Use of a Biomimetic Chromatographic Stationary Phase for Study of the Interactions Occurring between Inorganic Anions and Phosphatidylcholine Membranes

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ABSTRACT A liquid chromatographic method for the study of ion-membrane interactions is reported. A phosphatidylcholine biomimetic stationary phase was established by loading dimyristoylphosphatidylcholine (DMPC) onto a reversed-phase octadecylsilica packed column. This column was then used to study the interaction of some inorganic anions with the stationary phase by UV and conductivity detection. Ten inorganic anions were selected as model ions and were analyzed with the proposed chromatographic system. Anion-DMPC interactions of differing magnitudes were observed for all of the model anions. Perchlorate-DMPC interactions were strongest, followed by thiocyanate-DMPC, iodide-DMPC, chlorate-DMPC, nitrate-DMPC, bromide-DMPC, chloride-DMPC, fluoride-DMPC, and then sulfate-DMPC. Cations in the eluent, especially H^+ ions and divalent cations such as Ca^{2+}, showed strong effects on anion-DMPC interactions. The chromatographic data suggest that DMPC interacts with both the anions and the cations. Anion-DMPC interactions were dependent on the surface potential of the stationary phase: at low surface potentials anion-DMPC interactions were predominantly solvation dependent in nature whereas at more positive surface potentials anion-DMPC interactions were predominantly electrostatic in nature. Cation-DMPC interactions served to raise the surface potential, causing the anion-DMPC interactions to vary from solvation dependent to electrostatic. The chromatographic data were used to provide quantitative estimates of the enthalpies of the anion-DMPC interactions.

INTRODUCTION

Ion-membrane interactions can influence the effectiveness of some membrane-related physiological processes. As observed experimentally in both whole and single muscle fibers, ion-membrane interactions can cause an increase in the muscle twitch tension (Kahn and Sandow, 1950, 1955; Hodgkin and Horowicz, 1960). Membrane properties are sensitive to the membrane electric fields, and ion-membrane interactions alter these electric fields (Hodgkin and Horowicz, 1960). Although there has been a considerable number of studies (Tatulian, 1983, 1987; Cacace et al., 1997; Collins and Washabaugh, 1985; Clarke and Lüpfert, 1999; Grasdalan et al., 1977; Hauser et al., 1977; Horowicz, 1964; Jendrasiak, 1972; Rydall and Macdonald, 1992; Kalinin and Molotkovsky, 2000) to support this finding, the underlying principles of ion-membrane interactions are still not recognized fully.

Studies on ion-membrane interactions have conventionally been carried out by measuring the change in zeta potential on the surface of model membranes (Tatulian, 1983, 1987) or by detecting changes in certain chemical and/or physical properties of specific indicators using fluorescence (Paula et al., 1998; Clarke and Lüpfert, 1999; Kalinin and Molotkovsky, 2000) or NMR methods (Jendrasiak, 1972; Hauser et al., 1977; Grasdalan et al., 1977; Rydall and Macdonald, 1992) to indicate where the ion-membrane interaction has occurred. Although some ion-membrane interactions have been observed with these techniques, the direct observation of the interactions of specific ions by chromatographic methods has not been used.

If a chromatographic column can be established with a biomembrane as the stationary phase, then it should be possible to observe directly the ion-membrane interactions by monitoring the elution behavior of the ions. Over the past 10 years, we have been developing bifunctional zwitterionic columns for the chromatographic separation of ions (Hu et al., 1993, 1994, 1999; Hu and Haddad, 1998; Cook et al., 2001). In these studies we have immobilized a wide range of hydrophobic zwitterions onto a reversed-phase chromatographic support to produce the desired zwitterionic stationary phase. Because phosphatidylcholines, the chief components of biomembranes, are zwitterionic over a wide range of pH the same approach could be used to establish a biomimetic membrane column by immobilizing phosphatidylcholine onto a reversed octadecylsilica (ODS) support material. In this study we show that a model column of this type can be established successfully and can be used to investigate the interaction of inorganic anions with the biomimetic membrane by chromatographic techniques. Not only can direct ion-membrane interactions be observed, but

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also new insights into the mechanism of ion-membrane interactions can be obtained.

MATERIALS AND METHODS

Chemicals

Dimyristoylphosphatidylcholine (DMPC) of >99% purity was used to establish the stationary phase and was obtained from Funakoshi (Tokyo, Japan). Other reagents used to prepare samples and eluents were of analytical grade and were obtained from Wako Chemicals (Osaka, Japan). Deionized water used to prepare the sample and eluent was obtained by using a WG261 Autostill water purification system (Yamato Scientific Co., Tokyo, Japan).

Column preparation

DMPC (600 mg) was dissolved in 15 ml of chloroform in a round-bottomed flask. The DMPC was dried by rotary evaporation of the chloroform and the residue redissolved in 10 ml of diethylether. The ether was evaporated slowly to produce a DMPC film on the surface of the flask. The film was flushed with nitrogen kept under vacuum for at least 4 h and was then hydrated by dispersal in 10 ml of an aqueous/methanol (95/5, v/v) solution containing 10 mM $NaHCO_3$ to form multilamellar liposomes. The complete hydration was ensured by using a vortex mixer (Yamato, Tokyo, Japan). The multilamellar liposomes were passed 10 times through two stacked polycarbonate filters of 100-nm pore size (Nuclepore, Costar, Acton, MA) at room temperature in a high-pressure vesicle extruder (Lipex Biomembranes, Vancouver, Canada) to obtain the unilamellar liposomes. The unilamellar liposomes added to 2 L of the 10 mM $NaHCO_3$ aqueous/methanol (95/5, v/v) solution were then used to prepare the DMPC column by passing through an ODS-packed column (250 × 4.6 mm i.d., 5 μm particle size, 120 Å pore size, 17% C/Si, 300 $m^2\ g^{-1}$; Tosoh, Tokyo, Japan) at a constant flow rate of 1.0 ml/min for ~30 h. A column prepared in this manner (which was saturated by DMPC using the breakthrough method) was found to contain 0.68 mmol of DMPC/column by phosphorus elemental analysis. The breakthrough method (Hu and Haddad, 2000) was performed by monitoring the concentration of the zwitterionic species in the effluent during the column-coating procedure. When this concentration reached the same level as that in the column-coating solution, the column was considered to be saturated with the zwitterionic species. After completion of the HPLC separations in this study, 70 ml of chloroform was introduced into the column to remove DMPC from the ODS stationary phase. The effluent was collected and the solvent removed by rotary evaporation, and the residue was dried and weighed. The weight of the residue was 478.8 mg, and phosphorus elemental analysis of this residue confirmed it to be DMPC. These results indicate that the DMPC was immobilized by the ODS stationary phase.

HPLC system

Chromatographic separations were performed by a high-performance liquid chromatographic (HPLC) system obtained from Shimadzu (Kyoto, Japan). This consisted of a LC-10AT system comprising a Shimadzu LC-10AT pump, a Shimadzu SIL-10A auto-injector, a Shimadzu CTO-10A column oven, and a Shimadzu CR-6A Chromatopac data system. A Shimadzu SPD-6A UV-visible detector operated at 210 nm and a Shimadzu CDD-6A conductivity detector were used in tandem for the detection of the targeted ions.

RESULTS AND DISCUSSION

Anion-DMPC interactions observed with HPLC

The DMPC column was conditioned with an eluent composed of 10 mM $NaHCO_3$ aqueous solution (pH 8.67). This eluent was chosen because its background conductance can be reduced through conversion of $NaHCO_3$ to H_2CO_3 by inserting an electrolytic suppressor device between the UV-visible detector and the conductivity detector. The electrolytic suppressor (ASRS-1) used throughout this study was commercially available and was obtained from Dionex Corp. (Sunnyvale, CA). It operates on-line to electrically reduce the conductance of the electrolyte ($NaHCO_3$) before it enters the conductivity cell. This allows the model anions to be detected sensitively using conductivity detection. UV-visible detection was used as a supplementary detection method for the selective detection of the UV-absorbing model ions. Inorganic anions used as the model anions were sulfate, fluoride, chloride, bromide, nitrite, nitrate, chlorate, iodide, thiocyanate, and perchlorate, used as their sodium salts. When a mixture of these ions was injected onto the column, the chromatogram shown in Fig. 1 was observed. The void time (i.e., time for elution of an unretained analyte) for this system was 2.18 min, whereas the retention times determined for individual injections of the model anions were SO_4^{2-} (2.23 min), F^- (2.25 min), Cl^- (2.41 min), NO_2^- (2.57 min), Br^- (2.65 min), NO_3^- (2.77 min), ClO_3^- (2.85 min), I^- (3.28 min), SCN^- (5.13 min), and ClO_4^- (7.65 min). These retention data showed that the model anions interacted with the stationary phase to differing extents, with the DMPC-ClO_4^- interaction occurring most favorably, followed by DMPC-SCN^-, DMPC-I^-, DMPC-ClO_3^-, DMPC-NO_3^-, DMPC-Br^-, DMPC-NO_2^-, DMPC-Cl^-, DMPC-F^-, and then DMPC-SO_4^{2-}. The ranked order of the strengths of anion-DMPC interactions observed with the HPLC method developed in this study was exactly the same as the order of the strengths of the anion-phosphatidylcholine vesicles interactions observed with the fluorescence technique (Clarke and Lüpfert, 1999).

Use of PBS as eluent

To observe the anion-DMPC interactions under cell-viable conditions, PBS solution was used as eluent because this is a commonly used physiological buffer. PBS (pH 7.4) contains 8.10 mM Na_2HPO_4, 136.9 mM NaCl, 2.68 mM KCl, and 1.47 mM KH_2PO_4. Thiocyanate, iodide, nitrate, nitrite, bromate, and iodate were selected as the model ions because all of these species were UV-absorbing anions and could be detected directly by UV-visible detection. The conductivity detection could not be used when PBS solution was used as the eluent because of the high background conductance (because the conductivity suppression technique used in this study was not applicable for such electrolytes). Retention

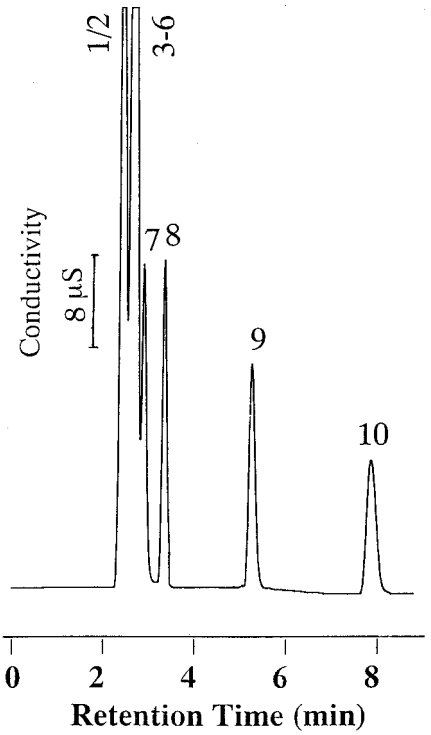

FIGURE 1 Anion-DMPC interactions observed using the HPLC approach. A DMPC column (250 × 4.6 mm i.d.) was formed by loading DMPC on the surface of a reversed-phase ODS support. An aqueous $NaHCO_3$ solution (10 mM) was used as eluent to condition the column. After the HPLC separation, $NaHCO_3$ was converted as H_2CO_3 using an electrolytic suppressor. The sample contained 10 μl of an aqueous solution containing 0.1 mM each of Na_2SO_4, NaF, NaCl, $NaNO_2$, NaBr, $NaNO_3$, $NaClO_3$, NaI, NaSCN, and $NaClO_4$, and the flow rate of the eluent was 1.0 ml/min. Peaks are identified as follows: 1/2 = SO_4^{2-} and F^-; 3–6 = Cl^-, NO_2^-, Br^-, and NO_3^-; 7 = ClO_3^-; 8 = I^-; 9 = SCN^-; and 10 = ClO_4^-.

times for SCN^-, I^-, NO_3^-, and NO_2^- were identical to those observed with the $NaHCO_3$ eluent, whereas retention times for IO_3^- and BrO_3^- were 2.29 and 2.35 min, respectively.

Influence of pH on anion-DMPC interactions

The DMPC column was then conditioned with aqueous 50 mM NaCl solutions having pH values in the range 3.15–7.41, adjusted by adding HCl or Na_2HPO_4/NaH_2PO_4 to the solution. Retention times for six model anions were obtained and used to construct plots of log k' versus pH (where k' represents the retention factor for the model anions, calculated as $k' = (t_i - t_0)/t_0$, where $t_0 = 2.18$ min and t_i is the observed retention time for a particular anion). The retention factor was used here because k' is a more convenient parameter than retention time for evaluating the mechanism of the interactions between the model ions and the stationary phase. As can be seen from Fig. 2, the strength of interactions of the model anions with the stationary phase increased as the pH value of the eluent was reduced below pH 5.

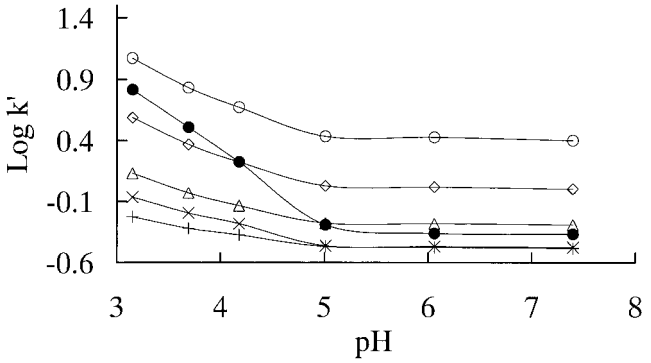

FIGURE 2 Influence of eluent pH on anion-DMPC interactions. Aqueous solutions containing 50 mM NaCl with different pH values were used as eluent. Other HPLC conditions were as in Fig. 1. ○, SCN^-; ◇, I^-; ●, NO_2^-; △, NO_3^-; ×, BrO_3^-; +, IO_3^-.

Aqueous solutions containing NaCl/HCl were prepared and were used as the eluent to separate the UV-absorbing model anions. The concentration of HCl in each eluent was maintained at 0.2 mM (pH 3.7), whereas the concentration of NaCl varied over the range 2–150 mM. Fig. 3 shows the observed changes in retention, plotted as log k' versus the logarithm of concentration of NaCl in the eluent. Straight lines with negative slopes were obtained for all of the model anions, with slopes for SCN^-, I^-, NO_3^-, BrO_3^-, and IO_3^- all being very similar (~ -0.42), with NO_2^- showing a value of -0.21. These values and the linearity of the plots indicated the existence of electrostatic effects (such as anion-exchange behavior) and suggested that NaCl had the ability to modify the adsorption of the model ions onto the DMPC stationary phase under HPLC conditions. On the other hand, the difference in slope between nitrite and the other ions suggested that nitrite-DMPC interaction occurred in a manner that differed from the other anion-DMPC interactions.

The effect of [NaCl] on retention of the model anions was also studied at pH 5.5 (NaCl dissolved in deionized water).

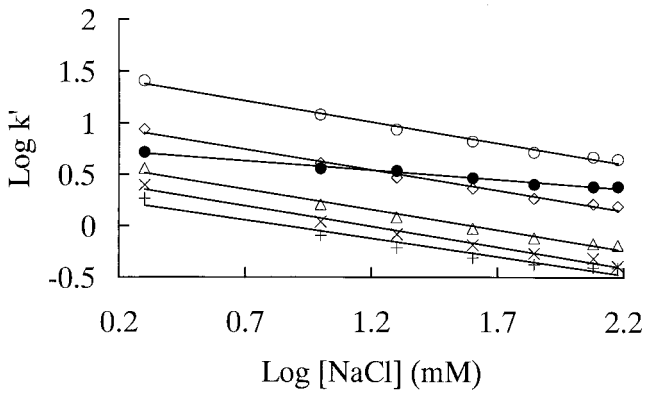

FIGURE 3 Effect of NaCl in eluents containing HCl at 0.2 mM (pH 3.71). Other HPLC conditions were as in Fig. 1. ○, SCN^-; ◇, I^-; ●, NO_2^-; △, NO_3^-; ×, BrO_3^-; +, IO_3^-.

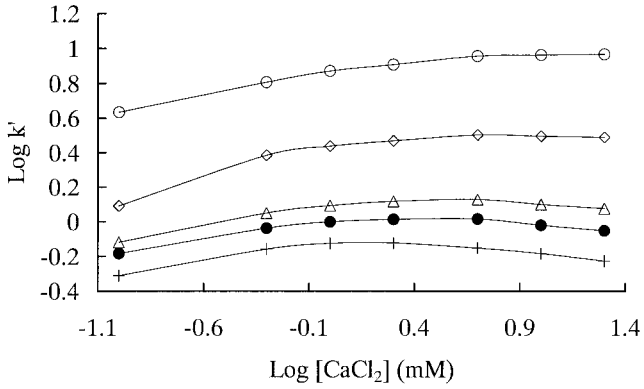

FIGURE 4 Effects of Ca^{2+} ions in the eluent. Aqueous solutions containing mixtures of NaCl and $CaCl_2$ were used as eluent. Concentration of NaCl in each eluent was 130 mM. Other HPLC conditions were as in Fig. 1. ○, SCN^-; ◇, I^-; △, NO_3^-; ●, NO_2^-; +, IO_3^-.

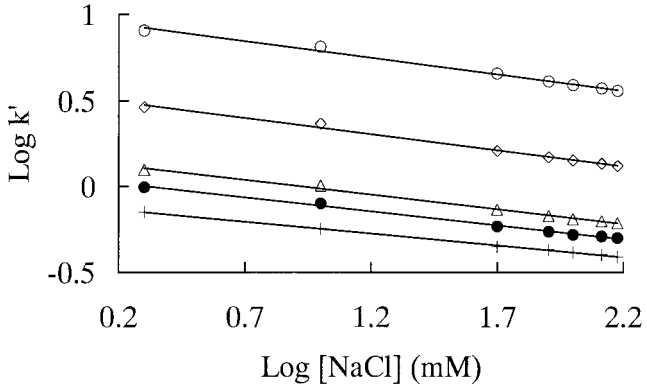

FIGURE 5 Effect of NaCl in eluents containing Ca^{2+} at 2.0 mM. Other HPLC conditions were as in Fig. 1. ○, SCN^-; ◇, I^-; △, NO_3^-; ●, NO_2^-; +, IO_3^-.

At this pH value, the retention times were independent of the concentration of NaCl in the eluent. That is, plots of log k' versus log [NaCl] were straight lines of zero slope for all of the model anions. This indicated that at this pH value, there was no electrostatic interaction occurring on the DMPC column. The retention times for the model anions obtained using the NaCl eluents at pH 5.5 were identical to those obtained using PBS as the eluent.

Influence of Ca^{2+} on anion-DMPC interactions

Aqueous NaCl/$CaCl_2$ solutions were used as the eluent, under conditions where [NaCl] was maintained at 130 mM and [$CaCl_2$] was varied over the range 0.1–20 mM, with the pH maintained at 5.5. Fig. 4 shows plots of log k' versus log [$CaCl_2$] and indicates that the addition of $CaCl_2$ to the eluent caused a general increase in retention and hence an increase in strength of anion-DMPC interactions. Next, mixed NaCl/$CaCl_2$ eluents were prepared in which [$CaCl_2$] was maintained at 2.0 mM, while [NaCl] was varied over the range 2–150 mM, again with the pH maintained at 5.5. Plots of log k' versus log [NaCl] are shown in Fig. 5. These plots all showed a slope of −0.42, including for NO_2^-. Experiments were also performed using $MgCl_2$/NaCl mixed electrolyte solutions as the eluent, with similar results to those obtained using $CaCl_2$/NaCl mixed electrolyte solutions as the eluent.

Mechanism of anion-DMPC interactions

The above results indicated that the interaction of the model ions with the DMPC stationary phase was influenced by pH, the concentration of NaCl, and the presence of Ca^{2+} or Mg^{2+} ions. These experimental data suggested that the DMPC interacted with both anions and cations and the cation-DMPC interactions had a strong effect on the anion-DMPC interactions. An explanation of this behavior can be derived from previous studies on the interaction of inorganic ions with sulfobetaine-type zwitterionic stationary phases (Cook et al., 2001) and uses the electrostatic surface potential existing at zwitterionic stationary phases.

DMPC contains a quaternary ammonium group (N^+) and a phosphate moiety (P^-). The net surface potential of the DMPC stationary phase, Ψ, is influenced by the positive potential ($+\delta\Psi$) produced by N^+ and the negative potential ($-\delta\Psi$) produced by P^-. The value of Ψ will be changeable, depending on the cation-DMPC interactions that take place. If the eluent has a higher pH value (pH 5; Fig. 2) and the cations in the eluent are monovalent (Na^+, K^+, and NH_4^+ were tested in this study), then Ψ has an extremely small value because the headgroup (P^--N^+) can be expected to lie almost parallel to the stationary phase surface (in accordance with membrane studies by Büldt et al., 1978) and the positive charge of the N^+ group is strongly offset by the negative charge of the P^- group. Under these conditions, interactions of the model anions with the DMPC stationary phase are typical anion-zwitterion interactions, and their strength is dependent chiefly on the solvation-dependent properties of the anion involved, as suggested by Clarke and Lüpfert (1999). Anions having a low free energy of formation in aqueous solution (such as I^- and ClO_4^-), and therefore having a high propensity to transfer from the eluent to the stationary phase, can easily approach the headgroup of DMPC and interact readily with DMPC. On the other hand, anions having high free energies of formation in aqueous solution (such as F^- and SO_4^{2-}), and therefore having a high propensity to remain in the aqueous phase, have difficulty in reaching the headgroups of DMPC and therefore show weaker interaction with the DMPC stationary phase. Iodate (IO_3^-) was eluted much earlier than chlorate (ClO_3^-), and this was attributed to the fact that the free energies of formation in aqueous solution are −8.0 kJ mol^{-1} and −128.0 kJ mol^{-1} for chlorate and iodate, respectively

(Lide, 1997). The chromatographic data obtained using aqueous solutions containing $NaHCO_3$, NaCl alone, or PBS as eluent give experimental evidence to support this conclusion.

H^+ ions and Ca^{2+} ions interact strongly with the P^- groups of DMPC, because of protonation and electrostatic interactions, respectively. As the cation-DMPC interaction increases, the value of $-\delta\Psi$ at the P^- group also decreases, leading to Ψ attaining a more positive value. Under these conditions, anion-DMPC interactions behave according to a conventional electrostatic interaction mechanism. The pH effect on the anion-DMPC interactions, as shown in Fig. 2, appeared at much higher pH values than would be expected from consideration of the pKa value of the phosphate group of DMPC (pKa $<$ 1 (Marsh, 1990)). This suggested that the local $[H^+]$ at the surface of the DMPC stationary phase may be many orders of magnitude higher than that of the $[H^+]$ in the eluent. The retention behavior shown in Fig. 4 for $NaCl/CaCl_2$ eluents reflects both the increased retention resulting from increasing Ψ caused by higher concentrations of Ca^{2+} and the decreased retention caused by the ionic strength effects on diminishing the strength of electrostatic interactions as the concentration of chloride in the eluent is raised. The plots in Fig. 4 therefore show a maximum retention for each model anion at $[CaCl_2] \approx 4$ mM. At lower $[CaCl_2]$, adsorption of Ca^{2+} on the P^- groups leading to increased Ψ is the predominant effect, whereas at higher $[CaCl_2]$, the ionic strength effects of the added chloride in weakening the electrostatic interactions predominate. Nitrite is a pH-dependent species and forms neutral nitrous acid (pKa = 2.8) at low pH values. This reduces the DMPC-NO_2^- electrostatic interactions and explains the anomalous behavior of nitrite in Fig. 3.

Thermodynamic emulation of anion-DMPC interactions

The strengths of the anion-DMPC interactions can also be ranked by other techniques, such as the fluorescence (Paula et al., 1998; Clarke and Lüpfert, 1999; Kalinin and Molotkovsky, 2000) or the NMR approaches (Jendrasiak, 1972; Hauser et al., 1977; Grasdalan et al., 1977; Rydall and Macdonald, 1992). However, the HPLC approach introduced here is much simpler and faster and allows the precise calculation of the free enthalpies of anion-DMPC interactions. The dependence of retention factor for the model anions on temperature at which the separation is performed is given in Eq. 1, which is generally used to derive ΔH^0 for the stationary-phase/model-ion interactions (Weiss, 1995).

$$\ln k' = -\Delta H^0/(RT) + \Delta S^0/R + \ln \Phi, \qquad (1)$$

where k' is the retention factor, ΔH^0 and ΔS^0 are the overall enthalpy and entropy changes for the anion-DMPC interaction for a specific anion, and Φ is the phase volume ratio of the stationary phase to the mobile phase. If it is assumed that ΔH^0 and ΔS^0 are independent of temperature (T), then plots of ln k' versus $1/T$ (i.e., van't Hoff plots) are straight lines from which the value of ΔH^0 can be calculated from the slope. Such van't Hoff plots were constructed using retention factor values for NO_3^-, I^-, SCN^-, and ClO_4^- obtained using 10 mM $NaHCO_3$ aqueous solution as eluent at 25°C, 30°C, 35°C, and 40°C (Fig. 6). Linear plots (r^2 0.998) were obtained, and the derived values of ΔH^0 were −25.85, −22.18, −10.61, and −2.91 kJ/(mol K) for the DMPC-ClO_4^-, DMPC-SCN^-, DMPC-I^-, and DMPC-NO_3^- interactions, respectively. As can be seen from these thermodynamic data and the rank-ordered series of retention times, an anion having a higher propensity to interact with DMPC was also observed to have a larger value of the enthalpy change. This order of ΔH^0 for I^- and SCN^- obtained using aqueous NaCl (5.0 mM, pH 5.5) solution and aqueous $NaClO_4$ (5.0 mM, pH 5.5) solutions as eluent were also calculated and were −22.20 and −6.85 kJ/(mol K) for thiocyanate and −10.58 and 3.22 kJ/(mol K) for iodide, with the NaCl and $NaClO_4$ eluents, respectively. These values of ΔH^0 were the average values of three measurements and standard errors relative standard deviation (RSD) were better than 1.4%. Generally, the value of ΔH^0 determined using the van't Hoff plots is not identical to the enthalpy changes of the analyte-stationary-phase interactions (ΔH). Other contributors, such as the standard enthalpy change associated with changes in the hydration of the model anions during the procedure of anion-DMPC interactions might also be included in ΔH^0. However, because the electrostatic contribution to anion-DMPC interaction was not observed under higher pH conditions and the anion-phospholipids interactions do not cause the anion to lose its water of hydration (Paula et al., 1998), it can be assumed that ΔH^0 is very similar to ΔH. The enthalpy changes obtained with the present method are similar in magnitude to the values determined previously with other techniques. It should be noted that the thermodynamics of ion-membrane interactions are measurable by using other

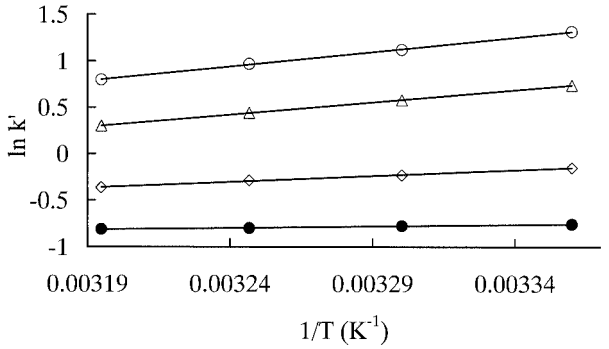

FIGURE 6 van't Hoff plots for ClO_4^-, SCN^-, I^-, and NO_3^- obtained with 10 mM $NaHCO_3$ aqueous solution as the eluent. ○, ClO_4^-; △, SCN^-; ◇, I^-; ●, NO_3^-.

techniques, such as the titration calorimetric method. The study by Blume and Tuchtenhagen (1992), which addresses cation interactions with liposomes made from phosphatidic acid, is a good illustration of this fact.

CONCLUSION

A simulated biomembrane formed from adsorbed DMPC was found to interact with both cations and anions. The cation-DMPC interactions exerted a very strong influence on the anion-DMPC interactions and can be explained by considering the surface potential of the stationary phase. The chromatographic approach introduced in this study can provide useful data regarding the behavior of ion-biomembrane interactions but gives no information regarding changes in orientation of the quaternary groups and the phosphate moieties during the ion-DMPC interactions. Finally, it is noted that the headgroups (P^--N^+) of DMPC are considered to be the primary sources of the DMPC layer electric potential in this study. Other possible contributors, such as the carbonyl groups and oriented water molecules, are not considered.

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