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RESEARCH PAPER

Bilayer Composition, Temperature, Speciation Effects and the Role of Bilayer Chain Ordering on Partitioning of Dexamethasone and its 21-Phosphate

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ABSTRACT

Purpose Models to predict membrane-water partition coefficients (K_p) as a function of drug structure, membrane composition, and solution properties would be useful. This study explores the partitioning of dexamethasone (Dex) and its ionizable 21-phosphate (Dex-P) in liposomes varying in acyl chain length, physical state, and pH.

Methods DMPC:mPEG DMPE, DPPC:mPEG DPPE, and DSPC:mPEG DSPE (95:5 mol%) liposomes were prepared by thin film hydration. K_p values for Dex and Dex-P were determined from pH 1.5–8 by equilibrium dialysis and equilibrium solubility (Dex).

Results Dex K_p values at 25°C were 705 ± 24, 106 ± 11, and 58 ± 9 in DMPC, DPPC, and DSPC, increasing to 478 ± 20 in DPPC liposomes at 45°C. Both neutral and anionic species contributed to the K_p of Dex-P *versus* solution pH (1.5–8). A linear correlation was found between the natural logarithm of K_p and the inverse of bilayer free surface area ($1/a_{free}$) where a_{free} is a parameter reflecting chain ordering that depends on bilayer composition and temperature.

Conclusions Models of the pH dependence of partitioning of ionizable compounds must include contributions of both neutral and ionized species. Bilayer free surface area may be an important variable to predict K_p of drug molecules *versus* lipid composition and temperature.

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ABBREVIATIONS

Dex	Dexamethasone
Dex-P	Dexamethasone Phosphate
DMPC	1,2-dimyristoyl-sn-glycero-3-phosphocholine
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
mPEG-DSPE	1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
mPEG-DMPE	1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
mPEG-DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]

INTRODUCTION

The partitioning of drugs into lipid bilayers and biological membranes is a significant factor governing their tissue distribution and bioaccumulation (1–4). These properties, in turn, directly influence efficacy or toxicity when the mode of drug action involves interaction with membrane components (5,6) and may also affect efficacy and toxicity indirectly through their role in modulating drug delivery, pharmacokinetics, metabolism and clearance (7,8). As drug candidates have become more lipophilic and less water soluble there is a greater need for rapid and reliable screening methods and/or computational approaches to allow pharmaceutical scientists to select lead candidates based on their developability, as

indicated by properties such as their potential to exhibit good oral bioavailability (9,10). Guided by the perception originating in Overton's rule and the solubility-diffusion model that the equilibrium membrane-water partition coefficient should correlate with permeability across biomembranes (9,11,12) numerous researchers have attempted to devise experimental systems and computational models that link drug permeation to membrane affinity. Models that quantitatively predict either biomembrane partitioning or transport solely from a knowledge of the structure of the drug and composition of the membrane would be very useful.

For decades, it has been a common practice to predict membrane-water partitioning of drugs and also *in vivo* absorption based on various bulk solvent-water partition coefficients (10,13,14). Although different bulk solvents have been used to determine the partition coefficient, octanol has been the most referenced system (13). However, with time, deficiencies of bulk solvents as models for membranes have become more widely appreciated. Conceptually, these deficiencies mainly reflect the fact that bulk solvents are homogeneous and isotropic while lipid bilayer membranes are anisotropic interfacial phases with properties that vary as a function of internal distance from the bilayer-water interface (15–18). One striking experimental discrepancy noted in several recent publications is that linear free energy relationships based on bulk solvent/water partitioning fail to predict lipid bilayer/water partitioning. This has been particularly apparent in the lack of correlation between octanol/water partition coefficients and membrane water partition coefficients for drugs containing ionizable substituents (9,19–21). Ionized solutes may have higher affinity for lipid bilayer membranes than octanol due to the anisotropy of lipid bilayers, their high surface area-to-volume ratios, and electrostatic interactions of ionized solutes with charged moieties in the bilayer headgroups (19,20,22–24). Within the anisotropic microenvironment of the bilayer interface, solutes preferentially adopt conformations and orientations that maximize hydrophobic and van der Waals interactions between non-polar regions of the solute and acyl chains within the bilayer interior while retaining electrostatic interactions with the polar head groups and water molecules (25–28). The complexity of factors leading to that preferred solute conformation, orientation, and position within the bilayer that constitutes a minimum in free energy appears to have no close correlate in bulk solvent/water partitioning.

Another discrepancy between lipid bilayer/water *versus* bulk solvent/water partitioning is the well-known dependence of lipid bilayer/water partition coefficients on bilayer phase behavior and chain ordering. For example, decreasing the temperature below the phase transition temperature, inclusion of cholesterol in the bilayer, or elongating the phospholipid chain length, cause significant decreases in partition coefficient values (29–31) that are not easily rationalized using only bulk solvent models for the partitioning process.

In addition to their importance in drug delivery, liposomes are widely used as model membrane systems for studying the partitioning behavior of drugs (5,15,32–34). Determination of the liposome/water partition coefficient is essential for characterizing drug loading and the driving force governing release kinetics from liposomal delivery systems (35–37). More generally, such information can contribute to an understanding of several biological phenomena including passive transport and biodistribution (10,38).

A unified quantitative mathematical model that could predict the membrane-water partition coefficient as a function of drug structure, membrane composition, and the local aqueous microenvironment would be valuable both from a practical standpoint in assessing drug developability and for understanding drug biodistribution, clearance, efficacy, and toxicity. Such a model would need to take into account the concentrations of the various neutral and ionized drug species that may exist as a function of the pH of the aqueous microenvironment; the hydrophobic regions of the solute, the nature and location of various polar functional groups in the solute molecule and its conformational flexibility; and the surface charge, chain ordering and phase behavior, and other properties of the bilayer that in turn depend on lipid composition and the temperature, pH and ionic strength of the solution.

In the present study, we have explored the influence of bilayer composition and phase structure on the partitioning behavior of the anti-inflammatory steroid dexamethasone (Dex). These studies demonstrate a quantitative dependence of the partition coefficient of dexamethasone on the free surface area of the bilayer, a property related to acyl chain ordering that is modulated by changes in bilayer composition and temperature. Comparison of the present results to previous literature studies of drug partitioning into lipid bilayers suggests that this concept may have general applicability, as we have previously shown for drug permeability across lipid bilayer membranes (31,39,40). Phosphorylation of dexamethasone to produce the water soluble prodrug dexamethasone 21-phosphate (Dex-P) complicates its membrane binding behavior. The apparent partition coefficient of Dex-P as a function of pH, after correction for drug concentration effects, was determined in order to generate species-specific partition coefficients. Comparison of these species-specific partition coefficients for Dex-P to those for Dex in bilayers varying in lipid composition suggests that the influence of chain ordering as quantified by free surface area on partitioning behavior affects both nonpolar and polar solutes.

MATERIALS AND METHODS

Phospholipids including DSPC (1,2-distearoyl-sn-glycero-3-phosphatidylcholine, >99% purity), DPPC (1,2-dipalmitoyl-

sn-glycero-3-phosphocholine, >99% purity), DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, >99% purity), m-PEG-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]), mPEG-DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000]) and mPEG-DMPE (1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]) were purchased as powders from Avanti Polar Lipids (Alabaster, AL). Dexamethasone (Dex) (≥98%, powder) was purchased from Sigma-Aldrich Co., St. Louis, MO. Dexamethasone sodium phosphate (Dex-P), USP, was from Spectrum Chemical Mfg. Corp., New Brunswick, NJ. Dialysis flat sheets (Spectra/Por® RC Membrane, MWCO: 3.5 kD) were purchased from Spectrum Laboratories (Rancho Dominguez, CA). HPLC grade solvents and other chemicals were purchased from Fisher Scientific (Florence, KY). Deionized water was used for all experiments.

Liposome Preparation

Blank liposomes were prepared based on a previously reported method (36). Briefly, DMPC: mPEG-DMPE, DPPC: mPEG-DPPE and DSPC:mPEG-DSPE (95:5 mol%, 120 mg) were separately dissolved in 2 mL of chloroform. The solvent was evaporated under a stream of nitrogen while rotating the container and the resulting film was dried overnight in a vacuum oven. For determination of partition coefficient of Dex in different lipid systems, the lipid film was hydrated with 2 mL of phosphate buffered saline (PBS) (pH 7.4) and for Dex-P 20 mM acetate buffer (pH 4) was used. For the pH-dependent partition coefficient of Dex-P in DMPC liposomes, several buffers at different pH were used (20 mM KCl-HCl buffer, pH 1.5; 20 mM glycine buffer, pH 2; 20 mM acetate buffer, pH 4 and 5; 20 mM MES buffer, pH 6; 20 mM phosphate buffer, pH 8). To uniformly suspend the lipid, the suspension was alternately vortexed and heated in a water bath at a temperature above the phase transition temperature of the respective lipids. The lipid suspension was then extruded 10 times through two stacked 100 nm polycarbonate membranes (GE Water and Process Technologies, Trevose, PA) using an extrusion device (Liposofast®, Avestin, Canada) at 30, 50 and 60°C for DMPC, DPPC and DSPC, respectively. Liposomes were allowed to cool to room temperature for 3 h and stored at 4°C until further use. Particle sizes of blank liposomes were measured at 25°C by dynamic light scattering (DLS) using a Delsa™ Nano submicron particle size analyzer (Beckman Coulter Inc., Brea, CA). The liposomes were diluted in the same buffer as that used in their preparation. DMPC, DPPC and DSPC lipids are generally assumed to be neutral (41,42), but since the pKa of the headgroup of phosphatidylcholine is approximately 0.8 (43), the membrane surface charge of phosphatidylcholine bilayers becomes more positive as pH is decreased. However, Moncelli *et al.* (43) estimated that only 2.6% of the phosphatidylcholine groups would be protonated at pH 2.5.

Lipid Analysis

Lipid concentrations in the liposomal suspensions were determined by high performance liquid chromatography (HPLC) using an evaporative light scattering detector (ELSD) as reported previously for DSPC liposomes (44). Briefly, separation was achieved using an Allsphere™ silica column (Grace Davison Discovery Sciences, Deerfield, IL) (5 μ, 4.6×150 mm) with a guard column (Allsphere silica, 5 μ, 7.5×4.6 mm). A linear gradient method was employed starting with 100% (v/v) mobile phase A (80% chloroform:19.5% methanol:0.5% (v/v) ammonium hydroxide solution (30%)) and changing to 80% mobile phase A:20% mobile phase B (80% methanol:19.5% water:0.5% (v/v) ammonium hydroxide solution (30%)) over 3 min. This composition was maintained from 3 to 7 min followed by a return to 100% mobile phase A by 14 min. The total run time was 15 min at a flow rate of 1 mL/min with the ELSD setting at a gain of 8, pressure of 3.4 bar, and temperature of 40°C. The sample compartment temperature was set at 4°C and the column was at ambient temperature. Standards of DSPC, DPPC and DMPC were prepared in mobile phase A and linearity was observed between log concentration and log peak area.

Aliquots of liposomal suspension (10 μL) were dried under nitrogen and reconstituted in mobile phase A for lipid analysis before the start of each experiment. Since 95% of the phospholipid content in the liposomes was non-pegylated and the chain lengths for the pegylated and non-pegylated components were the same, the liposomes were assumed to contain 100% DMPC, DPPC or DSPC, respectively, for the determination of lipid content. Five mol% of pegylated lipid has been shown to be the optimum concentration for thermodynamic stability of the liposomes (45,46). Lipid stability was assessed under two representative conditions (Dex-P, pH 1.5 and 6, DMPC liposomes). Stability samples were withdrawn at different time points during dialysis for lipid analysis.

Determination of Membrane-Water Partition Coefficients

Equilibrium Dialysis Method

Membrane-water partition coefficients of Dex and Dex-P (Fig. 1) were determined at 25°C and varying lipid concentrations in three different liposomal systems (DMPC, DPPC and DSPC) by equilibrium dialysis using one mL Teflon® cells (Equilibrium Dialyzer (Spectrum Labs)). A liposome stock suspension of 60 mg/ml was diluted to varying concentrations and mixed with a drug solution having a fixed

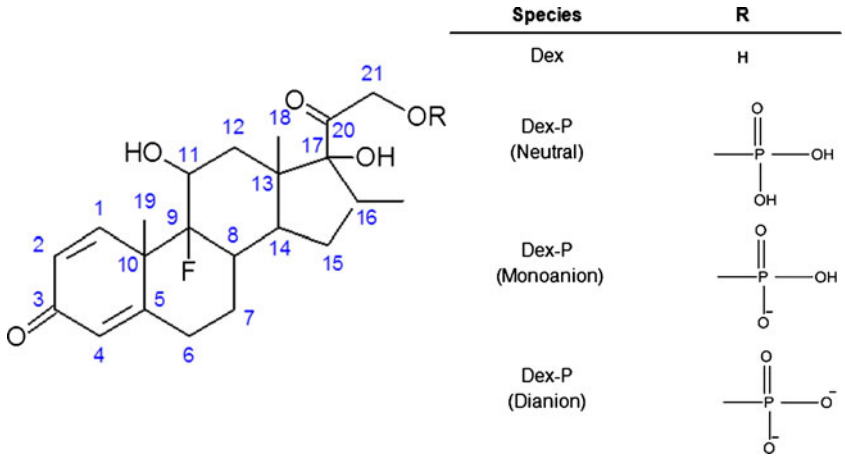

Fig. 1 Structures of Dex and Dex-P with different ionization states of the 21-phosphate.

concentration (Dex was 50–80 μM, Dex-P was 1–2 mM). For pH-dependent partition coefficients of Dex-P, the same buffers were used as that for liposome preparation and the pH of the Dex-P solution was adjusted after addition of the drug, if necessary. One ml of this mixture was introduced into one compartment (donor) of the equilibrium dialyzer and one ml of the corresponding blank buffer was added to the other compartment (receiver). The dialyzer, which consists of five pairs of one mL cells, was then placed in an incubator at 25°C and allowed to equilibrate. Aliquots (100–150 μL) were withdrawn at 24, 48 and 72 h from each compartment, diluted in 900 μL methanol and analyzed by HPLC. Equilibrium was considered to have been established when two subsequent time points yielded constant values (i.e., differences of < 7%). Partition coefficients of Dex were also determined in DPPC liposomes at 37°C and 45°C following the same procedure. The temperature was varied to study the effect of changes in bilayer phase structure on the partition coefficient.

Equilibrium Solubility Method

Partition coefficients of Dex in DMPC, DPPC and DSPC liposomes were also determined by the equilibrium solubility method. Excess solid (Dex) was equilibrated with blank liposome suspensions increasing in the liposome concentration (3–30 mg lipid/ml) at 25°C. After equilibration, samples were filtered through 0.45 μ PVDF filters and the filtrates were analyzed for drug concentration by HPLC after a 10-fold dilution in methanol. Errors due to filter adsorption were eliminated by analyzing successive aliquots of the filtrate to attain constant values. Aliquots of filtrate (10 μL) were dried under nitrogen and reconstituted in mobile phase for lipid analysis.

Simultaneous Determination of Dex and Dex-P by HPLC

A new isocratic HPLC method was developed and validated for the simultaneous determination of Dex and Dex-P with UV detection at 240 nm. Four independent standards for Dex-P (100–800 μM) in water and Dex (100–800 μM) in methanol were prepared. A Waters Alliance 2695 Separations Module coupled to a UV detector (Waters 996, Photodiode Array Detector) at 240 nm was employed. A Waters Symmetry® C18 column (5 μm, 3.9×150 mm) and guard column (3.9×20 mm) were used at an injection volume of 10 μL with a mobile phase composition of 32% acetonitrile and 68% (v/v) pH 5.5 triethylamine acetate (2%) buffer. The retention times for Dex-P and Dex at a flow rate of 1 mL/min were 2.5 and 6.4 min, respectively. The stability of Dex-P during equilibrium dialysis was monitored using this method as it allowed the simultaneous determination of Dex and Dex-P. The response factor for both analytes yielded a coefficient of variation less than 3% intraday and interday.

THEORY

Membrane-Water Partition Coefficient

At equilibrium, the total mass of drug in the donor (liposomal) compartment is the sum of the masses in the bilayer membrane and the aqueous phase:

$$C_d V_d = C_w V_w + C_m V_m \tag{1}$$

where C_d is the total drug concentration in the donor compartment, C_m and C_w are the drug concentrations in the lipid and aqueous phases, respectively, and V_d, V_m, V_w are the respective volumes. The aqueous concentration in the donor compartment (C_w) should equal the aqueous concentration in the receiver compartment (C_r) at equilibrium.

The volume based partition coefficient can then be defined by

$$K_p = \frac{C_m}{C_w} = \frac{C_d V_d - C_w V_w}{C_w V_m} = \frac{C_d V_d - C_r V_w}{C_r V_m} \tag{2}$$

Saturable Binding Correction for Neutral Dex-P

The concentrations of membrane bound Dex divided by the lipid concentration were proportional to the free drug concentration in solutions approaching infinite dilution. However, deviations from linearity were observed for Dex-P partitioning at pH 1.5 and 2, necessitating a correction for membrane saturation. At pH 1.5 and 2, membrane bound Dex-P exists predominantly as the neutral species (the reported first pKa of Dex-P in aqueous solution is 1.9 (47)). Therefore, the following equation was employed to fit the ratios of bound drug to lipid concentration (B) as a function of the unbound drug concentration (C_u):

$$B = \frac{K_p^{app} C_u}{1 + Const.\ C_u} \tag{3}$$

where K_p^{app} is the partition coefficient at infinite dilution at the solution pH specified. Unbound drug concentration (C_u) is the free aqueous drug concentration at equilibrium. *Const.* in Eq. 3 accounts for non-linearity at higher drug concentrations.

Gouy-Chapman Correction for the Partition Coefficient of Bound Monoanion

For membrane binding of ionized molecules, the partition coefficient decreases with the adsorbed concentration and needs to be adjusted to infinite dilution using Gouy-Chapman theory (48–53). At pH 4–6 in this study, the membrane bound drug was assumed to be predominantly monoanionic. Monoanion binding contributes to the development of a charge on the membrane surface, which in turn changes the surface potential as given by the Gouy-Chapman equation:

$$\sigma^2 = 2000 \in_0 \in_r RT \sum C_i \left(\exp\left(\frac{-z_i F \Phi_0}{RT} \right) - 1 \right) \tag{4}$$

where σ is the surface charge density, $\in_0$ is the permittivity of vacuum, $\in_r$ is the relative permittivity of water, R is the gas constant, C_i is the molar concentration of the ith electrolyte in the bulk solution, z_i is the signed charge number of that electrolyte, F is the Faraday constant and Φ_0 is the surface potential in volts.

Based on the number of moles bound per mole of lipid and the surface area of the phospholipids, σ was calculated and then using Eq. 4 the surface potential, Φ_0, was calculated. The apparent partition coefficient at infinite dilution K_p^{app} was calculated from the observed K_p^{obs} by the following equation,

$$K_p^{app} = K_p^{obs} \exp\left(\frac{z_D F \Phi_0}{RT} \right) \tag{5}$$

where z_D is the signed charge number of the drug.

pH Dependence of the Partition Coefficient (for Dex-P)

The apparent partition coefficient of Dex-P obtained at each pH value can be related to the concentrations of species D (unionized), D^- (monoanion) and D^{2-} (dianion) (Fig. 1) in the membrane and aqueous phases as follows,

$$K_p^{app} = \frac{D_m + D_m^- + D_m^{2-}}{D_w + D_w^- + D_w^{2-}} \tag{6}$$

$$k_p^{app} = \frac{K_{p1} + K_{p2} K_{a1}/H^+ + K_{p3} K_{a1} K_{a2}/(H^+)^2}{1 + K_{a1}/H^+ + K_{a1} K_{a2}/(H^+)^2} \tag{7}$$

where the subscripts m and w refer to the membrane and aqueous phases, respectively, K_{a1} and K_{a2} are the two ionization constants of Dex-P; and K_{p1}, K_{p2} and K_{p3} are the intrinsic partition coefficients of the three species D, D^- and D^{2-}, respectively. Over the pH range of interest in this study (pH 1.5–8) the properties of the DMPC bilayer itself were assumed to be independent of pH because the intrinsic pKa of the phosphatidylcholine head group of 0.8 (43) is still well below pH 1.5.

Bilayer Surface Density/ Free Surface Area

The normalized surface density is a parameter related to relative acyl chain ordering in the bilayer. It is defined as $\sigma = A_0/A$, where A is the area occupied per phospholipid molecule and A_0 is its area in the crystal (40.8 Å^2) (29,30,40). The normalized surface density varies from 0 to 1 with the value of 1 representing a completely ordered crystalline state. In the present study, the surface density values for the various liposome compositions were taken from a single compilation in the literature (40) and quantitatively related to the generated partitioning data.

Xiang and Anderson (40) related the surface density to the free surface area a_{free} of the bilayer by the following equation:

$$a_{free} = A - A_0 = A_0(1/\sigma - 1) \tag{8}$$

They showed that the free surface area served as a unified fundamental molecular packing parameter in lipid bilayers ($A_0/a_{free} = \sigma/(1-\sigma)$) for describing the permeability of solutes across both liquid crystalline and gel phases of the bilayer. An observed dependence of the lipid bilayer permeability coefficient on the inverse of free surface area was attributed, at least in part, to the sensitivity of solute partitioning into the interior hydrocarbon barrier domain of the bilayer to chain ordering as expressed by the following equation (40):

$$K_p = K_0 \exp\left(-a^*/a_{free}\right) \quad (9)$$

K_p in Eq. 9 refers to the barrier domain/water partition coefficient, not the overall bilayer/water partition coefficient that is of interest in this study, and K_0 is the partition coefficient for the same solute in a bulk solvent (e.g., a hydrocarbon) that was found to most closely mimic the chemical selectivity of the bilayer barrier domain and water. Xiang and Anderson (39) later examined the lipid bilayer permeability coefficients of a series of mono-carboxylic acids varying in the size of their alkyl portion to probe the dependence of bilayer transport on molecular size. When relative bilayer permeability coefficient measurements were ascribed to relative changes in apparent partitioning of the permeant into the barrier domain with increasing solute size, the quantity a^* in Eq. 9 was demonstrated to be proportional to the minimum cross-sectional area of the solute. Thus, sensitivity of lipid bilayer permeability coefficients to free surface area of the bilayer is a function of the permeant size.

A few previous studies of small molecule partitioning into lipid bilayers have revealed correlations between partition coefficients and the normalized surface density (29–31). However, the solutes employed in previous studies (i.e., hexane, benzene, and acetic acid) are hardly representative of typical drug molecules either in terms of their size or complexity. Herein, we seek to ascertain whether or not a dependence of the partition coefficient on bilayer free surface area is a more general phenomenon applicable to a broader array of drug molecules.

RESULTS

Liposome Characterization

Blank liposomes of DMPC, DPPC and DSPC, prepared for partitioning studies, had particle sizes in the range of 85–100 nm, with polydispersity indices in the range of 0.05–0.09, indicating monodisperse formulations. The extrusion procedure employed in this study was previously shown by ^{31}P-NMR, after correcting for the area difference between the inner and outer monolayers (~8%), to produce nearly unilamellar vesicles having a mean of 1.12 ± 0.03 bilayers per vesicle (54).

A simple analytical method using gradient HPLC and ELSD for evaluation of lipid concentration was developed for three different lipids DMPC, DPPC and DSPC. The retention times for DSPC, DPPC and DMPC were 7.9, 8.0 and 8.2 min, respectively. Lipid concentrations were determined from log-log plots of peak area *versus* concentration which were linear over the concentration ranges of 150–800 μM for DMPC, 100–400 μM for DPPC, and 150–400 μM for DSPC while the limit of quantification was about 80–100 μM for all the lipids.

Lipid concentrations in the liposomal samples were analyzed prior to equilibrium dialysis and used for calculation of volume based partition coefficients. The analyzed lipid concentrations were typically 25–35% lower than the theoretical concentrations indicating some lipid loss during the extrusion process. This highlights the necessity for analyzing the lipid concentration in order to obtain reliable partition coefficient values rather than just assuming no lipid loss. In order to assess lipid stability during the time required for equilibration (48 h), lipid concentrations were analyzed versus time for up to 72 h in representative experiments in DMPC liposomes (Dex-P) at pH 1.5 and 6 (Fig. 2). No significant change was observed in lipid concentration as judged by the 95% confidence interval of the slope from a first-order fit at pH 6. However, some loss of the lipid was detected at pH 1.5, with a first-order rate constant of 0.006 ± 0.0003 h^{-1} corresponding to a half-life of 115 ± 5 h, consistent with previous evidence that phospholipid hydrolysis is acid catalyzed (55,56). Importantly, no change in partition coefficients was discernible with time after equilibration indicating that a small percentage of lipid hydrolysis did not alter the lipid volume within the liposomes or the membrane affinity for the solute.

Membrane-Water Partition Coefficients

Dexamethasone

The membrane-water partition coefficient of Dex in DMPC, DPPC and DSPC liposomes at 25°C was determined at varying lipid concentrations by two different methods—equilibrium

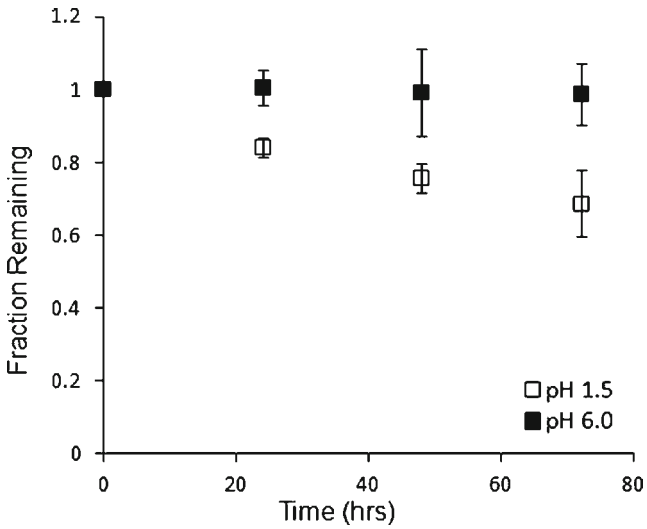

Fig. 2 Fraction of DMPC remaining *vs.* time during equilibrium dialysis experiments at pH 1.5 and 6. Each data point is the average of five different concentrations and the *error bars* are the standard deviations.

dialysis and equilibrium solubility. In the equilibrium dialysis experiments, the total drug concentrations were kept low and approximately constant with increasing lipid concentration (drug to lipid ratios varied from 0.0006 to 0.0052) to determine the partition coefficient in the linear region of the binding isotherm (Fig. 3). The bound drug to lipid ratios increased linearly with increasing unbound drug concentration (Fig. 3 inset) and the partition coefficients (705±24 in DMPC, 106±11 in DPPC and 58±9 in DSPC liposomes) were constant in the lipid concentration range studied. In the equilibrium solubility method, excess solid Dex was equilibrated with liposomes resulting in linear increases in solubility as a function of lipid concentration for DMPC, DPPC and DSPC liposomes (Fig. 4). The membrane-water partition coefficients determined from the equilibrium solubility method were also independent of the lipid concentration (Fig. 4 inset).

Partition coefficients of Dex in all three lipid systems determined by the equilibrium dialysis and solubility methods were combined in Fig. 5 to examine the effect of drug-to-lipid ratio on the partition coefficient, since the drug-to-lipid ratios at equilibrium were significantly higher in the equilibrium solubility method relative to the equilibrium dialysis method. In DPPC and DSPC liposomes, no significant differences in partition coefficients were observed at low *vs* high drug/lipid ratios as judged by the overlap of 95% confidence intervals for the average values from two methods. However, the drug-to-lipid ratios for Dex in DMPC liposomes were substantially higher for the equilibrium solubility method (~0.07) than those employed in equilibrium dialysis (<0.01), and in this case the partition coefficients differed significantly between the two methods.

The dependence of the partition coefficients on temperature was determined in DPPC liposomes using the equilibrium dialysis method, as shown in Fig. 6. Partition coefficients

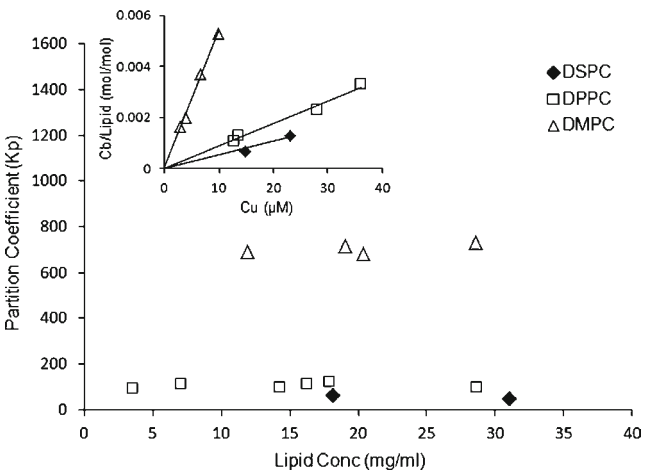

Fig. 3 DMPC, DPPC and DSPC membrane-water partition coefficients of Dex in PBS buffer at pH 7.4 as a function of lipid concentration determined by equilibrium dialysis at 25°C. *Inset* shows the corresponding binding isotherms.

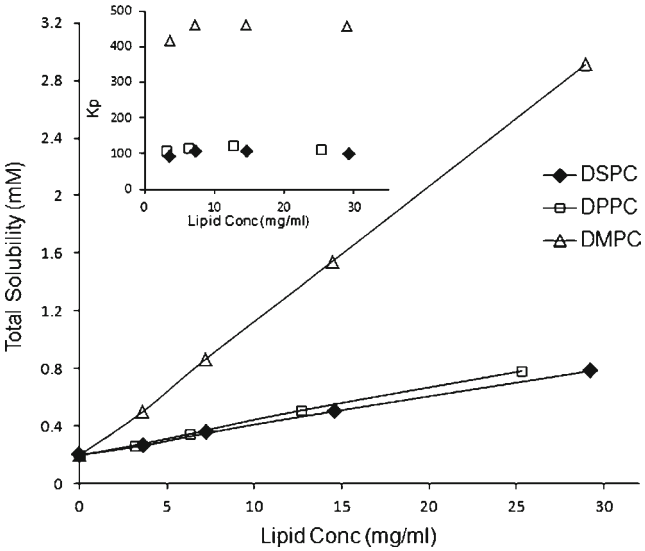

Fig. 4 Relationship between total equilibrium solubility of Dex and the lipid concentration in DMPC, DPPC, and DSPC liposomes at 25°C. *Inset* shows the effect of lipid concentration on the partition coefficients.

increased with temperature, varying from 106±11 at 25°C to 478±20 at 45°C (Fig. 6a). Linear binding isotherms were observed (Fig. 6a inset) in all cases, indicating that the partition coefficients represent the infinitely dilute region. The slope of a linear least squares fit of the van't Hoff plot shown in Fig. 6b yielded the molar enthalpy ($\Delta H° = 58.6$ kJ/mol) and entropy of transfer ($\Delta S° = 235.1$ J/mol.K) of Dex from water to DPPC liposomes.

Dexamethasone Phosphate

The 21-phosphate prodrug of Dex is ionizable and therefore partition coefficients of Dex-P were generated in DMPC

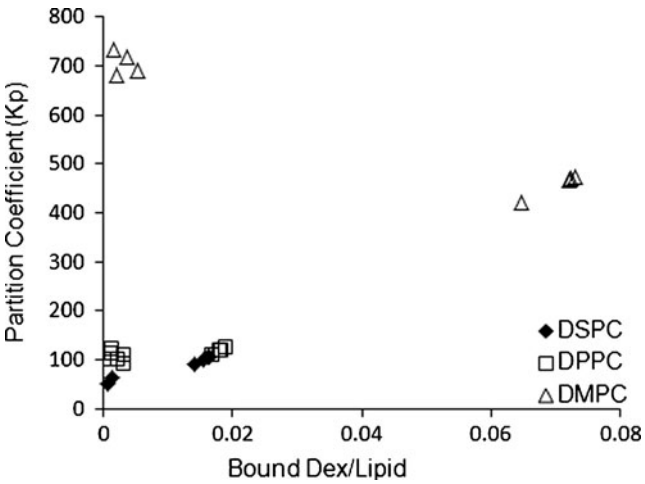

Fig. 5 DMPC, DPPC and DSPC liposomal membrane-water partition coefficients of Dex at 25°C *vs*. the bound drug-to-lipid ratios. The partition coefficients at bound Dex/lipid ratios below 0.01 were obtained by equilibrium dialysis and those above 0.01 were obtained by equilibrium solubility.

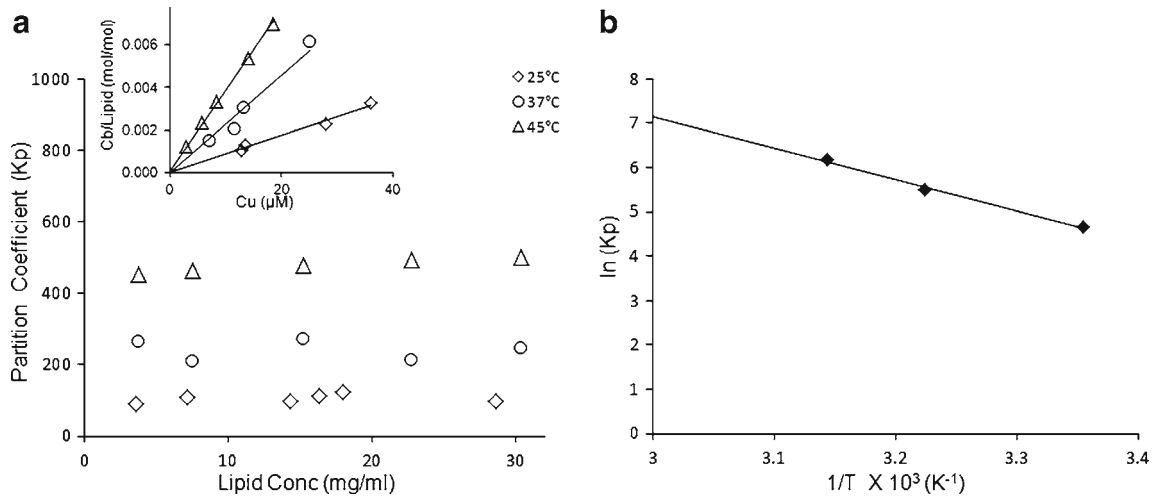

Fig. 6 (**a**) Influence of lipid concentration on the DPPC membrane-water partition coefficients of Dex at different temperatures. *Inset* shows the corresponding binding isotherms. (**b**) Van't Hoff plot of the DPPC membrane-water partition coefficients for Dex.

liposomes at 25°C as a function of aqueous solution pH from 1.5 to 8.0 to study the species dependence of its partitioning behavior. The partition coefficients generated at a fixed concentration of Dex-P and at various pH values are plotted *versus* lipid concentration in Fig. 7. At pH 1.5 and 2, systematic increases in the partition coefficients with increasing lipid concentration are evident. In this pH region, neutral Dex-P is the predominant bound species present because, although the first pKa of Dex-P is 1.9 (47), preferential binding of the neutral species relative to the monoanion results in a pKa shift for the membrane bound drug of approximately one unit. The binding isotherms (drug/lipid (mol/mol) ratio *versus* unbound drug concentration) for the data at pH 1.5 and 2 displayed in the inset to Fig. 7 suggest that membrane saturation may have been responsible for the decreasing partition coefficients with increasing unbound drug concentrations that accompany a decrease in liposome concentration. The solid lines in Fig. 7 (inset) represent fits of the data at pH 1.5 and 2 to Eq. 3. K_p^{app}, representing the partition coefficient at infinite dilution at the solution pH of interest, could be obtained from the linear portion of these fitted lines.

The partition coefficients at pH 4–6 are nearly constant with increases in lipid concentration (Fig. 7), though a slight upward drift is discernible at pH 4. In this pH region, the predominant membrane bound species is Dex-P monoanion. This conclusion is based on the experimentally determined pKa_2 value for Dex-P of 6.27 ± 0.13 (pH solubility method, our unpublished data) in aqueous solution combined with the observation in Fig. 7 that the partition coefficient for Dex-P at pH 8 is not significantly different from zero indicating that the concentration of Dex-P dianion in the membrane between pH 4–6 should be negligible. Anion partitioning to neutral phospholipid membranes

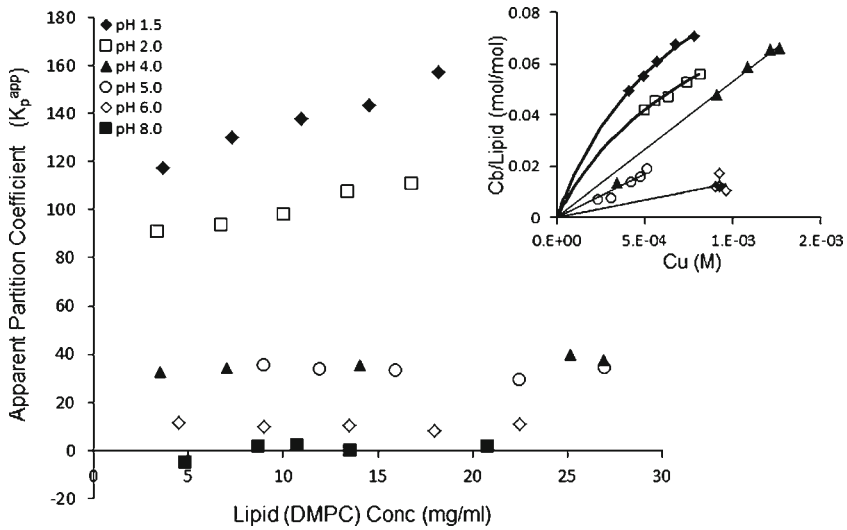

Fig. 7 Effect of lipid concentration on the apparent DMPC membrane-water partition coefficients of Dex-P at different pH values (25°C). *Inset* shows the binding isotherms (bound drug/lipid ratio *versus* unbound aqueous drug conc.) for data at pH 1.5, 2, 4, 5 and 6. The *solid curves* at pH 1.5 and 2 represent non-linear least-squares fits of the data to Eq. 3. Data at pH 4, 5 and 6 were corrected using the Gouy-Chapman equation (Eq. 5).

imparts a negative charge to the membrane that increases with the bound drug/lipid ratio. Typically, the Gouy-Chapman theory can be employed to correct for the effects of charge repulsion on the activity coefficient of the membrane bound anion in order to obtain a partition coefficient at infinite dilution (48–53). Such a correction, as described in Eqs. 4 and 5, was applied to the monoanion partitioning data at pH 4–6 resulting in the plots of drug/lipid ratio *versus* unbound drug concentration shown in the Fig. 7 inset.

After the appropriate corrections, K_p^{app} values for each pH applicable at infinite dilution were obtained and plotted in Fig. 8. These data were fit to Eq. 7 by nonlinear regression to generate the solid curve shown. For this analysis, pKa_1 and pKa_2 were fixed at their previously determined solution values of 1.9 and 6.27, respectively, and the partition coefficient for Dex-P dianion was assumed to be equal to zero. The partition coefficient for the neutral species was estimated to be 241±38 (95% C.I.) and for the monoanion, it was 48±9 (95% C.I).

Membrane water partition coefficients of Dex-P (at pH 4) were determined in DMPC, DPPC and DSPC liposomes as a function of lipid concentration by equilibrium dialysis (25°C) as shown in Fig. 9. The highest partitioning was observed in DMPC liposomes (62±8) (after Gouy-Chapman correction) while the values were dramatically lower in DPPC (2±4) and DSPC liposomes (6±2).

Effect of Bilayer Free Surface Area on Membrane-Water Partition Coefficients

The membrane water partition coefficients were significantly higher in DMPC liposomes as compared to DPPC and DSPC liposomes for both Dex and Dex-P (at pH 4). At 25°C DMPC, with a gel- > liquid crystalline phase transition, Tm, of 23°C is liquid crystalline while both DPPC (Tm=41°C) and DSPC (Tm=55°C) are in their gel states. Therefore, the acyl chain region in DPPC and DSPC bilayers is more highly ordered at 25°C in comparison to those in DMPC. To quantitatively probe the dependence of the partition coefficient on the degree of bilayer chain ordering, the natural logarithms of the partition coefficients (K_p) are plotted *versus* the inverse of free surface area as suggested by Eq. 9. For Dex, a linear relationship was observed between $ln(K_p)$ and free surface area of the bilayer ($1/a_{free}=\sigma/(1-\sigma)$) as illustrated in Fig. 10. In one of the partitioning systems (Dex in DPPC liposomes), the free surface area of the bilayer was altered by varying the temperature from 25°C to 45°C, through the phase transition temperature (41°C) of DPPC. Figure 10 shows the combined data

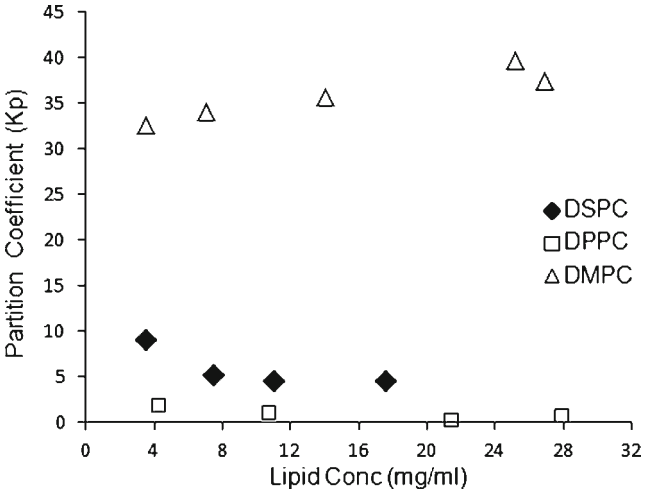

Fig. 9 DMPC, DPPC and DSPC liposomal membrane-water partition coefficients of Dex-P at pH 4 and 25°C determined by equilibrium dialysis *versus* lipid concentration.

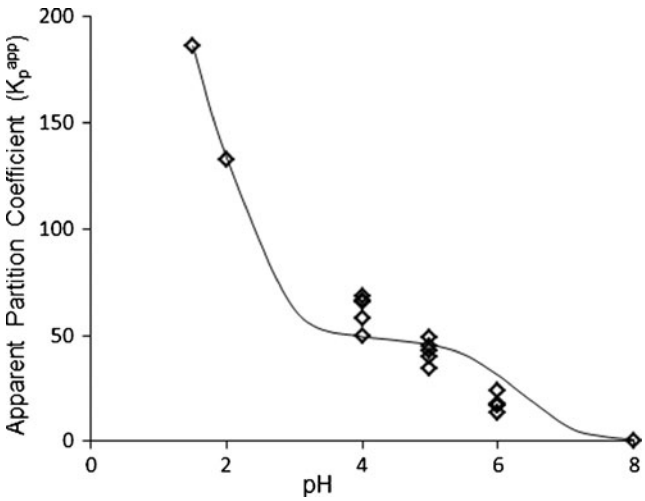

Fig. 8 Effect of pH on apparent partition coefficients of Dex-P in DMPC liposomes. The *solid curve* represents the best fit of Eq. 7 to the data by nonlinear least-squares regression analysis.

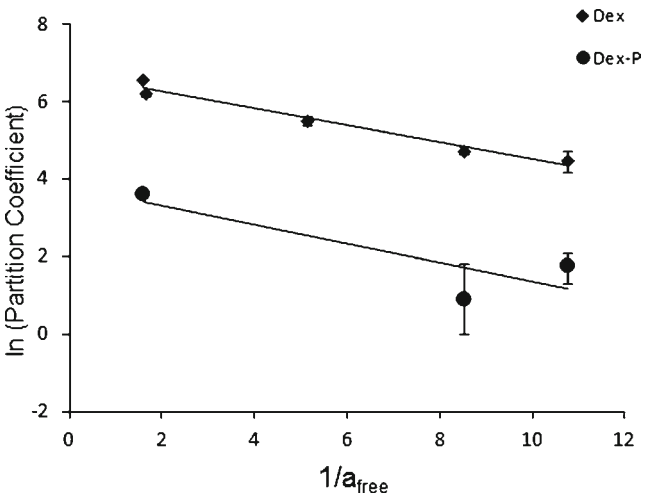

Fig. 10 Natural logarithms of the liposomal membrane-water partition coefficients of Dex and Dex-P *versus* the inverse of the bilayer free surface area, a measure of chain ordering as altered by phospholipid chain length and temperature.

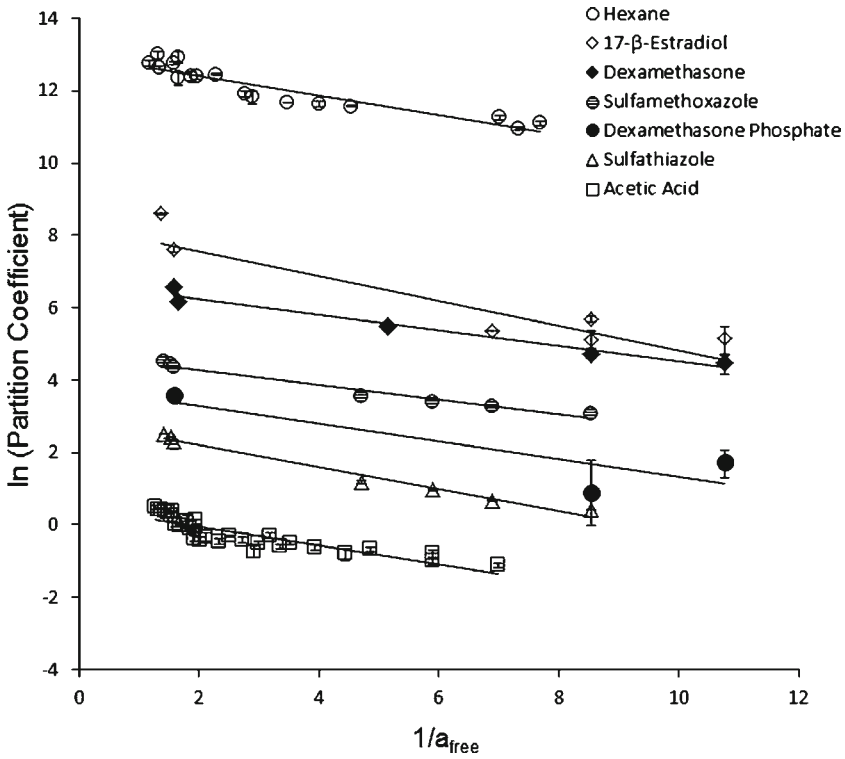

Fig. 11 Natural logarithms of membrane-partition coefficients for various solutes *versus* the inverse of free surface area ($1/a_{free}$). Key: hexane (30) (*white circle*, slope = −0.28 ± 0.03); 17-β-estradiol (60) (*white diamond*, slope = −0.34 ± 0.08); Dex (*black diamond*, slope = −0.22 ± 0.02); sulfamethoxazole (57) (*circle with horizontal lines*, slope = −0.21 ± 0.02); Dex-P (*black circle*, slope = −0.24 ± 0.15); sulfathiazole (57) (*white triangle*, slope = −0.30 ± 0.02); and acetic acid (31) (*white square*, slope = −0.26 ± 0.03). (The partition coefficient values are in mole fraction for hexane and in molarity for the other solutes). The slopes ± S.D. were obtained by linear regression according to Eq. 9.

for Dex partition coefficients when free surface area was varied either by varying phospholipid chain length (DMPC, DPPC, and DSPC) or varying temperature (DPPC, 25–45°C). The Dex-P partition coefficients at pH 4 appear to show a similar trend. Numerous studies in the literature have reported lipid bilayer/water partition coefficients of various solutes in similar fully saturated phospholipids as a function of either phospholipid chain length or temperature. Some representative examples taken from the literature (30,54,57–59) conducted in liposomal systems for which surface density data were available from the same source used in the present study (40) are plotted in Fig. 11 along with the partition coefficients generated for Dex and Dex-P in the present study *versus* the inverse of free surface area. In all cases explored, a similar trend of decreasing partition coefficient *versus* the inverse of bilayer free surface area was found with slopes in the plots of ln (K_p) *vs.* $1/a_{free}$ (see Fig. 11 legend) varying over a narrow range of approximately −0.2 to −0.3. The slope of each plot provides an estimate of the value of a* in Eq. 9 for the compound represented in the plot.

DISCUSSION

Liposome/water partitioning studies appear to offer clear advantages in predicting the biodistribution of drugs because ordered lipid bilayers more accurately mimic biological membranes than bulk solvents. These advantages are particularly apparent for ionizable compounds (19,20,22). Additionally, a quantitative understanding of liposomal partitioning is essential for designing liposomal drug delivery systems with predictable loading and tunable release characteristics (35,36,44,61). However, the interfacial nature of lipid bilayers adds complexity both in terms of generating reliable experimental data and in interpreting the values obtained. Proper correction for surface charge effects on binding of ionized species (48) and saturation phenomena for neutral species (52,62) may be necessary with increasing drug concentration. For ionizable drug molecules, quantitative mathematical models describing the contributions of neutral and ionized drug species are needed to account for the pH dependence of membrane partitioning. Finally, the experimental partitioning system employed in terms of liposome composition (e.g., head group, acyl chain length, degree of unsaturation, presence of cholesterol, etc.), size and lamellarity, temperature, and the like can be significant factors in determining the results obtained. Presently, a different LFER equation is required for each lipid system (59), highlighting the need for unifying relationships that could quantitatively predict membrane-water partitioning from a knowledge of drug structure, membrane composition, and the environmental conditions.

Some of the pioneering work on bilayer/water partitioning has revealed that factors such as decreasing the temperature below the phase transition temperature, inclusion of cholesterol in the bilayer, or elongating the phospholipid chain length cause decreases in partition coefficients that can be

correlated with order parameters such as surface density of the bilayer chains (29,30) or free surface area of the bilayer (31). The solutes typically employed in these studies have been simple, small molecules such as benzene, hexane, and acetic acid. Whether or not such relationships apply to larger and more structurally complex drug molecules has not been established. The present study of the partitioning behavior of Dex and Dex-P provides an opportunity to begin to explore the hypothesis that the bilayer free surface area is a "universal" variable useful in relating membrane-water partition coefficients to bilayer composition.

Factors Governing the Membrane-Water Partition Coefficient

Drug Concentration & Drug-to-Lipid Ratio

Determination of reliable membrane-water partition coefficients requires measurement of the bound drug-to-lipid ratio at equilibrium. As noted by De Young and Dill, it is very important to extrapolate partitioning data to infinite dilution for their proper interpretation. Unlike adsorption to solids that might be construed as having a fixed number of binding sites, solute binding to bilayer membranes may involve partial or complete insertion into the membrane with accompanying changes in both bilayer surface area and the chemical nature of the surface, with an increase in the bound drug/lipid ratio. These alterations with increasing solute uptake may lead to either increases or decreases in the apparent partition coefficient with drug concentration (29,50,63,64). Alternatively, self-association of drug molecules at high aqueous drug concentrations may reduce the activity of the free drug available for partitioning (65).

The importance of determining partition coefficients at concentrations approaching infinite dilution is evident from the difference in the partition coefficients obtained for Dex from the two different methods employed in this study. In the equilibrium dialysis method, the drug-to-lipid ratio could be varied with values approaching infinite dilution (~0.001) (Fig. 3 inset) whereas in the equilibrium solubility method, the drug to lipid ratio in DMPC was constant at 0.07 (±0.004) even with varying lipid concentration as dictated by the equilibrium solubility of Dex and the DMPC-water partition coefficient. The equilibrium solubility method could not be used to probe DMPC partitioning in a concentration region approaching infinite dilution and therefore the values obtained using this method differed from those obtained by equilibrium dialysis (Fig. 5). This was not the case for Dex partitioning in DPPC and DSPC where the drug-to-lipid ratios at equilibrium were <0.02 using the solubility method. For DPPC and DSPC, the partition coefficients were the same by either method.

While generally higher drug-to-lipid ratios are possible for neutral molecules in comparison to ionized compounds before deviations from linearity in sorption isotherms become apparent, the borderline appears to vary with the compound and lipid. Escher found no deviations from linearity in neutral compound sorption isotherms up to drug-to-lipid ratios of 0.1 (24) while Austin *et al.* suggested that the ratio of lipid to bound neutral compound should be greater than 60 (19) which would translate to a drug-to-lipid ratio of <0.017. As noted above, we found a significant deviation in the partition coefficient for Dex at a drug-to-lipid ratio of 0.07 but using equilibrium dialysis at drug-to-lipid ratios <0.007 the partition coefficients were independent of drug-to-lipid ratio (Figs. 3 and 6 insets).

Deviations in the sorption isotherms for Dex-P at pH conditions where the bound drug is predominantly neutral were clearly evident at drug-to-lipid ratios above 0.04. Eq. 3 was therefore used to extrapolate these results to infinite dilution. Langmuir-type adsorption models are commonly employed to account for saturation effects in membrane partitioning of drug molecules (66,67). While the assumption of a fixed number of binding sites may not accurately reflect the nature of the binding of molecules to flexible bilayer membranes, it might be justifiable for drugs that bind predominantly at the membrane surface (51). Changes in partitioning at high drug concentration are more likely due to changes in the membrane or in the affinity of the drug molecule for the modified membrane surface rather than a depletion of binding sites (50). However, at infinite dilution, partitioning and association models are equivalent.

Sorption isotherms for ionized molecules become nonlinear mainly because of the repulsive forces between the charged species upon membrane binding (48,49,51–53,62). Austin *et al.* suggested that for ions also, a ratio of lipid to bound ion >60 should be sufficient to render surface charge effects insignificant (19), although the Gouy-Chapman theory can be used to correct for surface charge effects at higher drug concentrations (Fig. 7 inset) (17,24,48,49,51,52,62,68,69). Although Austin *et al.* (48) found that ion-pair formation was negligible in partitioning of ionized drugs in liposome–water systems compared to octanol-water, others have invoked ion-pair formation to account for membrane transport of ionized species (70–72).

Temperature Dependence of Partition Coefficients

The fundamental driving force for transfer of nonpolar solutes from water to a lipid environment is generally assumed to be the hydrophobic effect but the degree of chain ordering in the bilayer modulates the overall thermodynamics of the process (73,74). The dependence of the membrane-water partition coefficient of Dex was measured in DPPC liposomes as a function of temperature from 25 to 45°C, a range that crosses the gel- > liquid crystalline transition temperature of 41°C. These results are displayed in the van't Hoff

plot in Fig. 6b. The standard free energy change (ΔG°) upon transfer of Dex from water to DMPC liposomes was negative at all temperatures indicating an energetically favorable process, driven by a positive entropy (ΔH° = 58.6 kJ/mol and ΔS° = 235.1 J/mol.K). The thermodynamic parameters for the transfer of Dex compare reasonably well qualitatively with values reported elsewhere for corticosteroid transfer into DMPC bilayers both above and below its Tm, supporting the observations of an entropy driven process for the partitioning of steroids into saturated phospholipids (58,75). One contribution to the positive entropy is that associated with the removal of the steroids from water (15,75,76).

Kwon *et al.* obtained the enthalpy and entropy changes for partitioning of several endocrine disruptors between water and liposomes formed from DOPC, POPC, DMPC, DPPC, and DSPC at temperatures ranging from 11–37°C (60). Over this temperature range DOPC and POPC liposomes are liquid crystalline and DPPC and DSPC liposomes are in a gel phase. DMPC undergoes a phase transition within the middle of this temperature range at 23°C. Generally, they found negative enthalpies of solute transfer from water to liquid crystalline bilayers consisting of unsaturated lipids and positive enthalpies for the transfer of solutes from water to gel phase membranes composed of saturated phospholipids. Others have reported similar findings (77,78). Such disparities illustrate the greater complexity of the membrane binding process relative to that for solute partitioning from water to bulk solvents and the importance of bilayer chain ordering.

Other factors may also contribute to the thermodynamic driving force for membrane binding depending on the location of binding and the structure of the solute (60,79). While the transfer of a hydrophobic solute from water into the hydrophobic core of a bilayer is entropically driven, favorable enthalpic contributions may dominate if a drug is preferentially bound to the interface due to electrostatic interactions between ionized groups on the drug and phospholipid. For example, cationic drugs have exothermic partitioning as compared to neutral compounds (22). Thus, the relative contributions of entropy driven hydrophobic effects, chain ordering effects, and electrostatic interactions between the drug and membrane interfacial regions all combine to determine whether a given transfer process will be primarily entropy or enthalpy driven.

Structure-Partitioning Relationships—21-Phosphate Group Contribution & Ionization

As reported in Table I, the Kp value for Dex at 25°C varies from 705 ± 24 in liquid crystalline DMPC to 106 ± 11 in DPPC and 58 ± 9 in DSPC bilayers, both of which are in their gel state at room temperature. These results illustrate the dramatic effect that simply changing the chain length of the phospholipid can have on the partition coefficient. Given such differences, it is understandable that a different LFER equation has been required for each lipid system (59).

The contribution of the polar phosphate group at the 21-position of dexamethasone to the free energy of transfer from water to DMPC depends on its state of ionization. Fitting the K_p^{app} *vs.* pH data in Fig. 8 to the model described in Eq. 7 generated values of K_p for the neutral and monoanion forms of Dex-P of 241 ± 38 (95% C.I.) and 48 ± 9 (95% C.I.), respectively. Comparing the neutral K_p values for Dex *vs.* Dex-P, phosphorylation of Dex reduces its partition coefficient by only 3-fold, giving a group contribution for the unionized phosphate residue to the transfer free energy from water to DMPC of only Δ(ΔG°) = 2.66 kJ/mol (0.64-kcal/mol). Monoanion formation reduces K_p by another 5-fold and dianion formation leads to a further decrease such that at pH 8, K_p is very small.

Several reports indicate that amphiphilic molecules orient themselves at the interphase of bilayers such that charged or polar groups interact with lipid head groups while their non-polar regions are embedded in the hydrocarbon region of the bilayer (27,34,80,81). Vijayan *et al.* (81) conducted MD simulations of the location and orientation of cortisone, a corticosteroid similar in structure to Dex, in a model lipid bilayer. They observed that, unlike cholesterol which aligns itself approximately parallel to the bilayer chains, cortisone adopts an orientation that is nearly parallel to the bilayer surface such that its polar groups are able to maximize favorable contacts in the heterogenous bilayer interface region. With the increase in charge and hydrogen bonding potential accompanying 21-phosphorylation, the molecule is likely pulled further towards the interface such that particularly the phosphate monoanion residue remains solvated by water. The reduction in the partition coefficient reflects the greater amount of energy needed to break the hydrogen bonds and remove the hydrophobic portions of the solute from water (20,82).

Effect of Bilayer Free Surface Area

Unlike bulk solvents, lipid bilayers are interfacial phases having properties that depend on surface density or free surface area. The acyl chains in bilayers are more highly ordered near the headgroups and the order diminishes with distance towards the bilayer center (83). The free surface area and the length of the phospholipid acyl chains are the principal independent variables that determine the chain organization (30). We studied the effect of changes in free surface area that accompanied alterations in phospholipid acyl chain length and variations in temperature.

At the temperatures involved in the current study, DSPC was in a gel phase and DMPC was in its liquid crystalline

Table I Membrane-Water Partition Coefficients of Dex and Dex-P Under Different Conditions

Drug	Liposome	pH	Temperature (°C)	Kp (Avg. ± SD) (Eq. Dialysis)	Kp (Avg. ± SD) (Eq. Solubility)
Dex	DMPC	7.4	25°C	705 ± 24 ($n = 4$)	458 ± 25 ($n = 4$)
	DPPC	7.4	25°C	106 ± 11 ($n = 6$)	118 ± 6 ($n = 4$)
	DPPC	7.4	37°C	241 ± 30 ($n = 5$)	–
	DPPC	7.4	45°C	478 ± 20 ($n = 5$)	–
	DSPC	7.4	25°C	58 ± 9 ($n = 2$)	102 ± 7 ($n = 4$)
Dex-P	DMPC	1.5	25°C	186 ± 10 ($n = 5$)	–
	DMPC	2	25°C	133 ± 9 ($n = 5$)	–
	DMPC	4	25°C	62 ± 8 ($n = 5$)	–
	DMPC	5	25°C	42 ± 5 ($n = 5$)	–
	DMPC	6	25°C	18 ± 5 ($n = 5$)	–
	DMPC	8	25°C	0.07 ± 3 ($n = 5$)	–
	DPPC	4	25°C	2 ± 4 ($n = 4$)	–
	DSPC	4	25°C	6 ± 2 ($n = 4$)	–

state ,while DPPC was in a gel state at 25 and 37°C, and liquid crystalline at 45°C. Irrespective of the ordering mechanism (temperature or lipid chain length), the partition coefficient of Dex was found to be negatively dependent on the inverse of free surface area (Fig. 10). A linear relationship was obtained between $ln\ (K_p)$ and the inverse of free surface area ($1/a_{free} = \sigma/(1-\sigma)$) for Dex (slope = −0.22 ± 0.02 (S.D.)). Dex-P at pH 4.0 exhibited a similar dependence on free surface area with a similar slope (−0.24 ± 0.15 (S.D.)). Dex-P, the prodrug of Dex having the same steroidal parent structure and differing only in the presence of a phosphate at the 21-position, is predominantly monoanionic at pH 4.0. The similarity in slopes indicates that the sensitivities of the partition coefficients of the two molecules, one neutral and one anionic, to bilayer chain ordering as quantified by the free surface area parameter are similar.

There are numerous reports in the literature of decreases in membrane partition coefficients of various solutes with increases in phospholipid chain length, changes in the degree of chain unsaturation, decreases in temperature, or incorporation of cholesterol into liquid crystalline bilayers (29–31,58,59,64). Yamamoto and Liljestrand (59) observed a 20-fold decline in the room temperature partition coefficient of estradiol when the liposome composition was changed from POPC to DPPC. This substantial effect of lipid composition on solute partitioning was attributed to the influence of chain saturation on the main transition temperature. At room temperature POPC liposomes exist as a liquid crystalline phase while DPPC is in a gel phase.

The lattice theory developed by Marqusee and Dill (16) predicts decreases in solute partitioning with increasing bilayer surface density due to an increasingly unfavorable entropy associated with solute insertion as orientational ordering of the phospholipid chains is increased. Experimentally, correlations have been observed between the partition coefficient for small molecules (hexane, benzene and acetic acid) and surface density of the lipid bilayer (29–31). Xiang and Anderson combined statistical mechanical theory with molecular dynamics simulations to show that the lateral pressure in a bilayer increases steeply with surface density (i.e., decreasing free surface area) and that increased lateral pressure correlates with higher local order and exclusion of solutes from the interphase (18). In other words, increased chain-chain interactions at higher chain density inhibits solute incorporation (59,64,65,84).

The magnitude of solute exclusion accompanying chain ordering may depend on the size or other features of the solute. To explore whether a general, quantitative relationship can be found between bilayer chain ordering and membrane-water partition coefficients, we plotted in Fig. 11 the natural logarithms of the bilayer-water partition coefficients of several compounds from studies published by other labs against the inverse of free surface area, $1/a_{free}$. These studies employed similar phospholipids as those in the present study. The average of the slopes was −0.26 with a 95% confidence interval of −0.22 to −0.30, thus establishing that a negative relationship exists between membrane binding and the inverse of free surface area of the bilayer.

The effect of bilayer chain ordering can be expected to be more dramatic for trans-bilayer transport *versus* membrane binding as in the latter event a given solute may be only partially inserted into the bilayer at equilibrium. Xiang and Anderson (40) previously determined both partition coefficients and permeability coefficients for acetic acid as a function of surface density. They found a slope for the natural logarithm of the partition coefficient of acetic acid *versus* the inverse of free surface area in Fig. 11 of −0.26. This compares to a much steeper slope (−0.71) when their permeability data for acetic acid are plotted in the same manner, a 2.7-

fold difference. This is attributed to the requirement for passage of the solute across the bilayer barrier region for permeation whereas partitioning involves only partial insertion into the interfacial region of the bilayer for acetic acid (31,40). In all cases, there is a dependence of the partitioning on the available free surface area, as demonstrated by Fig. 11 for several different solutes varying in size and chemical properties. Generally, drug molecules having both polar and hydrophobic residues may bind in the interfacial region of bilayers with only portions of their structures embedded into the ordered alkyl chain region depending on their lipophilicity. The similarity of the slopes obtained for all of the representative solutes illustrated in Fig. 11 suggests that the dependence of partitioning on chain ordering is not highly sensitive to either the overall size or chemical structure of the solute. The relatively uniform but modest dependence of membrane binding on bilayer free surface area may reflect the availability of hydrophobic surface area on the membrane, which is the surface property that increases with an increase in free surface area. While the above relationship appears to be valid for partitioning to a sub-set of bilayers composed of saturated phospholipids both above and below their phase transition temperatures, further studies in a more diverse set of membranes are necessary to determine the universality of this relationship.

CONCLUSIONS

The bilayer/water partition coefficient of a drug is dependent upon several factors such as the bound drug-to-lipid ratio, pH, temperature, membrane composition, etc. Appropriate corrections for neutral and ionized species at high concentrations are required in order to determine a reliable partition coefficient value (at infinite dilution). The membrane-water partition coefficient of Dex at infinite dilution was studied in liposomes of varying composition. Increasing the negative charge of Dex-P gradually reduced the partition coefficient, ultimately overcoming the hydrophobic contribution to membrane insertion provided by the steroid rings. In order to explore the possible existence of a relationship between membrane-water partition coefficients and bilayer chain ordering, the partition coefficients were related to the inverse of bilayer free surface area. For both, Dex and Dex-P, the natural logarithms of the partition coefficients in liposomes decreased linearly with the inverse of free surface area in the bilayer as modulated by increasing phospholipid chain length or decreasing temperature. Inclusion of membrane partitioning data in saturated lipid systems for other solutes from the literature, showed a similar dependence on the free surface area. These results may be useful in the development of more comprehensive quantitative models relating membrane-water partition coefficients of drug molecules to bilayer properties.

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