HYDROPHOBIC ION INTERACTIONS WITH MEMBRANES

Thermodynamic Analysis of Tetraphenylphosphonium Binding to Vesicles

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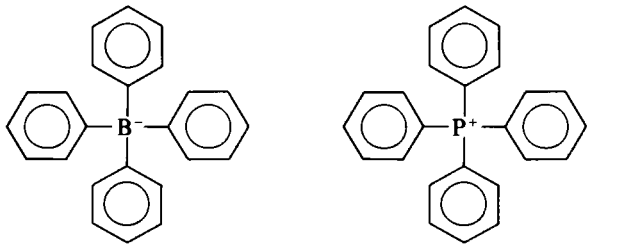
ABSTRACT The thermodynamic properties for the interaction of the hydrophobic ion tetraphenylphosphonium (TPP^+) with egg phosphatidylcholine vesicles were studied in detail by equilibrium dialysis and spin label techniques. A partition coefficient of $\beta = 4.2 \pm 0.4 \times 10^{-6}$ cm ($K \simeq 100$) was determined. Electrostatic saturation sets in at ~ 600 μM (about one adsorbed TPP^+ molecule per 100 lipids), and is not screened by salt. The temperature dependence of binding was determined, which reveals that the binding is entropy-driven with a positive (repulsive) enthalpy of binding, a result to be compared with hydrophobic anions in which the binding enthalpy is negative. The membrane dipole potential may be responsible for this binding difference. Activity coefficients are determined and shown to be significantly different from those of most common salts, an important result that should be considered in all hydrophobic ion studies. Comparison of the TPP^+ results with those of its anionic structural analogue, tetraphenylboron (TPB^-), permits a general analysis of hydrophobic ion interactions with membranes. A theoretical model consistent with the entire set of data is developed in an accompanying article.

INTRODUCTION

Hydrophobic (or, equivalently, lipophilic) ions are a class of charged molecules with the unique property that they readily permeate pure lipid bilayers, a characteristic that distinguishes them from small ions and most other charged molecules, which are instead virtually lipid impermeable. Favorable hydrophobic partitioning into the membrane hydrocarbon contributes to this ability. But even more important, hydrophobic ions possess large effective ionic radii—either because the charge is surrounded by hydrophobic groups or because it is significantly delocalized—and consequently the otherwise enormous electrostatic barriers to membrane transport are reduced. Examples of such ions include uncouplers of oxidative phosphorylation (e.g., $FCCP^-$, $CCCP^-$, DNP^- and TNP^-),[1] charge carriers (e.g., valinomycin, nonactin), many charged spin label probes (e.g., triphenylalkylphosphonium nitroxides) and some potential sensitive dyes (e.g., ANS^-). Protein insertion into membranes also often involves transport of charged groups across membranes, and in this sense may also share some features of hydrophobic ions.

The first successful theoretical model accounting for hydrophobic ion interactions with membranes was that of Ketterer, Neumcke, and Läuger (27), based on earlier work of Neumcke and Läuger (39). Their model combined repulsive (positive inside the membrane) electrostatic energy terms with an attractive (negative inside) hydrophobic energy term, to give deep potential wells (binding sites) near the membrane surface and a potential barrier in the interior (Fig. 1). Many experiments have since confirmed this basic potential profile (see references 1, 25, 26, and 29 for reviews).

Nevertheless, a fundamental feature of these molecules has never been adequately resolved. In the very earliest studies (31, 34) it was observed that hydrophobic anions interact with membranes in a dramatically different quantitative way than structurally similar cations, even for very close analogues such as TPB^- and TPP^+



Tetraphenylboron (TPB^-) Tetraphenylphosphonium (TPP^+)

Scheme I

[1] *Abbreviations used in this paper*: ANS^- (1-anilino-8-napthalenesulfonate); $CCCP^-$ (carbonylcyanide *m*-chlorophenylhydrazone); 2,4-D, (2,4-dichlorophenoxyacetic acid); DOPC, (dioleoylphosphatidylcholine); DNP^-, (2,4-dinitrophenol); DPA^-, (dipicrylamine); DPPC, (dipalmitoylphosphatidylcholine); EPR, (electron paramagnetic resonance); $FCCP^-$, (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone); GMO, (glycerylmonooleate); MOPS, (3-(*N*-morpholino)-propane-sulfonic acid); PC, (phosphatidylcholine); PCB^-, (phenyldicarbaundecaborane); PS, (phosphatidylserine); PV, (proline valinomycin = cyclo-[D-Val-L-Pro-L-Val-D-Pro-]$_3$); TEMPO, (2,2,6,6-tetramethylpiperidine-1-oxyl); TEMPOL, (4-hydroxy-TEMPO); THF, (tetrahydrofuran); TNP^-, (2,4,6-trinitrophenol); TPB^-, (tetraphenylboron); TPP^+, (tetraphenylphosphonium).

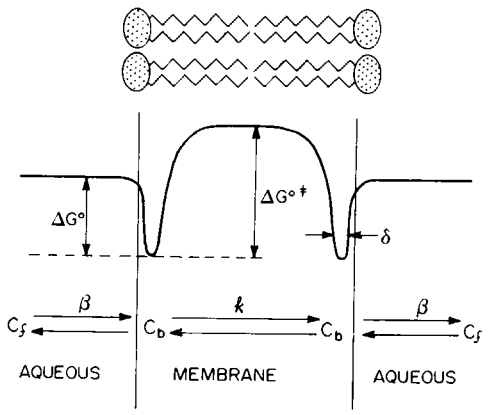
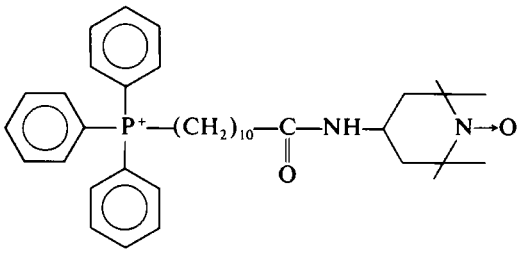


FIGURE 1 Hydrophobic ion membrane potential profile according to the model of Ketterer, Neumcke and Läuger (27). The free energies of binding ($\Delta G°$) and activation ($\Delta G°‡$) are as shown. See text for definition of other parameters.

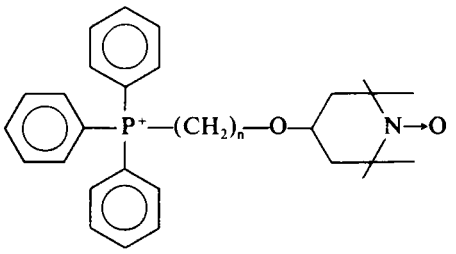
It has consistently been observed that hydrophobic anions bind several orders of magnitude more strongly to and translocate several orders of magnitude more rapidly across bilayers than structurally similar cations. Despite this fact, the Ketterer, Neumcke, and Läuger model (27) makes no distinction on the basis of the sign of the charge. It has been pointed out that lipid bilayers possess a substantial membrane dipole potential, positive inside, which is responsible for the permeability difference (e.g., 25, 45, 46), yet no self-consistent model has yet been developed that takes full account of this. The reasons for this shortcoming are mainly practical. Virtually all hydrophobic ion studies have been charge-pulse experiments in planar bilayers, a method that requires that ion translocation times be fast compared to the water-to-membrane exchange rate—a condition satisfied for hydrophobic anions, but rarely satisfied for hydrophobic cations (except for the very largest).

A full understanding of hydrophobic ion interactions with membranes thus requires that (*a*) the gap in knowledge about cations be filled, and (C_b) a self-consistent model be developed that accounts for both anions and cations. In this and the accompanying paper we aim to achieve these goals. TPP^+ and TPB^-, nearly identical analogues differing primarily in the sign of their charge, will be the focus of attention. By virtue of their relatively large ionic radii (4.2 Å), their electrostatic Born and hydrophobic energies should be nearly identical, as described in detail in the accompanying paper (21). The dramatic differences between these two molecules is presumably due to the membrane dipole potential, a hypothesis that the present studies will evaluate.

Here we report on the thermodynamic properties and interactions with lipid bilayers of the hydrophobic cation, TPP^+, and the spin-labeled analogues I and II(*n*) shown below



I



II(*n*)

Scheme II

Studies of binding, saturation, temperature, ionic strength, and activity coefficients will be reported. Of special interest is the temperature dependence of membrane partitioning, which permits evaluation of the separate enthalpic and entropic contributions to the free energy of binding. We expect the entropies for TPP^+ and TPB^- binding to be approximately the same (by virtue of their similar hydrophobic structure), whereas their enthalpies should differ largely as a result of the dipole potential contribution, other electrostatic contributions being of the same approximate magnitude and sign. This is indeed what is found.

The structural analogues TPP^+ and TPB^- are representative of hydrophobic ions in general, and elucidation of their comparative properties should provide an assessment of the salient features of all hydrophobic ion interactions with membranes. Armed with the new information to be presented here we then develop and evaluate in the accompanying article (21) a total potential model for hydrophobic ion interactions with bilayers that explicitly includes the membrane dipole potential in a self-consistent manner. The totality of these results not only provides a detailed description of hydrophobic ion properties, but also reveals the fundamental energetic structure of lipid bilayers, with applications to small and chaotropic ion interactions, as well as to membrane protein insertion and energetics. Some of this material has already appeared in a preliminary form (19, 20).

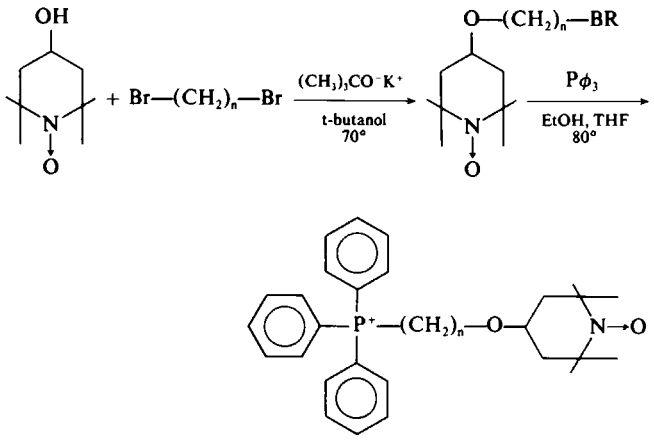
EXPERIMENTAL

Materials and Methods

Egg phosphatidylcholine (PC) was prepared according to the method of Singleton et al. (43) from local hen eggs, and stored as a 200 mM stock

solution in chloroform under argon at −20°C. The lipid 1,2-diphytanoyl-3-*sn*-phosphatidylcholine was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). All lipids were periodically checked for purity by thin layer chromatography on commercial silica gel plates with chloroform/methanol/water (65:25:4).

Tetraphenylphosphonium chloride and sodium tetraphenylboron were purchased from Aldrich (Milwaukee, WI). Potassium tetraphenylboron (K^+TPB^-) was prepared by mixing equimolar solutions of Na^+TPB^- and KCl, collecting and extensively washing the precipitate. The [alkyl-(*N*-tempoylcarbonamido)]triphenylphosphonium spin label I was synthesized by the method of Cafiso and Hubbell (13). The (tempoxyalkyl)triphenylphosphonium spin labels II(*n*) were newly synthesized for various *n* according to the following scheme:



To 1 g (5.8 mmol) TEMPOL and equimolar *t*-butoxide (5.8 mmol) in 30 mL dry *t*-butanol was added a twofold excess of Br-$(CH_2)_n$-Br (12 mmol). The solution was refluxed (at ~70°C) several hours until the reaction was complete, as assayed by thin layer chromatography on silica gel G plates, developed in chloroform and detected by rhodamine spray ($R_f \simeq 0.38$). Potassium bromide precipitate was removed by centrifugation, the solvent removed by rotary evaporation, and the product resuspended in ether. The ether solution was washed three times with water and then dryed over sodium sulfate. After removing the solvent, the product was resuspended in chloroform and purified on a silica gel column eluted with chloroform, the first yellow band containing the desired product (0.5 g yield). To 0.5 g (1.6 mmol) of the purified bromotempoxyalkyl in 2 mL ethanol and 1 mL THF, a threefold excess of triphenylphosphine was added, and the mixture allowed to reflux for ~8 h. The solvent was then removed by rotary evaporation, the product resuspended in ether, washed several times with water, and the supernatant discarded. A silica gel column was used for final purification, eluted first with pure chloroform and then with increasing methanol up to a final composition of 5%. About 0.7 g of an orange spin label product was obtained. It was found to be pure by thin layer chromatography on silica gel G plates developed in chloroform/methanol 10:1 as detected with sulfuric acid spray followed by charring. The product precipitated with TPB^- and had characteristic phosphorous-phenyl IR absorption bands at 9 and 10 μm. Assays by weight and total phosphorous were consistent with one nitroxide spin signal per molecule.

Vesicle Preparation

Unless otherwise noted, small sonicated unilamellar vesicles were used. Typically a 1–2 mL aliquot of stock egg PC in chloroform (~200 mM), handled under argon, was transferred to a 10 mL pear-shaped flask and evaporated to dryness, followed by further drying at <0.1 mmHg vacuum for 12+ h. It is extremely important to remove trace chloroform if the sample is to be sonicated; it appears that chloroform decomposes to HCl upon sonication with subsequent hydrolysis of PC to lyso-PC and fatty acid in some cases (see reference 18 for details). Approximately 1 mL of buffer was then added to the lipids and the mixture vortexed for 2–5 min until dissolved, followed by sonication under argon in an ice bath with a model W185 Branson cell disruptor (Branson Sonic Power Co., Danbury, CT) fitted with a 3 mm diameter tip tuned for maximum energy transfer at 30–35 W total output power. Sonication was continued until a clear, opalescent solution was obtained, typically 20–50 min, depending on lipid concentration and sonication efficiency. Following centrifugation at 16,000 rpm (31,000 *g*) for 20 min to remove unsonicated lipid and titanium dust (from the sonicator tip), the solution was passed through a 0.2 μm diameter Millipore (Millipore/Continental Water Systems, Bedford, MA) or Nuclepore filter to remove remaining unsonicated lipid and other particulates, and finally diluted with filtered buffer to the final lipid concentration. Bath sonication in a cylindrical bath sonicator (Laboratory Supplies Co., Hicksville NY) was used for samples of not more than a few hundred microliters and of low concentration. While bath sonication is generally preferred over tip sonication, the large lipid volumes needed for most of the studies reported here made this impractical. Results were essentially the same in either case although the sample temperature during sonication could be controlled better with the bath method. Unless otherwise indicated the buffer used was 100 mM NaCl, 20 mM MOPS adjusted to pH 7.0 with NaOH.

Phosphorous Assay

Total phosphorous in lipid and other phosphorous-containing solutions was assayed photometrically by a Fiske-Subbarow method based on a modification of the procedure of Bartlett (5). In addition a more convenient and more reliable method, using a lipid/ferrothiocyanate complex was developed based on the method of Stewart (44) with details given in reference 18. The general protocol was to prepare two separate dilutions of a phosphorous-containing solution and perform four or five assays on each, with the entire procedure repeated at least twice. By careful application of this method, ~50 nmol of phosphorous can be assayed to better than 3% overall.

Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) experiments were conducted with a Varian E-109 spectrometer system (Varian Associates, Inc., Instrument Group, Palo Alto, CA) in the X-band (~9.5 GHz) at a klystron power of 10 mW, and interfaced with a Nicolet 1280 computer (Nicolet Instrument Corp., Madison, WI). For most studies a quartz EPR microcell was used with an effective cavity volume of ~40 μL. Typically, aqueous samples with nitroxide reporter groups were used with spin concentrations in the 20–200 μM range. For temperature control a nitrogen gas flow was regulated with either an in-house design temperature controller or a Varian E-257 temperature accessory (Varian Associates, Inc., Instrument Group), both of which regulated sample temperature to within 0.5°C, as monitored with a 0.13 mm diameter copper-constantan calibrated thermocouple placed directly in the sample. The temperature gradient along the sample cell did not vary by more than 0.5°C.

One of the principal quantities to be obtained in this series of experiments is the partition coefficient, β, which is the ratio of the bound probe molecule surface density to the probe free concentration: $\beta = N_b/C_f$. Thus β has the units of length, and is the quantity most directly obtained from experiment. A derived quantity is the dimensionless binding constant, K, which is the ratio of the probe effective concentration in the membrane to the free concentration: $K = C_b/C_f$. These quantities are related by a parameter that is a measure of the thickness of the volume containing the bound population: $\delta = N_b/C_b = \beta/K$. As discussed below, bound hydrophobic ions are restricted to a narrow region near the membrane surface, and thus throughout this paper reported values of K are based on an assumed value of 4 Å for δ, unless otherwise noted. However we emphasize that most of the analysis presented here is

independent of the choice of δ, and instead only depends on the partition coefficient β.

Standard EPR techniques for spin label partitioning and relaxation were used (for reviews see references 14 and 37). For a series of different lipid concentrations, C_L, the EPR spectrum gives the ratio of the number of vesicle-bound spin labels to the number free in solution: $\lambda = N_b/N_f$. These quantities are related to the partition coefficient as follows

$$\frac{1}{C_L} = \beta A_L \frac{1}{\lambda} + V_L. \quad (1)$$

The parameters A_L and V_L are the average lipid surface area and the lipid specific volume, respectively, and are characteristic of the lipid in a given membrane preparation. For egg PC in small sonicated vesicles, the best values are $A_L = 66 \pm 4$ Å^2 and $V_L = 1{,}255 \pm 10$ Å^3. A linear least squares fit of $(1/C_L)$ vs. $(1/\lambda)$ then gives β.

Equilibrium Dialysis

Spectrapor 2 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA; 12,000–14,000 molecular weight cutoff) was washed according to manufacturers specifications. Microdialysis chambers were filled with 200 μL of appropriate aqueous solution and mixed by slow rotation. Temperature regulation and accuracy was shown to be no worse than ±1°C at and above 20°, ±0.5° at 10° and ±0.3° at 3°, as determined by NBS-certified thermometers or a calibrated thermocouple. Normally TPP^+ was added to both sides with equal initial free concentrations. Vesicles were added to one side only, and after attainment of equilibrium, the solution on the side without the vesicles was withdrawn and its UV absorbance measured to determine the equilibrium free concentrations. TPP^+ has a UV absorption spectrum consisting of three peaks at approximately 262.5, 268.5, and 275.5 nm. The highest amplitude peak at 268.5 nm was used throughout for relative absorbance measurements and, in conjunction with other methods, for absolute concentration determinations ($\epsilon_{268.5} = 3{,}700\ M^{-1}cm^{-1}$).

Equilibration time was determined by filling a series of dialysis chambers with 200 μM TPP^+ on one side only and measuring the TPP^+ concentration on both sides over time. With the 200 μL chambers and Spectrapor 2 dialysis membrane, the approach to equilibrium was exponential with a $1/e$ time of ~40 min, which agrees favorably with manufacturer specifications. A total dialysis time of ~4 h was therefore taken to ensure full equilibrium. TPP^+ concentrations were prepared by weight and then checked by both phosphorous assay and UV absorbance. Above 100 μM TPP^+, concentration reproducibility and overall spectrophotometric reproducability were good to better than 1%. Repeated equilibrium dialysis experiments with TPP^+ and buffer only, showed overall standard deviations for the procedure of not more than 5%. Vesicle-only controls were found to be necessary to correct for small but significant background UV absorbance due to a contaminant in the vesicle preparations. This background absorbance varied between 3 and 20% of the TPP^+ absorbance, depending on TPP^+ concentration, and increased with dialysis time and lipid concentration. Buffer-only dialysis did not show such an absorbance, and diphytanoyl-PC, which has completely saturated fatty acid chains and cannot be oxidized, reduced but did not eliminate it.

Equilibrium dialysis experiments give directly the free TPP^+ concentrations in the presence (C_f) and absence (C_f°) of membranes. Similar equilibrium experiments with triphenylphosphonium spin labels I and II allow for detection of both bound and free populations simultaneously and provide an independent check on this method. The fundamental quantities obtained from these experiments are the partition coefficient, β, and the binding constant, K, as defined above. Vesicle morphology and the constraints of equilibrium dialysis give the fundamental relation among the experimental observables and the partition coefficient

$$\frac{C_f^\circ}{C_f} = 1 + \frac{A_L}{2}(\beta - \lambda_L)C_L. \quad (2)$$

Here $\lambda_L = V_L/A_L$ is a lipid length parameter defined as the ratio of the lipid specific volume, V_L, to the average lipid surface area, A_L, in the membrane ($2\lambda_L$ is essentially the membrane thickness), and C_L is the effective lipid concentration. The best values for A_L and V_L were specified above. To increase the precision in the determination of β, (C_f°/C_f) for a series of C_L values can be obtained and fit by a linear least squares analysis, the slope gives β and the intercept should be 1, which provides a check on the formulation.

Binding of TPP^+ to chamber and dialysis tubing is insignificant, as determined by the constancy of TPP^+ absorbance for free-only controls, even following repeated transfers of a single control to successive dialysis chambers. Since TPP^+ is permeable to lipid bilayers whereas its Cl^- counterion is much more slowly permeable, it is possible that a positive inside transmembrane potential could develop across the vesicles which would preclude a true equilibrium measurement. But since all experiments were well buffered (20 mM MOPS), and since on the 4-h time scale of these experiments H^+/OH^- transport is appreciable (15), any transmembrane potential should, in fact, be collapsed (Cl^- ion relaxation across these vesicles has also been measured with a $1/e$ time of ~40 min, further arguing against a transmembrane potential; unpublished observations). By virtue of TPP^+ binding to the vesicles ($\sim 10^6$ D molecular weight per vesicle), a Donnan potential across the dialysis membrane need also be considered. Calculations for the typical conditions used in these experiments shows this to be <1 mV, and therefore not of significance.

Activity Coefficient Determination

The solubility of the sparingly soluble salt, K^+TPB^-, was determined for two separate preparations of 12 different NaCl salt solutions covering the range from 0 to 2 M. To a small flask containing 40 mL of each salt solution was added excess (~10 mg) K^+TPB^-, which was mixed vigorously for several minutes and then placed in a rocking constant temperature bath in the dark at 20 ±1°C for 40 h. Three to five samples from each flask were removed, filtered, and the absorbance measured at the UV absorbance peaks of TPB^-, 265, and 273 nm. Measurements taken before and after 40 h indicated that a true equilibrium had been obtained. Longer times are not advisable since TPB^- begins to decompose after more than a few days in aqueous solution at room temperature (16, 17).

Since the K^+ and TPB^- concentrations are equivalent, the mean ionic activity coefficient for K^+TPB^-, $\gamma_\pm$, is simply related by $K_{sp} = a_K a_{TPB} = \gamma_\pm^2 C^2$, where a is the activity and C the concentration. If A_o is the absorbance in the limit of zero ionic strength (where $\gamma_\pm = 1$), then the mean activity coefficient is obtained directly from the measured absorbance, A: $\gamma_\pm = (A_o/A)$.

RESULTS

Binding Constant and Saturation

Despite the extensive use of hydrophobic cations as probes of membrane electrical properties, no careful studies of cation binding constants or conditions of saturation have previously been made. Here we report the binding and saturation properties of TPP^+ at 20 ±1°C in 100 mM NaCl, 20 mM MOPS buffer at pH 7.0. Equilibrium dialysis experiments give the ratio of free TPP^+ concentration in the absence to that in the presence of vesicles, (C_f°/C_f), for several different total effective lipid concentrations, C_L. A least squares fit to the data gives a good straight line fit with typical correlation coefficients of $r \geqq 0.99$, and an intercept of 1 within 4% for all experiments, as expected from Eq. 2.

The results of a series of 12 different lipid and TPP^+ concentration combinations (below saturation levels), each of which represents four or more identical samples with appropriate controls as described in the methods section, give as the best overall value for the binding coefficient of TPP^+ to egg PC vesicles, $\beta = 4.2 \pm 0.4 \times 10^{-6}$ cm. This binding coefficient is the most directly obtained experimental result. Taking the binding region thickness to be $\delta = 4$ Å, the binding constant is then $K = 100 \pm 10$. However, reasonable values of δ could be 3–6 Å, in which case K is unknown to about ± 30.

Further experiments at increasingly higher concentrations of TPP^+ were performed to evaluate the conditions for saturation of binding. Twenty different combinations of TPP^+ and lipid concentrations were studied covering a range from 100 μM to 2 mM free TPP^+ and approximately 10 to 85 mM egg PC. The effects of saturation are best illustrated by a plot of bound surface density vs. free concentration as shown in Fig. 2. Saturation, defined most simply as when deviations from the linear $N_b = \beta C_f$ curve are evident, or equivalently where the binding coefficient becomes concentration dependent, is shown to set in at ~600 μM. The immediate practical result of this observation is that all subsequent studies be conducted below this level to avoid an additional (presumably electrostatic) contribution to the binding—typically C_f° was ≦400 μM. In addition, analysis of the saturation phenomena can provide information on the character of the electrostatic interactions of hydrophobic ions in the binding region, and may for example provide information on the effective local dielectric constant, as presented in the Discussion.

Temperature Studies

One of the principal goals of this work is to evaluate the individual entropy and enthalpy contributions to the binding of TPP^+ to membranes. Since the free energy of binding, ΔG°, is related to the binding constant by $K = \exp(-\Delta G^\circ/RT)$, and since $K = \beta/\delta$ and $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, the following relation results:

$$\ln \beta = [\ln \delta + (\Delta S^\circ/R)] - (\Delta H^\circ/R)/T. \qquad (3)$$

Thus a plot of $\ln \beta$ vs. $1/T$ will give ΔH° directly, with ΔS° obtained after selecting reasonable values for δ.

The results of a series of (C_f°/C_f) vs. C_L plots, which give β for each of six different temperatures between 3°C and 50°C, are shown in Fig. 3. The data clearly indicate a temperature dependence to ΔH° (and hence ΔS°). However, in the range of 3° to 40°C the values of ΔH° and ΔS° may, within experimental error, be taken to be constant. A linear least-squares fit to the data in this range gives a slope of $-1{,}770 \pm 140^\circ$ K and an intercept of -6.44 ± 0.47 ($r = 0.991$). The thermodynamic parameters are then determined (with the additional assumption $\delta \simeq 3$–6 Å for ΔS° and ΔG°)[2]:

$$\Delta G^\circ = -2.7 \pm 0.5 \text{ kcal/mol (20°C)}$$

$$\Delta H^\circ = 3.5 \pm 0.3 \text{ kcal/mol}$$

$$-T\Delta S^\circ = -6.2 \pm 0.4 \text{ kcal/mol (20°C)}$$

$$(\Delta S^\circ = 21 \pm 1 \text{ cal/mol-deg}).$$

One of the original motivations for this work was to establish the fundamental properties of hydrophobic ion interactions with lipid bilayers, and thereby to provide a firm foundation for using these molecules as probes of membrane electrical properties. Of particular interest are the use of hydrophobic ion spin labels, such as alkyltriphenylphosphonium nitroxides, as potential sensitive probes. It is apparent from the structure of these molecules that they should have properties quite similar to those of TPP^+. One

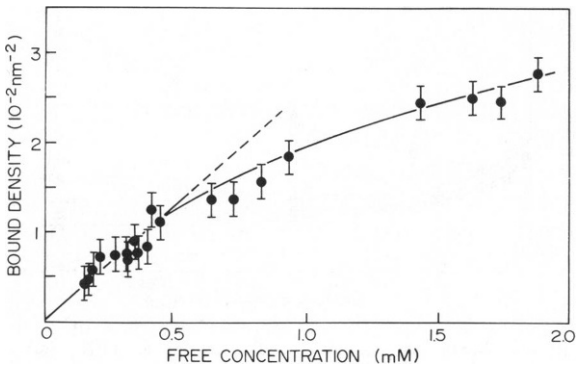


FIGURE 2 TPP^+ bound surface density (N_b) in egg PC vesicles as a function of free concentration (C_f). Dashed line is an extrapolation of a linear least-squares fit to the data below 0.6 mM at which point binding saturation is observed. The solid curve has no theoretical significance.

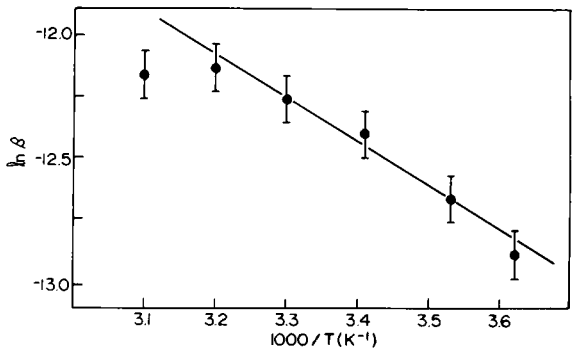


FIGURE 3 Natural logarithm of TPP^+ partition coefficient for binding to egg PC vesicles as a function of inverse temperature at 3°, 10°, 20°, 30°, 40° and 50°C. Least-squares fit to the data provides a determination of Hb° and Sb°; the 50°C point was omitted from fit because it begins to show a significant heat capacity effect (see text for details).

[2]The standard state choice for the thermodynamic quantities is the 1 M Henry's law reference state. A more satisfactory reference state would be in terms of mole fractions, but the former was chosen here to make comparisons with literature values for hydrophobic anion binding that are all based on the molar scale.

advantage of EPR spin label methods is that it is possible to observe both bound and free populations simultaneously in the presence of membrane. Comparison of the thermodynamic properties of the phosphonium spin label with those of TPP^+ is thus of interest.

The binding of spin label I to egg PC sonicated vesicles was measured at 10°, 25°, 40°, and 60°C (±1°C) in 100 mM KCl, 10 mM MOPS at pH 7. For a series of different lipid concentrations, C_L, the bound to free ratio, $\lambda = N_b/N_f$, was obtained from the EPR spectrum. The partition coefficient β is then determined by Eq. 1. As before, the enthalpy and entropy of binding can be obtained from a plot of β vs. $(1/T)$ and application of Eq. 3, with the following result

$$\Delta G° = -4.1 \pm 3.2 \text{ kcal/mol (20°C)}$$

$$\Delta H° = +3.9 \pm 0.8 \text{ kcal/mol}$$

$$-T\Delta S° = -8.0 \pm 3.1 \text{ kcal/mol (20°C)}$$

$$(\Delta S° = 27 \pm 11 \text{ cal/mol-deg}).$$

Thus within experimental error the enthalpy of binding for the phosphonium spin label I and for TPP^+ are equivalent. The enhanced binding of this spin label over that of TPP^+ can then be accounted for by the increased entropic contribution, as would be expected from the long alkyl chain. The similarity in their enthalpies of binding is consistent with the idea that the two hydrophobic cations bind in similar locations, a point that will be elaborated on in the Discussion.

Ionic Strength Effects on Partitioning

Electrostatic interactions, as between a hydrophobic ion and the membrane dipole potential or with the choline headgroup, could be screened by salt, providing the interaction is in a region accessible to the ionic medium. In particular, it is possible that the dramatic differences in hydrophobic anion vs. cation binding energies involve their electrostatic interactions with the choline headgroup (as suggested by the observation that hydrophobic anions precipitate with free choline in aqueous solution), and that therefore a change in ionic strength would affect these interactions. To check whether such an effect is observed, the binding of TPP^+ both above and below saturation levels were measured as a function of salt concentration in 10, 100, and 500 mM NaCl. In each case, plots of $(C_f°/C_f)$ vs. C_L were obtained and the partition coefficient determined, with the results as summarized in Table I. The data show that binding increases following either an increase or a decrease in ionic strength, relative to 100 mM NaCl.

Partitioning of spin label II ($n = 4$) to neutral egg PC vesicles was also determined for a series of NaCl and NH_4^+Acetate$^-$ concentrations as described in the Methods. Changes in binding with ionic strength obtained for this molecule were essentially identical to those found for TPP^+ (see Fig. 4).

TABLE I
TPP^+ BINDING TO EGG PC VESICLES UNDER DIFFERENT SALT CONDITIONS

Salt Conditions	β (10^{-6} cm) ± 6%	
	$C_f° = 400\ \mu M$	$C_f° = 2$ mM (Saturation)
10 mM NaCl 10 mM MOPS	4.4	2.85
100 mM NaCl 20 mM MOPS	4.1	2.7
500 mM NaCl 20 mM MOPS	5.1	3.45

If these hydrophobic cations bind in a region exposed to the aqueous solution it would be expected that increases in ionic strength would lead to screening of any electrostatic interactions, and therefore result in enhanced binding, whereas decreases in ionic strength would lead to the opposite effect. This was not observed. Thus the changes in binding with ionic strength are not simply due to screening effects. Below it is shown that nonideal solution behavior (activity coefficients) can quantitatively account for the effect.

Activity Coefficient Studies

An accurate method for determining activity coefficients of sparingly soluble salts is to measure solubility as a function of ionic strength. This is not possible with TPP^+ since its common salts are freely soluble in water. However, potassium tetraphenylboron (K^+TPB^-) is sparingly soluble in water ($K_{sp} \simeq 180\ \mu M$) and therefore its activity can be measured in this way. Mean ionic activity coefficients for K^+TPB^- were obtained as described in the Methods for various concentrations of NaCl, with the results as shown in Fig. 4. Activity coefficients for a few familiar inorganic salts are also shown for comparison.

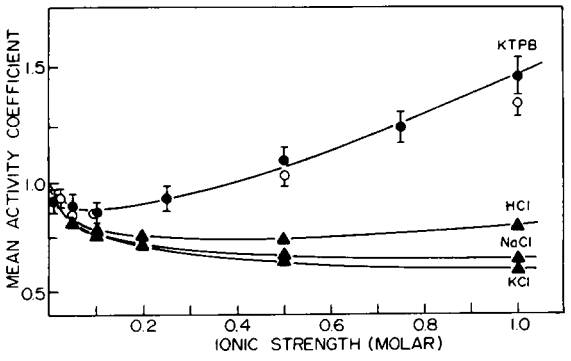


FIGURE 4 Mean ionic activity coefficients for K^+TPB^- (●) as a function of ionic strength. Selected standard inorganic ionic activity coefficients (▲) are shown for comparison. Relative partitioning of hydrophobic cation spin label II(n=4) (○) to neutral egg PC vesicles (normalized to the 0.1 M value) is also shown; TPP^+ partitioning data is virtually identical to that for the spin label.

Note that the mean ionic activity coefficient for K^+TPB^- is strikingly different from those of common salts, reaching a minimum value at ~0.1 M and then rising rapidly to values >1 at higher ionic strengths (common salts also rise above one, but only at ionic strengths >1 M). This unusual behavior indicates that an increase in hydrophobic ion binding to the membrane is expected with increasing or decreasing ionic strength about the minimum at 0.1 M NaCl, as observed experimentally. The data in Fig. 4 can then be used to quantitatively account for the salt dependence of the binding of TPP^+ given in Table I if the following plausible assumptions are made: (*a*) the unique behavior of the mean ionic activity coefficient of K^+TPB^- at high ionic strengths is due to the TPB^- ion; (*b*) the activity coefficients of TPP^+ and TPB^- are similar; and (*c*) the activity coefficient of membrane bound TPP^+ is independent of the ionic strength in the bulk solution. Assumption *a* appears justifiable from the rather constant mean ionic activity coefficients of simple inorganic salts at high ionic strength compared with the rapid rise for K^+TPB^-. Assumption *b* is reasonable since the anomalous salting out ($\gamma_\pm > 1$) is due to the hydrophobic nature of the ion, which is essentially identical for TPP^+ and TPB^-. Assumption *c* is implicit in the model for binding of the hydrophobic ions to bilayers, in which the binding site is located in a low dielectric region removed from contact with water or solution ions, a model consistent with all of the other data presented here. Thus the amount of TPP^+ bound to the membrane is directly proportional to the solution activity coefficient of the ion and the binding changes with ionic strength should parallel those of the activity coefficient shown in Fig. 4.

The data of Table I show an increase in binding in going from 100 mM to 500 mM salt, by ~24% below and 28% above saturation. But the differences observed in activity coefficient for this change in salt is ~28%, and thus can quantitatively account for the observed effects, within experimental error. Similarly in going from 100 mM to 10 mM salt, a small but significant increase in binding was observed, ~7% below saturation and 6% above. But the activity coefficient change in such a case was measured to be ~6%, and thus can entirely account for the observed effect. Upon correction for activity coefficient effects, we conclude that there is no ionic strength dependence to the intrinsic binding of TPP^+ to neutral membranes.

DISCUSSION

Thermodynamics of Binding

The thermodynamic contributions to binding determined here for TPP^+ can now be compared with the values for hydrophobic anions available in the literature (Table II). It is important to keep in mind that the anionic data is based on planar bilayer studies whereas that for TPP^+ is for sonicated vesicles, and that the type of lipid used varied throughout. Despite the differences in method and the large uncertainties in some cases, the results in Table II are fairly consistent and support a number of important conclusions. First, the entropic contribution to binding free energy for all these hydrophobic ions falls roughly within the range -5 ± 1 kcal/mol at 25°C. Particularly in comparing TPP^+ and TPB^-, this result supports the contention that their nonelectrical (i.e., hydrophobic or neutral energies) are nearly identical (although not all of it may be expressed at the binding site—see the accompanying article, reference 21). The second major observation is that the significant differences in the binding energies of anions vs. cations, ~4–7 kcal/mol, can be traced to the differences in their enthalpies. In fact, the enthalpy for TPP^+ binding is positive (repulsive), while that for the anions is negative (attractive). This constitutes a fundamental difference in their interactions with membranes.

TABLE II
THERMODYNAMIC PARAMETERS OF BINDING FOR HYDROPHOBIC IONS IN KCAL/MOL (MOLAR SCALE AT 25°C)

	CATION	ANIONS				
	TPP^+	TPB^-			DPA^-	
	(*)	(‡)	(§)	(‖)	(¶)	(**)
$\Delta G°$	-2.8 ± 0.5	-7.2	-7.6	-7.5	-6.7	-7.6
$\Delta H°$	3.5 ± 0.3	-1.8	-3.5	0 ± 2	-3.4	-3.6
$-T\Delta S°$	-6.3 ± 0.4	-5.4	-4.1	-7.5	-4.3	-4.0

(*) This work. Egg PC vesicles.
(‡) R. Benz (personal communication). Planar bilayer. (±0.6 kcal/mol)
(§) Reference 10. Temperature jump in planar PS bilayer; possibly subject to large errors due to necessary surface charge corrections that depend on an assumed charge density and Gouy-Chapman model. (±0.7 kcal/mol)
(‖) Reference 12. Planar di(22:1)-PC bilayer. (±2 kcal/mol)
(¶) Reference 9. Planar DPPC bilayer.
(**) Reference 28. Noise analysis on planar di(16:1)-PC bilayer

This significant difference in the enthalpies of binding could arise from any of several sources: difference in steric factors; different locations of their binding sites or their effective radii and therefore differences in the magnitude of the Born-image contribution; difference in their hydration energy; specific local electrostatic effects; and interactions with the membrane dipole potential. Several possibilities can be ruled out. The best evidence available does not support any significant difference in their effective ion size or hydration energy (see reference 21 for further discussion of this point). Further, given their almost identical structure and that they bind in approximately the same location (see below), it is unlikely that steric factors can account for the major portion of their enthalpy difference. This leaves electrostatic interactions as the most likely sources, either with the membrane dipole potential or with the lipid carbonyls, phosphate, or quaternary ammonium groups. That a wide variety of hydrophobic anions all have nearly the same very high binding constant suggests that specific

chemical interactions are unlikely. The absence of a salt dependence to binding is also consistent with this conclusion. On the other hand, it is possible to account for nearly all of the differences between cation and anion binding by the effect of the membrane dipole potential, an important conclusion discussed thoroughly in the accompanying paper (21).

TPP^+ and TPB^- Binding and Kinetic Parameters

TPB^- was one of the very first hydrophobic ions to be carefully studied in lipid bilayers (27, 30–34), and there soon followed many other papers and review articles on its membrane interactions. Essentially all of these were planar bilayer studies which, for practical considerations, were not suited for analogous hydrophobic cation studies. Thus for TPP^+ the kinetic parameters k (translocation rate; see Fig. 1) and β (partition coefficient) have previously only been determined as the product, βk. With the value for β obtained here, and the βk value from the literature, it is now possible to compare the complete set of thermodynamic parameters for TPP^+ and TPB^- interactions with membranes (Table III). While the anion data was obtained mainly in planar bilayers and the cation data mainly in small sonicated vesicles, it is believed that the comparison is still an apt one. In support of this note that comparison of hydrophobic molecule partitioning in organic solvent vs. small and large lipid vesicles shows only small differences (e.g., reference 42); and measurements of triphenylalkylphosphonium spin labels in small and large unilamellar vesicles show no dramatically different results (unpublished observations).

The actual value of the partition coefficient β depends on lipid type and composition (e.g., references 6–8) and on the presence of other molecules in the membrane such as phloretin and 2,4-D. But for most pure phospholipid bilayers, the partition coefficient generally falls within a very narrow range of values, typically between 1 and 4×10^{-2} cm for both TPB^- and DPA^- (3, 6–9, 12, 27, 38, 41). If one assumes a thickness of the binding region of ~4 Å, then binding constants in the range 10^5–10^6 are obtained. By comparison, TPP^+ in egg PC vesicles as determined here has $\beta = 4.2 \times 10^{-6}$ cm, or $K \simeq 102$; the TPB^- anion therefore binds ~5,000 times more strongly. The only other measures of actual binding constants for hydrophobic cations we are aware of are those for PV-K^+, a large potassium ion carrier, with a reported value of 4.4×10^{-4} cm in egg PC planar bilayers (7), and in a footnote to a table, a single value of 1.8×10^{-5} cm for TPP^+ in GMO membranes (41).

Taking the value of β above, and with $k = 8.5 \times 10^{-8}$ cm/s for DOPC planar measurements (41), a translocation rate of 0.020 s^{-1} is determined for TPP^+. This is to be compared with TPB^- values of 10–100 s^{-1} (e.g., 27, 41). Thus TPB^- translocates about 10^3–10^4 times faster than TPP^+, suggesting an activation energy difference of ~5 kcal/mol in the center of the bilayer. As discussed thoroughly in the accompanying article (21), this result can be entirely accounted for by a dipole potential of ~240 mV, inside positive.

TABLE III
SUMMARY OF THERMODYNAMIC AND KINETIC PARAMETERS FOR TPP^+ AND TPB^- INTERACTIONS WITH BILAYERS

	TPP^+ (vesicles)	TPB^- (planar)
Binding		
β	4.2×10^{-6} cm	$2\text{-}4 \times 10^{-2}$ cm
K	100	10^5–10^6
$\Delta G°$	−2.5 kcal/mol	−7.5 kcal/mol
$\Delta H°$	+3.5 kcal/mol	−2.5 kcal/mol
$-T\Delta S°$	−6.0 kcal/mol	−5.0 kcal/mol
Translocation		
βk	10^{-7}–10^{-8} cm/s	0.1–1.0 cm/s
k	10^{-2}–10^{-3} s^{-1}	10–100 s^{-1}
$\Delta G^{o‡}$	20 kcal/mol	14 kcal/mol
Saturation		
N_b^{sat}	2×10^{12} cm^{-2} ~1/100 lipids	5×10^{12} cm^{-2} ~1/100 lipids

Saturation and Local Dielectric Constant

Liberman and Topaly (34) and LeBlanc (30) first observed that a variety of hydrophobic ions—including TPB^-, PCB^- and triphenylmethylphosphonium$^+$—show saturation behavior in the form of a leveling-off of the membrane conductance with increasing hydrophobic ion concentration. The major models that have been suggested to explain saturation include: (*a*) space charge limitation (30, 40); (*b*) fixed number of binding sites (11, 27); (*c*) surface potential inhibition (48, 49); and (*d*) boundary potential inhibition (2, 3, 47). All of these theories give quantitatively similar results just at the onset of saturation, but diverge at higher adsorbed ion concentrations. Since the data is often unreliable at very high concentrations, it is difficult to evaluate the various models in detail, with none being entirely satisfactory (e.g., see reference 18). Further, mainly only data on the anions has previously been available, so a comprehensive analysis has remained elusive.

Apart from all of the differences in detail, several broad generalizations stand out from saturation studies. Most notably, all hydrophobic anions studied—TPB^-, DPA^-, and PCB^-—begin to show saturation at essentially the same surface density in the membrane: $\simeq 10^{12}$ $cm^{-2} \simeq 1/(10^4 Å^2) \simeq 1/100$ lipids. This clearly indicates a general underlying phenomenon that is made even more remarkable by the findings here that TPP^+ binding saturates at essentially the same surface density. That is, although the free TPP^+ concentration at saturation, $\simeq 6 \times 10^{-4}$ M, is much higher than that for anions, the much lower binding constant, $\simeq 4.2 \times 10^{-6}$ cm, gives the same saturation

surface density: $N_b^{sat} = \beta C_f^{sat} \simeq 1.5 \times 10^{12}$ cm$^{-2} \simeq 1/(7{,}000$ Å$^2) \simeq 1/100$ lipids. This result is consistent with the concept that hydrophobic ions bind in similar environments and are involved in similar membrane interactions. Furthermore, although the several models for saturation noted above vary in their details, they all reduce to essentially the same approximate result: saturation just sets in when the ion electrostatic interaction energy reaches the average thermal energy. In the simplest model, the Coulomb electrostatic energy, $q^2/[4\pi\epsilon_o]\epsilon r$, is equal to kT (0.59 kcal/mol) if the local effective dielectric constant, ϵ, is ~10 (more precisely, in the range 5–30), where r is the effective ion radius. This result is in agreement with the notion that these molecules bind in a region of dielectric strength between that of bulk water and of bulk hydrocarbon. A binding region in the vicinity of the lipid carbonyls is particularly attractive.

An overall conclusion of the above observations is that both hydrophobic anions and cations bind at the membrane-water interface, toward the membrane side, and are not freely accessible to the aqueous phase. That a dielectric constant significantly less than that of bulk water can account for the onset of saturation is just one fact in support of this conclusion. Equally significant is the observation that increases in ionic strength cannot screen ionic interactions which lead to saturation. The various anionic studies cited above all find essentially the same surface density for the onset of saturation despite, in those experiments, differences in salt concentrations of several orders of magnitude. Our results for TPP^+ binding with different salt conditions also revealed no significant differences in binding above saturation. That is not to say that there will not be a screenable effect at all. On the contrary, the work of Wang and Bruner (48, 49) clearly shows that at sufficiently high concentrations the surface potential self-inhibition of binding also plays a role, and this effect is screenable. But at the lower concentrations studied here we find there is a nonscreenable "boundary potential", a point also emphasized by Andersen et al. (2, 3).

Activity Coefficients for Hydrophobic Ions

The mean ionic activity coefficients for K^+TPB^- (and indirectly, hydrophobic cation spin labels) as shown in Fig. 4 demonstrate that these ions are significantly different from other simple inorganic ions (NaCl, KCl, HCl, etc.), with values greater than one above only a few hundred millimoles per liter of salt. This could have important consequences for some applications of hydrophobic ions, necessitating that experiments with varying salt conditions or with accessible compartments of differing ionic strength make appropriate corrections. Such activity coefficient effects are not unusual for compounds with hydrophobic-like characteristics. Qualitatively similar effects to those in Fig. 4 have, for example, been reported for DNP^- (24), tetraethyl- and tetrapropylammonium chlorides (35, 50), and tetrapropylammonium fluoride (50). However, hydrophobic ions as a class are somewhat unique in having these relatively large activity coefficients, and there is some theoretical justification for this (22, 23, 36, 50).

Thus the bulk of the data is consistent with the general conclusion that both hydrophobic anions and cations bind at the membrane interface, toward the lipid side, in a region of dielectric strength between that of bulk water and hydrocarbon. Furthermore, while their binding is entropy driven, their enthalpies of binding are of opposite sign. Armed with this comparative data it is now possible to develop and evaluate a more detailed theoretical model for hydrophobic ion interactions with membranes, as done in the accompanying article (21).

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