filtration with a Buchner funnel and rinsed with 150 mL of water at pH 2.0. The yellow solid was then dried in vacuo at 50EC to yield 1.9 g of product (mp 184-186EC); ^1H NMR (CD_3CN): δ 7.72 (d, 2H, *o*), 7.49 (d, 2H, *m*), 4.57 (s, 2H, ICH_2), and 4.02 (d, 2H, NCH_2). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 319$. Purity was >99% by HPLC.

*α -Methoxy-*p*-methylhippuric Acid (Figure 1 and Figure 2)*

Approximately 400 mg of α -iodo-*p*-methylhippuric acid was added to 20 mL of methanol, along with 700 mg of sodium hydroxide pellets. This solution was stirred at 50EC for 2 hours then dried in vacuo. Sufficient water was then added to dissolve the solid, and the pH was adjusted to 2.7 with H_2SO_4 . The desired product was then extracted into ethyl acetate, and the fractions were combined and evaporated. The resulting paste was then recrystallized using several drops of acetonitrile, resulting in needle-shaped crystals when viewed under a microscope and cross-polarized light (mp 127-129EC). ^1H NMR (CD_3CN): δ 7.78 (d, 2H, *o*), 7.41 (d, 2H, *m*), 4.48 (s, 2H, OCH_2); 4.03 (d, 2H, NCH_2), and 3.34 (s, 3H, OCH_3). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 223$. Purity was >97% by HPLC.

*α -Cyano-*p*-methylhippuric Acid (Figure 1 and Figure 2)*

To 40 mL of dimethyl sulfoxide (DMSO) was added 624 mg of NaCN. (Note that working with NaCN requires that the solution never come into contact with acid or poisonous HCN gas will be produced.) To this solution was added 6.3 mL of 1.0 N NaOH. This solution was then placed in an ice water bath, and 2.0 g of α -iodo-*p*-methylhippuric acid was added. Approximately 30 minutes were required for the α -iodo-*p*-methylhippuric acid to completely dissolve, leaving a pink/red solution. The reaction progress was monitored by HPLC, reaching

completion within 10 minutes after the α -iodo-*p*-methylhippuric acid had dissolved. The desired product was isolated by diluting the solution 3-fold with water, adjusting to pH 6.0 (where the solution turned yellow) and extracting the DMSO into ethyl acetate. The product was then isolated by preparative HPLC and dried in vacuo, yielding 0.7 g of needle-shaped crystals (mp 181-184EC); ^1H NMR (CD_3CN): δ 7.81 (d, 2H, *o*), 7.46 (d, 2H, *m*), 3.89 (s, 2H, NCCH_2), and 4.03 (d, 2H, NCH_2). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 218$. Purity was >99% by HPLC.

α -Hydroxy-*p*-methylhippuric Acid (Figure 1 and Figure 2)

To 40 mL of water was added 500 mg of α -iodo-*p*-methylhippuric acid, and the pH was adjusted to 13 using concentrated NaOH. The reaction progress was monitored by HPLC. The solution was stirred at room temperature, protected from light, for approximately 30 hours, at which time the pH was adjusted to 3.0 and the desired product was isolated by preparative HPLC. The desired product fractions were pooled and dried in vacuo to yield 208 mg of plate-shaped crystals (mp 156-159EC); ^1H NMR (D_2O): δ 7.68 (d, 2H, *o*), 7.37 (d, 2H, *m*), 4.57 (s, 2H, OCH_2), and 3.99 (s, 2H, NCH_2). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 209$. Purity was >99% by HPLC.

α -Carboxy-*p*-methylhippuric Acid (Figure 1 and Figure 2)

To 7 mL of 50% H_2SO_4 was added 300 mg of α -cyano-*p*-methylhippuric acid, and the solution was allowed to react at 70EC for approximately 21 hours. The reaction was monitored by HPLC, which indicated the appearance of an intermediate peak (α -carbamoyl-*p*-methylhippuric acid) that subsequently disappeared as the final desired product peak appeared. The solution was then placed in an ice bath, and 20 mL of water was slowly added, producing a white precipitate. The solution was adjusted to pH 8 with 10 N NaOH to dissolve the solid and then immediately adjusted to pH 3.0 with

10 N HCl for compatibility with the HPLC mobile phase. The desired product was then purified by preparative HPLC and dried in vacuo, yielding 175 mg of a white, crystalline solid (mp 210-212EC with decomposition); ^1H NMR (D_2O): δ 7.68 (d, 2H, *o*), 7.33 (d, 2H, *m*), 4.00 (s, 2H, NCH_2), and 3.70 (s, 2H, HO_2CCH_2). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 237$. Purity was >99% by HPLC.

α -Carbamoyl-*p*-methylhippuric Acid (Figure 1 and Figure 2)

To 7 mL of 96% H_2SO_4 was added 340 mg of α -cyano-*p*-methylhippuric acid. The solution was allowed to react at room temperature for approximately 5 hours while the course of the reaction was monitored by HPLC. The solution was then placed in an ice bath, 20 mL of water was added, and the pH was adjusted to 3.0 using 10 N NaOH. The product was isolated by preparative HPLC, and the fractions were dried in vacuo, yielding 225 mg of white, solid plate-shaped crystals, mp 231-234EC with decomposition; ^1H NMR (D_2O): δ 7.68 (d, 2H, *o*), 7.31 (d, 2H, *m*), 3.97 (s, 2H, NCH_2), and 3.57 (s, 2H, NH_2OCCH_2). The mass spectrum was consistent with the indicated structure, having a parent ion peak at $m/z = 236$. Purity was >99% by HPLC.

pKa Measurements

The ionization constants for the α -substituted-*p*-methylhippuric acids were measured by pH titration at 25EC at concentrations ranging from 0.004 to 0.4 M. A solution of each compound prepared in distilled/deionized water was stirred with dry nitrogen gas, and aliquots of an NaOH solution of known normality were delivered while the pH was monitored. Plots of pH versus volume of titrant were fitted to determine the pKa values using the appropriate theoretical model.

Partition Coefficient Determinations

Organic solvent (hexadecane, hexadecene, 1,9-decadiene, or octanol)/water partition coefficients for *p*-methylhippuric acid were determined by the shake-flask method (10) wherein 1 mL of a 5×10^{-3} M

solution of *p*-methylhippuric acid in 0.1 N HCl, I = 0.1 adjusted with NaCl, and 3-10 mL of organic solvent were allowed to tumble overnight at 25EC. The phases were separated and appropriately diluted or concentrated for analysis by HPLC.

Determination of Permeability Coefficients

LUV Preparation

The procedure employed to prepare LUVs was adapted from an earlier study (11). Briefly, egg-PC and egg-PA were accurately weighed and dissolved in chloroform in the desired 96/4 (egg-PC/egg-PA) ratio. Aliquots of this solution were then transferred into 12- × 75-mm culture tubes to provide 11.25 mg total lipid per tube. The chloroform was then removed under a dry nitrogen stream, and the lipid samples were further dried in vacuo at ~50EC for more than 30 minutes. An aqueous permeant solution (1.5 mL) in 0.04 M buffer, I = 0.1 (adjusted with NaCl) was added to the lipid film for a lipid concentration of 7.5 mg/mL. The aqueous solutions used the appropriate buffer species for each pH value, ie, 2-(N-morpholino)ethanesulfonic acid (MES), phosphate, carbonate, tris (hydroxymethyl) aminomethane (Tris), and borate. The lipids were suspended in the buffer solution by repeated vortexing at room temperature until the entire lipid film was removed from the glass surface. These lipid suspensions were then forced through 0.2- μ m polycarbonate filters (Nuclepore⁷, Pleasanton, CA) 17 times each at room temperature to form the LUVs.

The average vesicle hydrodynamic diameter, *d*, in each vesicle solution made was measured by dynamic light scattering (DLS). The DLS apparatus consisted of a goniometer/autocorrelator (Model BI-2030AT, Brookhaven, Holtsville, NY) and an Ar⁺ ion laser (M95, Cooper Laser Sonics, Palo Alto, CA) operated at 514.5-nm wavelength. The solutions used in the DLS measurements were prepared by adding 1 to 2 drops of LUV solution to 2.0 mL of the respective buffer solution. The sample was then placed in a cuvette holder with a toluene index-matching bath at room temperature. Auto-correlation functions were determined for a period of 100 seconds with a 20-

μ sec duration at a 90E angle and analyzed by the method of cumulants.

Transport Experiments

A 600- μ l aliquot of an LUV solution prepared as described above was loaded onto a size-exclusion column packed with Sephadex G-25M (PD-10 Sephadex G-25M, Supelco, Bellefonte, PA) in a 10-mL syringe, which was thoroughly equilibrated with the same buffer, without permeant, that was used to prepare the LUV solution to help ensure that the pH of the solutions inside and outside the LUVs were the same. The LUVs were then eluted by centrifugation (Model CL, IEC, Needham Heights, MA) at 2 successive speeds (2 minutes at 300g; 1 minute at g 500). The void volume of the size exclusion column contained the vesicles with entrapped permeant sufficiently separated from free permeant in solution. The LUV solution was collected into a 12-mL glass vial, which was capped and placed immediately in a 25EC water bath. A 600 :l aliquot of an LUV solution prepared as described above was loaded onto a size-exclusion column packed with Sephadex G-25M (PD-10 Sephadex G-25M, Supelco, Bellefonte, PA) in a 10 ml syringe which was equilibrated with the same buffer, without permeant, that was used to prepare the LUV solution. The LUVs were then eluted by centrifugation (Model CL, IEC, Needham Hts., MA) at two successive speeds (2 min at g 300; 1 min at 500g). The void volume of the size exclusion column contained the vesicles with entrapped permeant sufficiently separated from free permeant in solution. The LUV solution was collected into a 12 ml glass vial which was capped and placed immediately in a 25EC water bath. A 600 :l aliquot of an LUV solution prepared as described above was loaded onto a size-exclusion column packed with Sephadex G-25M (PD-10 Sephadex G-25M, Supelco, Bellefonte, PA) in a 10 ml syringe which was equilibrated with the same buffer, without permeant, that was used to prepare the LUV solution. The LUVs were then eluted by centrifugation (Model CL, IEC, Needham Hts., MA) at two successive speeds (2 min at 300g; 1 min at 500g). The void volume of the size exclusion column contained the vesicles with entrapped permeant sufficiently separated from free permeant in solution. The LUV

solution was collected into a 12 ml glass vial which was capped and placed immediately in a 25°C water bath. After elution of the vesicles from the size-exclusion columns, there existed a permeant concentration gradient resulting in a net flux across the LUVs from inside to outside. In order to measure this flux, the extra-vesicular permeant concentration was monitored at various time points. Aliquots (350 µL) of the LUV solution after gel filtration were loaded onto Centricon-100 filters (MWCO = 100,000; Amicon, Beverly, MA). The loaded filter was then centrifuged at 1600g for 6 to 8 minutes. The Centricon-100 filters did not significantly affect permeant concentration when the filters were pre-rinsed with water and dried prior to use. Concentrations of *p*-methylhippuric acid analogs in the collected filtrates were analyzed by HPLC, while concentrations of D-mannitol-[1-³H(N)] were determined by liquid scintillation counting (Beckman LS1801, Beckman Instruments, Fullerton, CA). The samples for HPLC analysis were analyzed without dilution except for the addition of a trace quantity of concentrated hydrochloric acid to lower the pH of the solution to less than 3 for optimal compatibility with the mobile phase. The concentrations of the *p*-methylhippuric acid analogs at t_4 were measured by lysing a 350-µL aliquot of the LUV solution after gel-filtration with 8 µL of Triton X-100 surfactant (Sigma, 10% in water) prior to HPLC analysis. The D-mannitol-[1-³H(N)] samples were prepared for liquid scintillation counting by adding an aliquot (100 µL) of filtrate to 3.5 mL of scintillation cocktail (Opti-Fluor, Packard Instrument B.V., The Netherlands).

HPLC Analyses

A modular HPLC system described previously (12) and a reversed-phase column packed with a 5-µm, C-18, 300 D stationary phase (Jupiter column 4.6 mm i.d. × 300 mm, Phenomenex, Torrance, CA) was used for all analyses. The mobile phase consisted of acetonitrile:water varying from 7% to 33% organic phase, depending on the lipophilicity of the compound. The aqueous phase was buffered to a pH of 3.0 using 0.01 M ammonium phosphate.

Mathematical Treatment of Transport Data

The permeant concentration in the vesicles varies with time according to the following kinetic equation:

$$-\frac{dC_{in}}{dt} = k_{obs}(C_{in} - C_{out}) \quad (\text{Eq. 1})$$

where C_{in} and C_{out} are permeant concentrations inside and outside the LUVs at time t , respectively, and k_{obs} is the first-order rate constant. Converting concentrations to mass, M , and assuming total mass of permeant to be constant, M_{total} , leads to the expression

$$-\frac{d(M_{total} - M_{out})}{dt} = k_{obs}(M_{total} - M_{out}) - k_{obs}\left(M_{out} * \frac{V_{in}}{V_{out}}\right) \quad (\text{Eq. 2})$$

where V_{in} and V_{out} are the total internal and external aqueous volumes, respectively. Solving this differential equation assuming $V_{in} \ll V_{out}$ and with the measured initial external concentration, C_{out}^0 , and infinity concentration, C_{out}^∞ , gives

$$-k_{obs}t = \ln\left(\frac{C_{out}^\infty - C_{out}}{C_{out}^\infty - C_{out}^0}\right) \quad (\text{Eq. 3})$$

Solving this equation for the external concentration of permeant at time gives

$$C_{out} = C_{out}^\infty - (C_{out}^\infty - C_{out}^0)e^{\left(-k_{obs}t\right)} \quad (\text{Eq. 4})$$

The transport data were fit to this equation by nonlinear least-squares regression analysis using Scientist⁷ (Micromath Inc., Salt Lake City, UT) to solve for k_{obs} . The average coefficient of determination for all of the data fit to this model was 0.986 with a standard deviation of 0.02.

The apparent permeability coefficient, P_{app} , can be obtained from this first-order rate constant and the ratio between the entrapped volume and surface area of the LUVs, V_{in}/A , from the equation

$$P_{app} = k_{obs} * \frac{V_{in}}{A} \quad (\text{Eq. 5})$$

The V_{in}/A ratio is obtained from the vesicle hydrodynamic diameter, d ($V_{in}/A = d/6$)

RESULTS AND DISCUSSION

Shown in Figure 2 is an image of a single molecule of *p*-methylhippuric acid (see structure in Figure 1) inserted into a DPPC bilayer consisting of 98 DPPC molecules and 2800 water molecules prepared using the Insight II/Discover software (Molecular Simulations Inc., San Diego, CA) and the cvff forcefield (T.-X. Xiang and B. D. Anderson, unpublished results). This image illustrates that the sizes of the permeants explored in this study are sufficiently small that the entire permeant may be accommodated within the acyl chain region of the bilayer. Deuterium NMR order parameters indicate that about 72% of the chain segments in the bilayer

interior lie within the highly ordered chain regions (13), which we have previously argued constitute the barrier domains located within each of the two halves of the bilayer (7,14). It appears that the length of *p*-methylhippuric acid is comparable to the thickness of these barrier regions.

Physicochemical Properties of p-methylhippuric Acid Analogs

Equilibrium ionization constants at 25EC of the *p*-methylhippuric acid compounds were needed to determine the molar fractions of neutral species HA, f_{HA} , at the pH values used in the transport experiments. These pKa values are listed in Table 1.

Table 1. Physicochemical Properties of the *p*-Methylhippuric Acid Series.

Compound ^a	PKa ^b	Partition Coefficient ^d			
		Hexadecane	Hexadecene	Decadiene	Octanol
2a	3.85 ± 0.01	(1.4 ± 1.4) × 10 ⁻⁴	(1.8 ± 0.1) × 10 ⁻⁴	(3.4 ± 1.2) × 10 ⁻⁴	12.2 ± 0.9
2b	3.70 ± 0.04	7.0 × 10 ⁻⁵	1.5 × 10 ⁻⁴	2.0 × 10 ⁻⁴	6.9
2c	3.66 ± 0.01	1.8 × 10 ⁻⁵	2.2 × 10 ⁻⁵	4.2 × 10 ⁻⁵	3.1
2d	3.66 ± 0.00	1.3 × 10 ⁻⁶	3.3 × 10 ⁻⁵	6.4 × 10 ⁻⁶	0.81
2e	3.69 ± 0.01	2.8 × 10 ⁻⁸	8.7 × 10 ⁻⁸	2.8 × 10 ⁻⁷	0.44
2f	3.53 ± 0.07 ^c	5.0 × 10 ⁻⁹	1.4 × 10 ⁻⁸	4.5 × 10 ⁻⁸	0.92
	4.54 ± 0.08 ^c				
2g	3.70 ± 0.02	8.0 × 10 ⁻¹⁰	2.4 × 10 ⁻⁹	1.5 × 10 ⁻⁸	0.11

^aFor structure see Figure 1. ^bValue ± 95% S-plane confidence interval ^cpKa₁ and pKa₂, respectively

^dExpressed as mean ± SD; PC data for substituted compounds are extrapolated from permeant **2a** and *p*-toluic acids in reference (9).

Also included in Table 1 are the organic solvent/water partition coefficient values (PC) for the series of *p*-methylhippuric acids at 25EC. The values for the unsubstituted *p*-methylhippuric acid were obtained using the shake-flask method described above, and the values in Table 1 for the substituted compounds were calculated from this value and functional group contributions obtained using the substituted *p*-toluic acid series (9).

Lipid Bilayer Membrane Permeability Coefficients

Transport experiments using the *p*-methylhippuric acid analogs as permeants were performed in the egg lecithin LUV system at 25EC. Figure 3 shows a representative plot of C_{out} versus time for *p*-methylhippuric acid at pH 9.97, along with the nonlinear fit to Equation 4, which was used to generate first-order rate constants. Apparent permeability coefficients were calculated from these first-order rate constants using the average diameter (from DLS measurements) of the LUVs of each

individual experiment, which ranged from 143 to 183 nm, and Equation 5.

Figure 3. Representative Extravesicular Concentration versus Time Plot Along with Non-Linear Fit for *p*-Methylhippuric Acid at pH 9.97.

Because the transport experiments of the *p*-methylhippuric acids use a wide range of pH values, it was necessary to determine the pH dependence of the properties of the lipid bilayer itself. Transport experiments using radio-labeled D-mannitol-[1-³H(N)] were performed in the egg lecithin LUV system in order to assess the possible pH dependence of the barrier properties.

Table 2. Apparent Permeability Coefficient for D-mannitol-[1-³H(N)] used to establish the Independence of Bilayer Resistance to pH.

pH	P_{app} (cm/sec) ^a
5.56	$(10.6 \pm 0.8) \times 10^{-11}$
5.98	$(10.4 \pm 0.9) \times 10^{-11}$
6.46	$(9.08 \pm 0.8) \times 10^{-11}$
6.97	$(11.5 \pm 1.2) \times 10^{-11}$
7.47	$(9.72 \pm 0.7) \times 10^{-11}$
8.03	$(10.0 \pm 0.7) \times 10^{-11}$
8.52	$(10.5 \pm 0.8) \times 10^{-11}$
8.87	$(10.5 \pm 0.5) \times 10^{-11}$
9.37	$(9.08 \pm 0.7) \times 10^{-11}$
9.67	$(8.98 \pm 0.6) \times 10^{-11}$
10.34	$(10.7 \pm 0.7) \times 10^{-11}$

^aExpressed as value $\pm$ SD

Mannitol is a nonionizable compound, so any change in its permeability with pH can be attributable to changes in the barrier resistance. Table 2 shows that the permeability coefficient for mannitol is independent of solution pH, in agreement with previous results obtained using egg lecithin bilayers and acetamide as the model permeant (14).

Figure 4 shows $\log(P_{app})$ versus pH plots for *p*-methylhippuric acid and the 6 methyl-substituted *p*-methylhippuric acids. The range of pH values selected for the permeability experiments was chosen such that the rate of transport is membrane controlled. A previously published study of *p*-toluic and its α -methyl-substituted analogs established that when the permeant of interest is a weak acid transport, it is completely governed by the fraction existing as unionized species over a wide range in pH (9). Equation 6 below expresses the apparent permeability coefficient in terms of the relative contributions of the unionized and ionized species

$$P_{app} = f_{HA}P_{HA} + (1 - f_{HA})P_A \quad (\text{Eq. 6})$$

where f_{HA} is the fraction of unionized species at each pH value. In Equation 6, the terms P_{HA} and P_A are the permeability coefficients for the unionized and ionized species, respectively. In the case of compounds 2a-2e and 2g, f_{HA} is given by

$$f_{HA} = \frac{[H^+]}{[H^+] + K_a} \quad (\text{Eq. 7})$$

For the dicarboxylic acid, 2f, P_{app} may include contributions from 3 species (ie, the unionized free acid, the monoanion and the dianion). The fraction of dicarboxylic acid in unionized form, f_{H_2A} , would be

$$f_{H_2A} = \frac{[H^+]^2}{[H^+]^2 + [H^+]K_{a1} + K_{a1}K_{a2}} \quad (\text{Eq. 8})$$

The permeability coefficients of the ionized species are considered to be many orders of magnitude smaller than that of the unionized species, P_{HA} or P_{H_2A} , because of their charge and the lipophilic nature of the lipid bilayer barrier domain.

Equation 6 suggests that the shape of $\log(P_{app})$ versus pH should be sigmoidal with an upper plateau region at low pH (below the pK_a of the permeant), reflecting the intrinsic permeability coefficient of the unionized species (in the case of highly permeable compounds, this region may represent unstirred layer controlled transport); a pH-sensitive region in which the overall transport continues to be governed by the unionized species, which is decreasing in concentration by a factor of 10 per unit increase in pH (this factor is 100 for the dicarboxylic acid, 2f, at pH values $> pK_{a2}$); and a lower plateau region, in which transport of the ionized species becomes an important contribution. The profiles in Figure 4 indicate that the data obtained in this study were within the pH-sensitive region. The slopes of the linear portions of these profiles were either -1 for compounds 2a-2e and 2g or -2 for compound 2f, consistent with Equation 6.

The permeability coefficients for each of the neutral species, P_{HA} (or P_{H_2A}), were determined by fitting the apparent permeability coefficient versus pH profiles to Equation 6 using Scientist⁷ and the expressions for f_{HA} in Equations 7 and 8. Although the fits were improved in some cases by including the term

representing the permeability of the ionized species, P_A , these values were considered unreliable because of the absence of sufficient data in the lower plateau region. The permeability coefficients of the neutral species for the compounds of the *p*-methylhippuric acid series diffusing through the egg lecithin lipid bilayers at 25EC are shown in Table 3, along with the same values obtained for the *p*-toluic acid series in a previous study (9).

Figure 4. Apparent Permeability Coefficients versus pH for the *p*-Methylhippuric Acid Series Along with Fitted Curves.

Table 3. Intrinsic Permeability Coefficients (P_{HA}) for Substituted *p*-Toluic Acids and *p*-Methylhippuric Acids.

Compound	$\underline{X}$	P_{HA} (cm/s) ^a	
		<i>p</i> -Toluic Acid ^b	<i>p</i> -Methylhippuric Acid
a	-H	1.1 ± 0.2	$(4.9 \pm 0.4) \times 10^{-4}$
b	-Cl	$(6.4 \pm 0.1) \times 10^{-1}$	$(3.5 \pm 0.5) \times 10^{-4}$
c	-OCH ₃	$(3.5 \pm 0.1) \times 10^{-1}$	$(1.0 \pm 0.1) \times 10^{-4}$
d	-CN	$(2.7 \pm 0.5) \times 10^{-2}$	$(9.2 \pm 1.0) \times 10^{-6}$
e	-OH	$(1.6 \pm 0.4) \times 10^{-3}$	$(5.5 \pm 0.4) \times 10^{-7}$
f	-COOH	$(1.8 \pm 0.3) \times 10^{-4}$	$(1.7 \pm 0.4) \times 10^{-7}$
g	-CONH ₂	$(4.1 \pm 0.4) \times 10^{-5}$	$(9.9 \pm 0.6) \times 10^{-9}$

^a Values expressed as mean $\pm$ SD.

^b Data from Reference (9)

Functional Group Contributions to the Standard Free Energy of Transfer into the Barrier Domain of the Lipid Bilayer

One of the main goals of these experiments was to verify that the functional group contributions to the

free energy of transfer of the compounds into the barrier domain of the bilayer are independent of the compound to which they are attached. This was done by comparing the contributions of the same functional group to the transfer free energies in 2 different series of compounds. The transfer free

energy of a given functional group, $\Delta(\Delta G^\circ)_{P,X}$, can be calculated using the equation

$$\Delta(\Delta G^\circ)_{P,X} = -RT \ln \left(\frac{P_{RX}}{P_{RH}} \right) \quad (\text{Eq. 9})$$

where P_{RX} and P_{RH} are the permeability coefficients of the substituted compound and reference or unsubstituted compound, respectively. The incremental free energy calculated in this way is primarily a measure of the contribution of the functional group to the transfer from an aqueous solution to the bilayer barrier domain. Values for $\Delta(\Delta G^\circ)_{P,X}$ previously reported were obtained using substituents attached to *p*-toluic acid and planar egg lecithin bilayers (9). The current study used α -substituted *p*-methylhippuric acids and egg lecithin lipid bilayers in the form of LUVs. LUVs are stable for several weeks, a requirement to obtain permeability data for the highly polar *p*-methylhippuric acid series. In order to minimize possible differences between the planar and vesicle systems, LUVs (143-183 nm) were employed as opposed to small unilamellar vesicles (SUVs), 40-70 nm, to minimize the possible effects of surface curvature (15).

The substituent free energy contributions obtained from permeabilities across egg lecithin bilayers at 25EC generated using both series of compounds listed in Table 4 and indicate that the values for each functional group are very similar.

Table 4. Functional Group Contributions to the Standard Free Energy of Permeant Transfer from Water Into the Egg Lecithin Lipid Bilayer Barrier Domain Determined from Transport Experiments.

Compound	X	$\Delta(\Delta G^\circ)_{P,X}$ (cal/mol)	
		<i>p</i> -Toluic Acid	<i>p</i> -Methylhippuric acid
a	-H	0	0
b	-Cl	330	200
c	-OCH ₃	690	930
d	-CN	2170	2360
e	-OH	3860	4030
f	-COOH	5170	4680
g	-CONH ₂	6060	6410

Location/Nature of the Barrier Domain.

The location of the barrier domain within the lipid bilayer (eg, polar head region versus hydrocarbon chain region) can be ascertained by determining the selectivity of the membrane to structural changes in the permeant. The permeability of a given compound reflects its partition coefficient into the barrier domain, $PC_{\text{barrier/water}}$, and its diffusion coefficient within the barrier, D_{barrier} , as described below

$$P = \frac{PC_{\text{barrier/water}} D_{\text{barrier}}}{d} \quad (\text{Eq. 10})$$

where d is the thickness of the barrier. In both bulk solvents (16) and lipid bilayers (17), the diffusion coefficient varies inversely with the minimum cross-sectional area of the permeant (ie, $D_{\text{barrier}} \propto [\text{molecular volume}]^{2/3}$). Figure 5 shows the minimum cross-sectional area view for each compound in the series of *p*-methylhippuric acids, along with the relative surface area normalized to that of the unsubstituted *p*-methylhippuric acid.

Figure 5. Minimum Cross-Sectional Surface Areas for Various Substituted *p*-Methylhippuric Acids Normalized to that of *p*-Methylhippuric Acid (assigned a value of 1.00)

Within each series examined, permeant size and cross-sectional surface area remain nearly the same, such that changes in diffusion coefficient can be neglected. This consistency suggests that a linear free energy relationship exists between the logarithms of permeability coefficients, P_{HA} , and bulk organic

solvent/water partition coefficients, $PC_{org/w}$ as described by

$$\log(P_{HA}) = s * \log PC_{org/w} + i \quad (\text{Eq. 11})$$

The slope, s , in this linear relationship is often referred to as the selectivity coefficient (8) and measures the relative chemical affinities of a series of solutes for the bilayer barrier domain versus the organic solvent chosen for the specific correlation. The organic solvent that most closely resembles the solvent nature of the barrier domain gives a value of s closest to 1. The intercept, i , is the value of $\log(P_{HA})$ when $PC_{org/w} = 1$.

Because the hydrocarbon/water partition coefficients for the substituted compounds of the *p*-methylhippuric acid series are so small (typically, $<1 \times 10^{-4}$), they could not be reliably determined using the shake-flask method. Therefore, they were estimated using the measured value for the unsubstituted compound, *p*-methylhippuric acid, and the functional group contributions obtained from the bulk solvent/water partition coefficients measured in the *p*-toluic acid series. The independence of functional group contributions to transfer free

energies in bulk solvents is well established (4,5). The values for 4 bulk organic solvent/water systems (hexadecane, hexadecene, 1,9-decadiene, and 1-octanol) are shown in Table 1. A given functional group's contribution to the standard free energy of transfer from water into a bulk organic solvent can be calculated from the partition coefficients of the substituted and unsubstituted compounds using Equation 12.

$$\Delta(\Delta G^\circ)_{PC,X} = -RT \ln \left(\frac{PC_{RX}}{PC_{RH}} \right) \quad (\text{Eq. 12})$$

A linear relationship between the functional group contributions calculated from permeability coefficients to those calculated from the partition coefficients for the *p*-methylhippuric acid series can be expected from Equations 9-11.

$$\Delta(\Delta G^\circ)_{P,X} = s * \Delta(\Delta G^\circ)_{PC,X} + i \quad (\text{Eq. 13})$$

In this equation, the term i is the intercept of the linear free energy relationship and the superscripts P and PC refer to values calculated from permeability and partition coefficients, respectively. The s values from Equation 13 are shown in Table 5.

Table 5. Selectivity Coefficients, s , Obtained from Correlations of Permeability Coefficients to Partition Coefficients in Various Bulk Solvent / Water Systems.

Model Solvent	<i>p</i> -Toluic Acid Series		<i>p</i> -Methylhippuric Acid Series	
	s	r	s	r
n-Hexadecane	$0.85 \pm 0.03^{a,b}$	0.999	0.86 ± 0.04	0.995
1-Hexadecene	0.91 ± 0.04^a	0.998	0.91 ± 0.04	0.995
1,9-Decadiene	0.99 ± 0.04^a	0.998	1.02 ± 0.06	0.991
Octanol	2.4 ± 0.5^a	0.961	2.4 ± 0.4	0.945
$-\Delta \log(PC)^c$	1.2 ± 0.1	0.986	1.2 ± 0.1	0.967

^aData from reference (9)

^bExpressed as mean $\pm$ SD.

^c $\Delta \log(PC) = \log(PC_{octanol/water}) - \log(PC_{hexadecane/water})$

A value for $s > 1$ implies that the barrier domain of the egg lecithin bilayer is more selective than the reference solvent to solutes varying in chemical structure. An s value of < 1 implies the opposite.

From Table 5 it is apparent that the barrier domain is most closely mimicked by the hydrocarbon 1,9-decadiene, which agrees well with previously published results from the transport of the *p*-toluic

acid series (9). Figure 6 shows the plot and correlation for $\log(P_{HA})$ versus $\log(PC)$ using 1,9-decadiene/water partition coefficients, including the compounds from both the *p*-toluic and *p*-methylhippuric acid series. The slope of this plot, which spans a permeability coefficient range of 8 orders of magnitude, is 1.00. This result firmly establishes the principle of independence for functional group contributions in permeants having molecular sizes that can be accommodated within the barrier domain.

Figure 6. Plot of $\log(P_{HA})$ versus $\log(PC)$ in 1,9-Decadiene / Water for Methyl-Substituted *p*-Toluic Acids and *p*-Methylhippuric Acids (Error bars represent standard deviations of P_{HA} measurements.)

1,9-Decadiene is a 10-carbon chain with double bonds at either end of the molecule. The alkyl chains within egg lecithin are generally from 16 to 18 carbons and have an average of 1 double bond per chain (18). In addition, the first double bond is most often located between carbons 9 and 10 from the polar head groups, which corresponds to regions of higher order (19,20). The barrier domain is likely to be located in this ordered alkyl chain region (9). It may be for this reason that the barrier domain is well modeled by 1,9-decadiene with its two double bonds. Octanol, a hydrogen-bonding solvent, poorly resembles the solvent nature of the barrier domain, yielding an *s* of 2.4. This value indicates that the barrier domain is much more selective to permeants than octanol, supporting the view that the barrier domain resides within the hydrocarbon chain region.

Also shown in Table 5 is the correlation of $\log(P_{HA})$ with $-\Delta\log(PC)$, which is the difference in the $\log(PC)$ values of the octanol-water and hexadecane-

water partition coefficient values. This term has been used by other authors (21) as a measure of the hydrogen bonding potential of a compound, which, they have argued, is the most important factor governing permeability in various biomembranes. Conceptually, correlations with $\Delta\log(PC)$ have been taken as evidence in support of the viewpoint that the rate determining step for solute transfer across biomembranes is the desolvation that accompanies the transfer of permeant adsorbed at the membrane interface into the hydrocarbon chain region. This conceptual picture implies that, as stated by Chikhale et al (22), lowering desolvation energies may be more useful than increasing lipophilicity for optimizing biomembrane permeability. Given the *s* value of 1.2 in Table 5 for the correlation of $\log(P_{HA})$ with $-\Delta\log(PC)$, however, the lipid bilayer permeability data suggest that 1,9-decadiene/water partitioning more closely mimics the selectivity of egg lecithin bilayers. Because decadiene/water partition coefficients reflect both permeant desolvation and lipophilicity, it appears that there is no need to invoke a hybrid partitioning scale such as $\Delta\log(PC)$ to fully account for the effects of permeant structure on bilayer permeability.

While the additivity and independence of individual constituent contributions to bulk phase partitioning are widely accepted, this study is the first, to our knowledge, to quantitatively establish for a variety of functional groups the principle of independence of group contributions to the free energies of transfer from water to the lipid bilayer barrier domain. The molecular size range over which functional group additivity is displayed remains unknown, however. Certainly, nonadditivity would be expected for 2 polar functional groups attached to opposite ends of a membrane-spanning alkyl chain, for example, as only 1 group at a time would reside in the barrier domain during the passage of such a molecule across a bilayer. Further studies are underway to explore the extent to which functional group additivity and independence hold with increases in molecular size.

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