# Effect of Membrane Association on the Stability of Complexes between Ionophore A23187 and Monovalent Cations†

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ABSTRACT: The monovalent cation complexation properties of ionophore A23187 in methanol–water (65–95% w/w) and bound to unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) are contrasted. In both solution and vesicle-containing systems, 1:1 complexes between the ionophore and $Li^+$ or $Na^+$ predominate. The analogous complexes with $K^+$, $Rb^+$, and $Cs^+$, which exist in methanol, are not detected on DMPC vesicles by changes in the absorption or fluorescence emission spectra of the ionophore. In solution, the logarithms of stability constants (log $K_{MA}$) for both the LiA and NaA complexes increase by 1.5 units over the range of solvent polarity encompassed by 65% methanol–water to methanol. Selectivity for $Li^+$ vs. $Na^+$ is constant at a ratio of 5 in these solutions. On DMPC vesicles, selectivity for $Li^+$ vs. $Na^+$ is improved 15-fold with log $K^b_{LiA}$ (3.23 ± 0.03, $T$ = 25 °C, $\mu$ = 0.05 M) being comparable to the value obtained in 80% methanol–water. In the latter solvent, increasing ionic strength (0.005–0.085 M) has little effect on log $K_{LiA}$ or log $K_{HA}$ but increases these constants by 0.4–0.5 unit in the DMPC vesicle system. Transition from the vesicle liquid-crystalline to gel-phase state reduces log $K^b_{LiA}$ and log $K^b_{NaA}$ by approximately 0.6 unit but has no effect on log $K^b_{HA}$. Thermodynamic parameters for formation of HA, LiA, and NaA in 80% methanol–water and on DMPC vesicles are reported. Analysis of these data and related considerations suggests that differences in the membrane interaction energies of particular ionophore species dominate in establishing the observed difference in complexation properties between the solution and vesicle-containing systems.

Cation transport catalyzed by carboxylic acid ionophores can be viewed as occurring through a series of consecutive reactions which, taken in sum, constitute the overall transport cycle (Pfeiffer et al., 1978). In general, each component reaction can be characterized by equilibrium and kinetic constants which, if available for each reaction, would allow a fairly detailed interpretation of the transport rates and specificities of these compounds. Historically, work directed at interpreting the transport behavior of carboxylic acid ionophores has employed solution models for the reactions of interest [see Taylor et al. (1982) for a review]. However, in practice, the component reactions are believed to occur at or near the aqueous phase–membrane interface which is an environment dissimilar to that of true solutions. Various interactions between ionophores and membrane components can be anticipated [see Painter & Pressman (1982) for a review] which, together with the inherent anisotropic nature of an interface, would be expected to affect both equilibrium and kinetic properties of the component reactions in ways not revealed by a solution model.

To assess the role of ionophore–membrane interactions on the transport properties of these compounds, we have compared the acid base properties of ionophore A23187 in solution to those observed when the compound is associated with phospholipid vesicles (Kauffman et al., 1982). Aspects of how A23187 interacts with vesicle membranes as a function of ionophore protonation state have also been described (Kauffman et al., 1983). The present study is an extension of that work in which the effects of membrane association on the stability of complexes between the ionophore and monovalent cations are examined. Monovalent rather than divalent cations have been investigated initially, in part because fewer complex species must be considered (see below) and in part because accurate stability constants for complexes between A23187 and monovalent cations will be needed to explain the unusual divalent cation transport selectivity of this ionophore. Aspects of these data have appeared in abstract form (Pfeiffer et al., 1983; Pfeiffer & Chapman, 1984).

## Materials and Methods

L-α-Dimyristoylphosphatidylcholine (DMPC)[1] was obtained from Sigma Chemical Co. (St. Louis, MO). The synthetic phospholipid was purified by chromatography on 2.5 × 25 cm columns of silicic acid which had been prewashed with methanol followed by diethyl ether. Columns were loaded with 0.5 g of DMPC in 200 mL of $CHCl_3$ and eluted with a step gradient of methanol–chloroform as follows: 200 mL 10:90, 200 mL of 15:85, 200 mL of 25:75, 400 mL of 50:50, and 200 mL of methanol. DMPC was obtained in the 50:50 fraction

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[1] Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DMPC, L-α-dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; AH, free acid of A23187; $A^-$, carboxylate anion of A23187; MA, 1:1 A23187–monovalent cation complex; $MA_2$, 2:1 A23187–monovalent cation complex; $\lambda_{ex}$, excitation wavelength of maximum fluorescence; $\lambda_{em}$, emission wavelength of maximum fluorescence; $\lambda_{max}$, wavelength of maximum absorbance.

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which was subsequently taken to dryness in vacuo, redissolved in chloroform, and then precipitated by the addition of 9 volumes of acetone. The final material was free of spectral impurities of impurities detectable by thin-layer chromatography in several solvent systems and was stored at −20 °C as a dry powder.

All solvents were distilled shortly before use. Reagent-grade methanol was distilled from zinc dust and potassium hydroxide. Distilled, deionized water (specific resistance ≥ 13 MΩ cm) and acid-washed glassware (sulfuric–nitric, 3:1 v/v) were used for all solutions. Tetraethylammonium perchlorate (Eastman Chemicals) was recrystallized 4 times from hot water before use. Reagent-grade perchloric acid (Fisher Scientific, Pittsburgh, PA) and tetraethylammonium hydroxide (Eastman Chemicals, Rochester, NY) were used without further purification. Ultrapure $LiClO_4$, $NaClO_4$, $RbClO_4$, $CsClO_4$, and KCl were obtained from Alfa Products or Aldrich. Standard solutions of these salts were prepared by weight. The free acid of A23187 was a gift from Dr. Robert Hamill of Eli Lilly Co. (Indianapolis, IN).

*Vesicle Preparation.* Small unilamellar vesicles of DMPC were prepared by sonication with the cup-horn apparatus (Heat Systems no. 431A) as described previously (Kauffman et al., 1982). Unless otherwise specified, the aqueous solutions contained 33 mM tetraethylammonium perchlorate, 5 mM each of β,β′-dimethylglutaric acid, Hepes, and Ches, and 0.1 mM each of EDTA and EGTA.

The vesicles were purified by ultracentrifugation employing the method of Barenholz et al. (1977) as modified by Gaber & Sheridan (1982). The nominal concentration of DMPC in the vesicle suspensions was determined by measurement of lipid phosphorus (Bartlett, 1959). The preparations were stored at 35 °C, under $N_2$, and were used within 2 weeks. No fusion could be detected by electron microscopic examination of negatively stained samples during storage for this length of time.

Vesicle preparation, purification, and storage were conducted at pH 8 with the pH desired for particular experiments established subsequently by the addition of tetraethylammonium hydroxide or perchloric acid as required. A combination glass electrode (Beckman 39030) calibrated against standard buffers (Fisher Scientific) was utilized for these pH measurements.

A23187 was added to liposomal suspensions as the free acid in ethanol. The final ethanol concentration was kept at less than 0.1 vol %, a concentration region in which this solvent does not perturb lecithin vesicles (Jain & Wu, 1977).

*Methanol–Water Solvents.* Solutions of varying percentages of methanol in water (w/w) were prepared graviemtrically. Unless otherwise specified, these solutions contained buffers, chelators, and tetraethylammonium perchlorate as described above for the aqueous phase of DMPC vesicle suspensions. Measurements of pH* in methanol–water mixtures were carried out by using a glass electrode (Corning 476024) in combination with a polymer body reference electrode (Markson 1881). Electrodes were calibrated against aqueous standard buffers (Fisher Scientific) prior to equilibration with methanol–water solution of interest. The operational pH* scales developed by deLigny et al. (1960a,b) and Gelsema et al. (1966, 1967) and extended by Bates et al. (1963) were then utilized to determine the value of pH*. The term pH* is defined as $-\log a_H^*$, where $a_H^*$ is the activity of $H^+$ in the mixed solvent. The term pH* when used in reference to a specific methanol–water mixture, therefore, has the same meaning as the term pH when used in reference to an aqueous solution [see Rorabacher et al. (1971) and references cited therein].

*Data Acquisition and Analysis.* Absorption spectra were recorded with either a Cary Model 118 or an American Instrument DW2a spectrophotometer. Data from these instruments were tabulated manually and fed into a microcomputer for analysis. Fluorescence spectra were obtained with an SLM 8000DS spectrophotometer equipped with double-grating excitation and emission monochromators and operated in the analog mode. A Schott KV390 filter was placed in front of the emission monochromator to assist in the separation of fluorescence emission from scattered light. This instrument was interfaced to a microcomputer system for data manipulation and storage. Complexes of 1:1 stoichiometry between A23187 and monovalent cations predominate under the conditions of this work (see below), and in most cases, pH conditions were utilized such that the free ligand existed as the carboxylate anion (>99%). In addition, for titrations involving vesicle-associated ionophore, the mole ratio of DMPC to A23187 was maintained at values such that >95% of the total ionophore was membrane associated [see Kauffman et al. (1983) for association constants of A23187 with DMPC vesicles]. The complexation reactions under investigation are then given by eq 1 and 2 for the solution model and vesicle–aqueous biphasic system, respectively.

$$M^+ + A^- \rightleftharpoons MA \tag{1}$$

$$M^+_{aq} + A^-_b \rightleftharpoons MA_b \tag{2}$$

In these expressions, $M^+$, $A^-$, and MA refer to the metal cation, the anion of A23187 and the 1:1 complex, respectively. The subscripts aq and b in eq 2 denote respectively an aqueous phase or membrane-bound location for the species in question.

Equilibrium expressions for reactions 1 and 2 are given by eq 3 and 4 which can be rewritten in their linear forms as eq 5 and 6.

$$K_{MA} = [MA]/[M^+][A^-] \tag{3}$$

$$K^b_{MA} = [MA_b]/[M^+_{aq}][A^-_b] \tag{4}$$

$$\log K_{MA} = pM^+ + \log ([MA]/[A^-]) \tag{5}$$

$$\log K^b_{MA} = pM^+_{aq} + \log ([MA_b]/[A^-_b]) \tag{6}$$

In these expressions, the subscripts aq and b have the same meaning as described above while the concentrations of bound species are nominal. Equilibrium constants were resolved from titration data expressed as plots of log ($[MA]/[A^-]$) or $[MA_b]/[A^-]$ vs. $pM^+$. The best fit to the experimental data was obtained by the least-squares method. In the case of fluorescence titrations, ratios of free to complexed ionophore at a given cation concentration were calculated by using the equation:

$$[MA_b]/[A^-_b] = \Delta F_{obsd}/\Delta F_{max} \tag{7}$$

where $\Delta F_{obsd}$ is the observed change in relative fluorescence compared to the value obtained in the absence of metal and $\Delta F_{max}$ is the maximal change obtained at saturation. In the case of absorbance titrations, an analogous expression was employed in which changes in absorbance replaced changes in relative fluorescence. It was usually not possible to obtain $\Delta F_{max}$ or $\Delta A_{max}$ experimentally due to the relative low affinity of this compound for monovalent cations. This quantity was therefore estimated by extrapolation of plots of $1/[M^+]$ vs. $1/\Delta F_{obsd}$ or $1/\Delta A_{obsd}$.

The methods employed for the determination of the ionophore protonation constant were similar to those used for

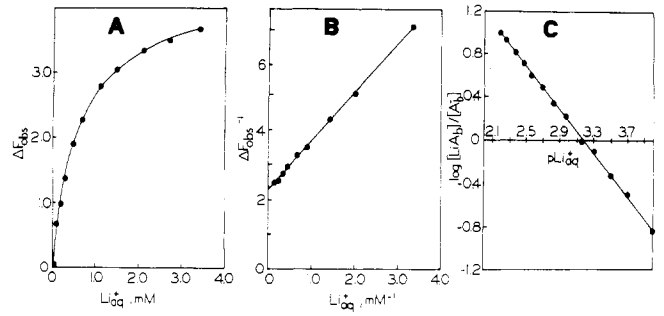


FIGURE 1: Determination of log $K^b_{LiA}$ by fluorometric titration. The nominal concentrations of DMPC and A23187 were 2.5 mM and 3.33 $\mu$M, respectively. The aqueous phase contained tetraethylammonium perchlorate, buffers, and chelators as described under Materials and Methods. The aqueous phase pH was 10.5, the temperature was 25 °C, and the ionic strength was approximately 50 mM. Samples were excited at 360 nm and emission was monitored at 425 nm which are $\lambda_{ex}$ and $\lambda_{em}$, respectively, for the complex $LiA_b$ under these conditions. Panel A shows the change in relative fluorescence intensity as a function of increasing aqueous phase concentration of $LiClO_4$. In panel B, the data are expressed as a double-reciprocal plot to illustrate the identification of $\Delta F_{max}^{-1}$ at the $y$-axis intercept. Some points from panel A are deleted in panel B for clarity of the presentation. In panel C, the data have been plotted according to eq 6. log $K^b_{LiA}$ is equal to $pLi^+_{aq}$ at log ($[LiA_b]/[A_b]$) = 0.

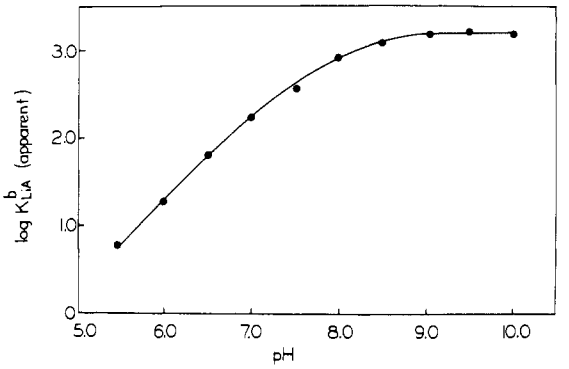


FIGURE 2: Effect of aqueous phase pH on the apparent value of log $K^b_{LiA}$. Stability constants were determined as described in the legend to Figure 1 except that the aqueous phase pH values were shown and the temperature was 30 °C. When the ionophore was titrated at aqueous phase pH values below approximately 9, the relative fluorescence in the absence of $Li^+$ was a function of pH due to the equilibrium beteen $A^-_b$ and $H^+$ [see Kauffman et al. (1982)]. This caused the value $\Delta F_{max}$ determined as illustrated in Figure 1B to vary with pH. Thus, when using eq 7 to determine $[LiA_b]/[A^-_b]$ ratios, it was necessary to utilize the value of $\Delta F_{max}$ observed for the pH in question.

complexation constants and have been described in detail previously (Kauffman et al., 1982).

## RESULTS

*Stoichiometry of Complexes between $Li^+$ and Membrane-Associated A23187.* Previous studies have shown that both 1:1 2:1 (ionophore:cation) complexes between A23187 and monovalent cations occur and that protonated forms of these complexes may also exist (Pfeiffer & Lardy, 1976). In addition, it has been reported that, in methanol, the second stepwise binding constant is larger than the first for some 2:1 A23187–divalent cation complexes (Tissier et al., 1979). Thus, initially it was necessary to demonstrate that eq 1 and 2 adequately describe the equilibria which pertain under the conditions of this study.

Figure 1A shows a fluorescence titration of vesicle-associated A23187 with increasing aqueous phase concentrations of $LiClO_4$. The nominal concentrations of DMPC and A23187 employed were chosen so that the fraction of ionophore partitioned into the aqueous phase is negligible (Kauffman et al., 1982, 1983). The aqueous phase pH employed (10.5) is sufficiently above the value of $pK^b_{HA}$ under these conditions (7.85 ± 0.05; Kauffman et al., 1982) to preclude formation of protonated complexes. Thus, the increase in fluorescence as a function of increasing $[Li^+_{aq}]$ in Figure 1A could represent the formation of the species $LiA_b$, $LiA_{2b}$, or a mixture of both species. When the data are expressed as a plot of $1/\Delta F_{obsd}$ vs. $1/[Li^+_{aq}]$ as in Figure 1B, a linear relationship is obtained demonstrating the expected first-order dependence of complex formation on $[Li^+_{aq}]$. When the data are expressed as a plot of log $[MA_b]/[A^-_b]$ vs. $pLi_{aq}$ (from eq 6), the relationship is again linear and displays a slope of −1.0 (Figure 1C). These findings support a model in which the predominant complex species formed is $LiA_b$. Analysis of the data according to eq 6 yields a value of log $K^b_{LiA}$ of 3.23 ± 0.03 ($n$ = 5). The value obtained by absorbance measurements was the same as that obtained by fluorescence within one standard deviation (data not shown).

To further define the stoichiometry of complexes between vesicle-associated A23187 and $Li^+$, stability constants were determined as a function of ionophore concentration and as a function of aqueous phase pH. Figure 2 demonstrates that, in the region of aqueous phase pH where a significant fraction of the vesicle-associated ionophore exists as the free acid (pH ≤9), the apparent stability constant $K^b_{LiA}$ decreases with decreasing values of pH. At lower pH values, the relationship between log $K^b_{LiA}$ and pH is linear and displays a slope of 1.0. These findings indicate the absence of protonated species and are further support for the predominance of the complex $LiA_b$.

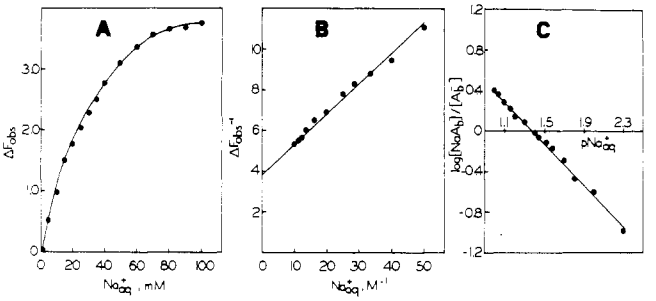


FIGURE 3: Determination of log $K^b_{NaA}$ by fluorometric titration. Data were obtained and analyzed as described in the legend to Figure 1 except that the titration was conducted with $NaClO_4$.

Table I: Effect of A23187 Concentration on the Stability of the Complex $LiA_b$[a]

| log $[A^-_b]$ (M) | log $K^b_{LiA}$ |
|---|---|
| −4.48 | 3.24 ± 0.02 |
| −5.48 | 3.17 ± 0.01 |
| −6.48 | 3.05 ± 0.05 |
| −7.48 | 2.99 ± 0.08 |
| −8.48 | 3.05 ± 0.06 |

[a] log $K^b_{LiA}$ was determined as described in the legend to Figure 1 except that the nominal concentration of ionophore was as indicated and the temperature was 30 °C. Values presented are the mean ± SD of five determinations.

The effects of varying ionophore concentration are shown in Table I. In the absence of complexes of the type $LiA_2$, no effect of ionophore concentration on complex stability would be expected. Table I shows that this condition is essentially met over 4 orders magnitude of ionophore level. There is an apparent slight trend toward increasing stability at higher concentrations of the compound. This effect could indicate that a small fraction of the 2:1 complex is formed at the higher ionophore concentrations although other explanations are more probable (see Discussion).

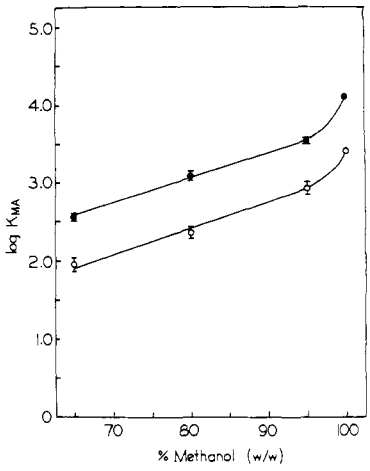


FIGURE 4: Effect of solvent composition on the stability of solution complexes of A23187 and monovalent cation. Values were determined at 25 °C in the indicated methanol–water mixtures which contained buffers and tetraethylammonium perchlorate as described under Materials and Methods. Ionic strength = 0.05 M; pH* >10; total concentration of A23187 = 30 μM. Complex formation was followed by recording absorption spectra over the region of 250–420 nm. The maximal absorbance change upon complexation of either cation occurred near 300 nm and was used to follow the reaction. A single set of isosbstic points was observed. In addition, the relationships between complex formation, [$Li^+$], the value of pH*, and the concentration of A23187 employed (data not shown) were the same as those described under Results with respect to the stoichiometry of complexes involving membrane-bound ionophore (80% methanol–water). These findings indicate that eq 1 describes the equilibria being observed.

Table II: Stability Constants for the Species LiA, NaA, and HA in Solution and Vesicle–Aqueous Biphasic Systems[a]

| media | log $K_{LiA}$ | log $K_{NaA}$ | log $K_{HA}$ |
|---|---|---|---|
| 65% MeOH | 2.53 ± 0.04 | 1.95 ± 0.08 | 7.19 ± 0.05 |
| 80% MeOH | 3.08 ± 0.05 | 2.36 ± 0.07 | 7.84 ± 0.05 |
| 95% MeOH | 3.54 ± 0.03 | 2.92 ± 0.07 | 9.43 ± 0.05 |
| 100% MeOH | 4.1 | 3.4 | 10.9 |
| DMPC vesicles | 3.22 ± 0.04 | 1.33 ± 0.04 | 7.85 ± 0.05 |

[a] Values were determined at 25 °C as described under Materials and Methods. Except for the 100% MeOH value, protonation constants were taken from Kauffman et al. (1982). All values in 100% MeOH were from Tissier et al. (1979) and were determined at an ionic strength of ≃0. Other values were obtained at an ionic strength of 0.05 M.

*Effect of Membrane Association on Selectivity of Complexation.* With membrane-associated ionophore, aqueous phase concentrations of $K^+$, $Rb^+$, and $Cs^+$ as high as 0.1 M give no evidence of complex formation by measurements of absorbance or fluorescence emission (data not shown). However, formation of the $Na^+$ complex can be observed as shown by the data in Figure 3. As described for Figure 1 above, the data in Figure 3 indicate that the 1:1 complex between A23187 and $Na^+$ predominates under the present conditions; log $K^b_{NaA}$ is 1.33 ± 0.04 ($n$ = 5) which, when compared to the value obtained for the $Li^+$ complex, indicates that a selectivity ratio ($K^b_{LiA}/K^b_{NaA}$) of approximately 80 pertains in the vesicle–aqueous biphasic system.

The stability constants of the complexes LiA and NaA were also determined in solutions of methanol–water as a function of percent methanol. The results are presented graphically in Figure 4 and numerically in Table II. The stability of both complexes increases by approximately 1.5 log units upon passing from 65% to 100% methanol. These findings indicate that both stability constants are markedly less dependent on solvent polarity than is the protonation constant which increases by almost 4 orders of magnitude over the same solution

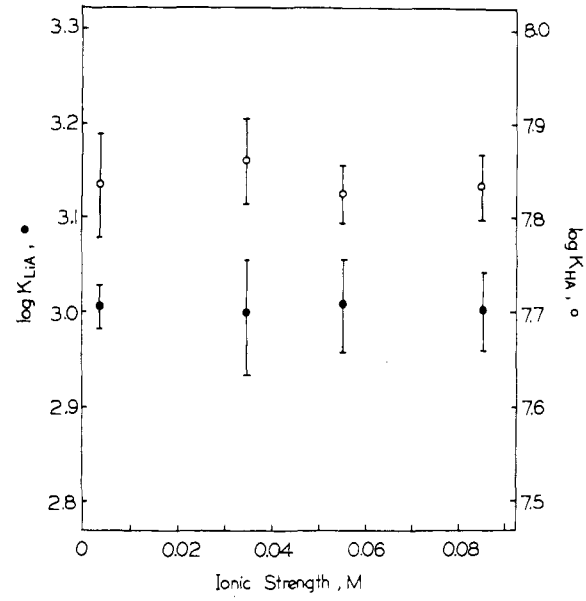


FIGURE 5: Effect of ionic strength on the stability constants of HA and LiA. Experiments were conducted in 80% methanol–water as described under Materials and Methods and in the legend to Figure 4. Ionic strength was maintained at the indicated value by varying the concentration of tetraethylammonium perchlorate. (○) log $K_{HA}$; (●) log $K_{LiA}$. Error bars associated with each point show the standard deviation obtained from five replicate titrations.

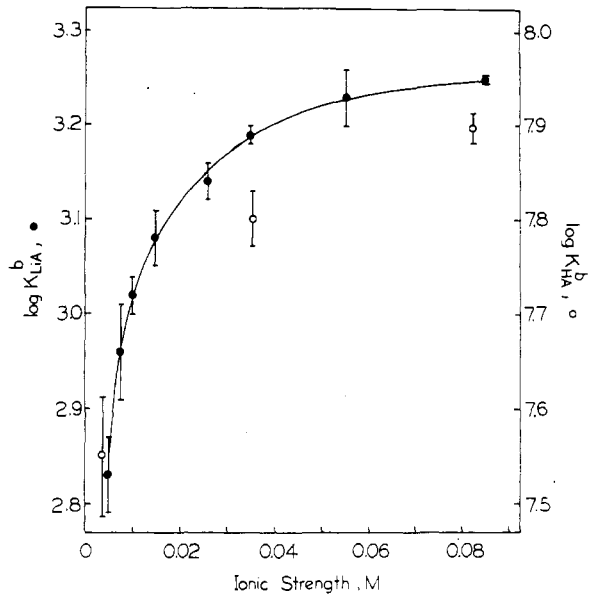


FIGURE 6: Effect of ionic strength on the stability constants of $HA_b$ and $LiA_b$. Experiments were conducted as described under Materials and Methods and in the legend to Figure 1, except that the ionic strength was maintained at the indicated value by varying the concentration of tetraethylammonium perchlorate in the media used for preparation of DMPC vesicles. (○) log $K^b_{HA}$; (●) log $K^b_{LiA}$. Error bars associated with each point show the standard deviation obtained from five replicate titrations.

range (Kauffman et al., 1982; Tissier et al., 1979, see Table II). In the case of the $Li^+$ complex, the stability constant of DMPC vesicles corresponds to the value that is observed in 80–85% methanol. This behavior is comparable to that seen previously for the protonation constant (Table II; Kauffman et al., 1982). However, a comparable pattern is not seen with the $Na^+$ complex. The species NaA is markedly more stable in 80–85% methanol–water than when associated with the vesicles. Over the solvent range examined, the $Li^+$ to $Na^+$ selectivity ratio is approximately 5. When this value is compared to the value of 80 observed on DMPC vesicles, it can

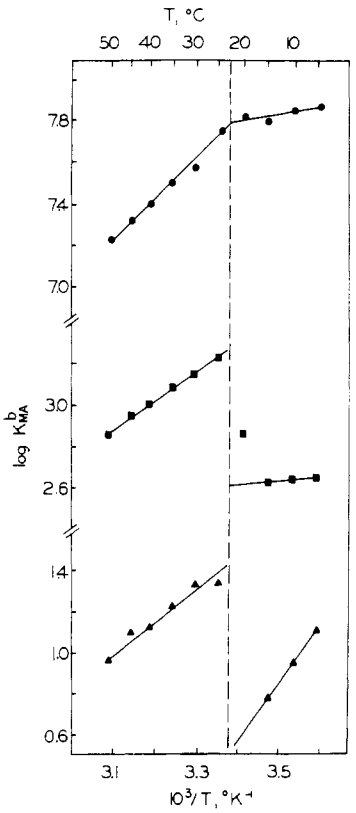


FIGURE 7: Effect of temperature and membrane phase state on the stability constants of $HA_b$, $LiA_b$, and $NaA_b$. Experiments were conducted as described under Materials and Methods and in the legends to Figures 1 and 3 except that the temperature was varied as indicated. (●) log $K^b_{HA}$; (■) log $K^b_{LiA}$; (▲) log $K^b_{NaA}$. The dashed line in the figure shows the midpoint in the phase transition temperature range of these vesicles at 23 °C (Kauffman et al., 1983).

be seen that membrane association increases the selectivity for $Li^+$ over $Na^+$ by approximately 15-fold.

*Effect of Ionic Strength on Stability of the Species HA and LiA.* Figures 5 and 6 compare the effects of ionic strength on the stability constants of LiA and HA in solution and in the membrane-bound states, respectively. In 80% methanol–water, the stability constants of both species are little affected by changes in the ionic strength from 5 to 85 mM. However, as shown in Figure 6, when membrane associated, the species LiA and HA are increasingly stabilized, compared to the dissociated reactants, over the same ionic strength range. The observed magnitude of this effect, expressed as changes in the stability constants, is 0.4–0.5 log unit. The true magnitude of the ionic strength effect is likely to be even larger because increasing ionic strength would decrease the activity of aqueous phase $Li^+$ and may diminish the effective negative surface charge of DMPC vesicles arising from bound $A^-$. Both of these effects would be expected to decrease the apparent value of log $K^b_{LiA}$. The effect of ionic strength on stability of the bound NaA complex could not be determined due to the low affinity of the compound for $Na^+$.

*Membrane Phase State and Stability of A23187 Complexes.* As shown in Figure 7, the phase state of DMPC vesicles is another factor which differentially affects the stability of vesicle-associated A23187 complexes. Upon passing below the critical temperature of these preparations ($T_c$ = 23 °C), both the LiA and NaA complexes are destabilized compared to the reactants. The magnitude of this effect, expressed as the change in the equilibrium constant, is 0.6–0.7 log unit. A comparable effect is not seen for the ionophore's protonation constant. Thus, upon passing from the liquid-crystalline to gel phase, both complexes are destabilized relative to the membrane-associated free-acid form.

Table III: Thermodynamic Parameters at 25 °C for Formation of the Species HA, LiA, and NaA in Solution and Vesicle–Aqueous Biphasic Systems[a]

| species | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ [cal/(mol·deg)] |
|---|---|---|---|
| HA | −10.7 | −1.2 | 32 |
| $HA_b$ | | | |
| above $T_c$ | −10.5 | −8.7 | 6 |
| below $T_c$ | −10.6 | −1.4 | 31 |
| LiA | −4.2 | −3.9 | 1 |
| $LiA_b$ | | | |
| above $T_c$ | −4.5 | −6.3 | −6 |
| below $T_c$ | −3.6 | −0.9 | 9 |
| NaA | −3.3 | −2.1 | 4 |
| $NaA_b$ | | | |
| above $T_c$ | −1.8 | −6.6 | −16 |
| below $T_c$ | −0.6 | −11.6 | −37 |

[a] Values for the bound species were calculated from the data in Figure 7. Values for the equivalent species in solution were determined in 80% methanol–water from titrations at several temperatures which were otherwise conducted as described in the legends to Figure 4 and Table II.

From the data in Figure 7, thermodynamic parameters can be calculated for the protonation reaction and the two complexation reactions as a function of membrane phase state. These values, together with the comparable data obtained in 80% methanol–water, are presented in Table III.

## Discussion

The present study illustrates aspects of how equilibrium complexation properties of an ionophore can be affected by association of the ligand with phospholipid bilayer membranes, compared to properties observed in homogeneous solutions. Examples of variations between the two systems include the thermodynamic characteristics of the reactions, the complexation selectivity, and the effect of ionic strength on complex stabilities. Before discussing these membrane-dependent effects, it is first useful to compare the solution data presented here to a previous solution study and to consider some special problems which arise when interpreting complexation data obtained with vesicle-associated ionophore.

Tissier et al. (1979) have determined stability constants for complexes of 1:1 stoichiometry between A23187 and several monovalent and divalent cations. They utilized methanol as the solvent and potentiometric techniques to monitor the reactions. The present findings, obtained by spectroscopic methods, extend their work to solvents of greater polarity and provide thermodynamic data for the reactions with $H^+$, $Li^+$, and $Na^+$. The selectivity of A23187 for $Li^+$ vs. $Na^+$ is constant at a ratio of approximately 5 over the solvent range of 65% methanol–water to methanol. The stability of complexes with larger alkaline cations was not investigated as a function of solvent composition since we could not detect complexation of these cations by the vesicle-associated compound. However, Tissier et al. (1979) found that, in methanol, the larger monovalent cations are complexed by the ionophore with log stability constants that decrease linearly as a function of ionic radius and which vary by only 2 orders magnitude between $Li^+$ (log $K \simeq 4$) and $Cs^+$ (log $K \simeq 2$). This range of stability constants is within that accessible to measurement by the procedurs employed here. Thus, the fact that complexes with $K^+$, $Rb^+$, and $Cs^+$ were not detected on vesicles suggests that the factors which destabilize NaA compred to LiA on vesicles (see below) also destabilize the analogous complexes with the larger cations.

Table IV: Thermodynamic Parameters for Carboxylic Acid Ionophore–Cation Complexes[a]

| cation | ionophore | log $K$ | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ [cal/(mol·deg)] | ref |
|---|---|---|---|---|---|---|
| $H^+$ | nigericin | 10.3 | −14.1 | −2.9 | 38 | *b* |
| $H^+$ | monensin | 10.3 | −14.1 | −2.4 | 39 | *c* |
| $H^+$ | grisorixin | 10.3 | −14.0 | −3.0 | 37 | *b* |
| $H^+$ | benzoic acid | 9.4 | −12.8 | −4.2 | 29 | *b* |
| $Na^+$ | nigericin | | −5.31 | 1.65 | 23.4 | *d* |
| $Na^+$ | monensin | | −9.2 | −5.5 | 12.4 | *c* |
| | | | −8.2 | −3.9 | 14.6 | *d* |
| $K^+$ | nigericin | | −7.7 | −1.0 | 22.2 | *d* |
| $K^+$ | monensin | | −6.2 | −3.7 | 8.4 | *d* |
| $K^+$ | lasalocid A | | −3.58 ± 0.02 | 4.1 ± 1.1 | 26 ± 4 | *e* |

[a] All values were determined in methanol at 25 °C. [b] Juillard et al. (1983). [c] Hoogerheide & Popov (1979). [d] Lutz et al. (1971). [e] Degani & Friedman (1974).

Thermodynamic parameters for reactions of A23187 in solution given in Table III can be compared to the available analogous data for other ionophores which are summarized in Table IV. For the protonation reaction, A23187 shows $\Delta H$ and $\Delta S$ values which are typical of those observed with other ionophores. For all compounds, protonation is driven primarily by entropic factors, suggesting that free-acid forms of these compounds are substantially desolvated compared to the carboxylate anions. For ionophores other than A23187, entropic factors also make substantial contributions to the free energy of complex formation. However, for the $Li^+$ and $Na^+$ complexes of A23187, only small values for $\Delta S$ are observed. These findings are consistent with incomplete desolvation and shielding of the cation by A23187 in complexes of 1:1 stoichiometry. Although the structures of the LiA and NaA complexes have not been determined, they are likely to involve the same cation-liganding atoms as the 2:1 complexes with divalent cations (Deber & Pfeiffer, 1976; Chaney et al., 1976; Smith & Duax, 1976; Anteunis, 1977; Pfeiffer & Deber, 1979) and to be equivalent to half of the 2:1 complex structure. If this is the case, $Li^+$ and $Na^+$ in 1:1 complexes with A23187 would be bound by only three donor atoms. This structure would be expected to produce incomplete shielding and to allow partial solvation of the bound cation which, in turn, could account for the marginally favorable $\Delta S$ values observed.

When interpreting data obtained with vesicle-associated ionophore, it is necessary to consider how thoroughly different pools of reactants and products can be expected to equilibrate under the conditions utilized. Equilibria of interest in this regard include the exchange reactions of free and complexed ionophore between individual phospholipid vesicles. Under the conditions employed for most of this work (3.33 $\mu$M A23187 and 2.5 mM DMPC) and assuming $3 \times 10^3$ phospholipid molecules per vesicle (Watts et al., 1978), an average of only four ionophore molecules are associated with each vesicle when the ionophore binding and exchange reactions are at equilibrium. The extent to which this equilibration is maintained during titrations is of interest in cases where complexes of multiple stoichiometries are possible as in the present work. The reason for this is that incomplete equilibration of ionophore between vesicles (i.e., slow exchange reactions) would alter the apparent magnitudes of stepwise binding constants as a function of the ligand to vesicle ratio. To visualize this, consider the titration of bound ionophore with aqueous phase cation under conditions where most vesicles are not occupied by a molecule of the ligand. Slow exchange rates under these circumstances would kinetically isolate individual ionophore molecules and thereby favor 1:1 complexes compared to those of higher stoichiometries. Furthermore, slow vs. fast exchange rates would not, in general, affect the apparent magnitude of the stability constant for the 1:1 species. At the opposite extreme, many ligand molecules per vesicle, the apparent stepwise binding constants would approach their true values without regard to the rates of intervesicular exchange. Presumably, a spectrum of apparent relative constants would be observed at conditions between the extremes if the exchange reactions were slow, but not if they were fast.

Rate constants for the binding and dissociation reactions of the various ionophore species of interest here have not been determined nor have the pathways for intervesicular exchange been studied. However, the equilibrium binding constants for the association of $A^-$, HA, and LiA with DMPC vesicles are only in the order of $10^3$–$10^5$ $M^{-1}$, and, in addition, no species involved here is extremely insoluble in water (Kauffman et al., 1983; unpublished experiments). Furthermore, Table I shows that the $Li^+$ titration data can be described by eq 6 and yields essentially invariant values for $K^b_{LiA}$ over a range of ionophore levels which vary from 1 molecule per 250 vesicles to 40 molecules per vesicle. It is therefore probable that intervesicular exchange is rapid and that the predominance of 1:1 complexes on vesicles indicated by Figures 1–3 and Table I reflects the true magnitudes of stepwise binding constants for formation of the 2:1 species and does not arise from kinetic limitations. As noted under Results and in Table I, there is a tendency for $K^b_{LiA}$ to increase as the ionophore level is increased. If this does not represent an increasing prevalence of the 2:1 complex as implied by the above discussion, then another explanation is required. It seems reasonable to explain the effect of ionophore level on the stability of bound complex as an effect of negative surface charge arising from bound $A^-$. As this parameter increases with increasing ionophore levels, the concentration of $Li^+$ near the membrane interface may be elevated sufficiently to account for the apparent small rises in complex stability.

The distribution of ionophore between the two monolayers of the vesicle membrane and the movement of cations from the extra- to the intravesicular aqueous phases represent additional reactions where the degree of equilibration during titrations could affect the magnitude of observed complex stability constants. The distribution of all ionophore species involved in the complexation equilibria and cation transport occurring both with and without ionophore catalysis could be of interest in this regard. The present data do not address the rates of these distribution reactions or reveal to what extent these processes may be affecting the apparent stabilities of bound complexes. However, since the titrations are conducted over relatively long time frames (promoting equilibration of the distribution reactions) and since the data from both solution and vesicle-containing systems are well described by analogous equilibrium expressions, it appears that processes of these types are not of major importance in establishing stabilities of the bound complexes.

It is clear from the results of this work that caution is warranted when chemical properties of ionophores observed

in solutions are utilized to interpret transport properties of the compounds and to predict their properties upon association with bilayer membranes. As shown in Table II and III, fundamental characteristics such as selectivity between cations and thermodynamic parameters of complexation reactions can be substantially altered by membrane association. Effects of ionic strength and membrane phase state on complex stabilities are also shown to be of interest in this regard. At present, too little is known regarding how the various chemical forms of A23187 (i.e., free acid, anion, and monomeric complexes) interact with vesicles to allow molecular-level interpretations of these membrane effects. However, from a general perspective, it is reasonable to propose that each form of the compound interacts with the membrane in a specific fashion which is compared of particular charge pairings, dipole–dipole interactions, hydrogen bonds, etc. and of nonspecific hydrophobic bonds. Transformations in the chemical form of the bound compound, such as protonation or complexation, then require alterations in these interactions which contribute to the energetics of the reaction and which are not components of the analogous reaction in solution. Stated in a simplified manner, differences in the membrane interaction energies of reactants and products must be accounted for when comparing solution to vesicle-containing systems.

Some support for the above view is obtained by comparing the differences in thermodynamic parameters for the protonation reaction in 80% methanol–water and the vesicle-containing media (Table III) to the differences in vesicle interaction energies of HA and $A^-$ reported previously (Kauffman et al., 1983). Table III shows that, for temperatures above $T_c$, $\Delta H$ and $\Delta S$ are altered by −7.5 kcal/mol and −26 cal/(mol·deg), respectively, when protonation is conducted on the vesicle instead of in solution. The previous data showed that the differences in $\Delta H$ and $\Delta S$ for the binding reactions of $A^-$ and HA to these vesicles are −7.5 kcal/mol and −20 cal/(mol·deg), respectively (Kauffman et al., 1983). The close similarity between the two sets of quantities indicates that transforming the membrane interactions of $A^-$ to those of HA during protonation of the bound compound is the major factor which alters the thermodynamic properties of the reaction compared to what is seen in solution. When the thermodynamic parameters for the binding of LiA and NaA to the membrane above and below $T_c$ are available, it will be possible to determine if the other membrane effects on complex stabilities and selectivity reported here have similar explanations.

Additional information on the interactions of A23187 with DMPC vesicles will also be necessary before definitive interpretations of ionic strength and membrane phase state effects on complex stabilities are possible. Regarding the effect of ionic strength, $ClO_4^-$, used here to control that parameter, is a chaotropic agent which would disrupt water structure in the aqueous phase and thereby diminish the strength of hydrophobic interactions between the various ionophore species and membranes [see Hatefi & Hanstein (1974) for a review]. If, as is the case for HA [see Kauffman et al. (1983)], LiA is less dependent than $A^-$ on hydrophobic effects for binding to vesicles, then the stabilization of $LiA_b$ by increasing chaotroph concentrations may be explainable in a fashion similar to the above discussion on the role of membrane interactions in altering the thermodynamic properties of the protonation reaction.

Finally, the destabilization of $LiA_b$ and $NaA_b$ but not of $HA_b$ by the liquid-crystalline to gel-state transition is an additional membrane-dependent effect on the complexation properties of A23187 which will require explanation. Our working hypothesis in this area is also based on previous studies on the acid–base properties of A23187 and on how HA and $A^-$ interact with DMPC vesicles (Kauffman et al., 1982, 1983). That work indicated that the benzoxazole chromophore of the ionophore, which contains two of the three cation-liganding atoms, including the ionizable carboxylic acid moeity, is located at the membrane interface for both the protonated and anionic forms. The data suggested further that with HA, but not $A^-$, portions, of the molecule distal to the benzoxazole group penetrate into the membrane acyl group region (Kauffman et al., 1983). With the carboxylic acid groups remaining at the interface, it is easy to visualize why transition to the gel state would not alter $K_{HA}^b$. This portion of the molecule is simply removed from the acyl chains and so is unaffected by the phase state of that membrane region. For the complex, $LiA_b$ and $NaA_b$ portions of the molecule important for complex formation may protrude into the hydrocarbon region of the membrane and thereby render the stability of complexes sensitive to the phase transition. Work designed to test this hypothesis is in progress.

**Registry No.** DMPC, 18194-24-6; A23187, 52665-69-7; MeOH, 67-56-1.

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# *n*-Alkanols and Halothane Inhibit Red Cell Anion Transport and Increase Band 3 Conformational Change Rate†

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ABSTRACT: The effects of halothane and *n*-alkanols on band 3, the anion-exchange protein of the red cell membrane, have been characterized by (1) radioactive sulfate exchange and (2) equilibrium and kinetic binding of a fluorescent anion transport inhibitor, 4,4′-dibenzamido-2,2′-stilbenedisulfonic acid (DBDS), with fluorescence and stopped-flow techniques. Ethanol, butanol, hexanol, heptanol, octanol, and decanol inhibit radioactive sulfate efflux from red blood cells in a dose-dependent manner with an average Hill coefficient of 1.3 ± 0.1. Over a $10^4$-fold range of buffer concentrations, the calculated membrane alkanol concentrations at which anion transport rates are reduced by 50% are 100–200 mM. At 100–300 mM membrane concentrations, halothane and the *n*-alkanols increase the apparent rate of DBDS binding to band 3 2–3-fold. Analysis of kinetic and equilibrium DBDS binding data shows that these drugs increase the rate of the DBDS-induced conformational change in the DBDS–band 3 complex. Equilibrium DBDS binding studies reveal differences between the actions of short-chain alkanols (ethanol and butanol) and those of long-chain alkanols (hexanol and longer). Short-chain alkanols reduce the equilibrium affinity of DBDS for band 3, while long-chain alkanols have no effect on equilibrium DBDS binding. The results for halothane and long-chain alkanols suggest a nonspecific, lipid-mediated mechanism of anesthetic action, which may be coupled to protein inactivation by an increase in the rate of protein conformational changes resulting in nonfunctional states. The results for short-chain alkanols indicate that they have the same nonspecific actions as the long-chain alkanols but also have specific effects on the stilbene binding site of band 3.

General anesthetics are a structurally heterogeneous group of compounds, whose potencies correlate with oil/water (or membrane/buffer) partition coefficients (λ; Janoff & Miller, 1982; Meyer, 1937). Proposed mechanisms of action of anesthetics include changes in membrane fluidity (Metcalfe et al., 1968; Hubbell et al., 1970), membrane volume (Miller et al., 1973; Seeman & Roth, 1972), and lipid phase transitions (Hill, 1974; Trudell, 1977). Membrane proteins are assumed to mediate anesthetic-induced changes in functional properties such as transmembrane transport (Makriyannis & Fesik, 1980), receptor–ligand binding (Young & Sigman, 1981; Boyd & Cohen, 1984), and electrophysiologically observable channel dynamics (Gage et al., 1975). At present, the bulk lipid hypotheses of anesthetic action (fluidity, volume, and phase transitions) remain unproven, and in some experimental systems, a number of general anesthetic agents produce unexpected, drug-specific effects (e.g., short-chain alcohols enhance activation of postsynaptic cholinergic receptors at the neuromuscular junction; Gage et al., 1975). Recently, Franks & Lieb (1984) showed that anesthetics competitively inhibit ligand binding to a water-soluble protein. Other proposed sites of anesthetic action include hydrophobic sites within membrane proteins (Richards et al., 1978) and the boundary lipids adjacent to protein (Richards, 1976).

We have examined the interaction of halothane and the *n*-alkanols[1] with the red cell membrane protein band 3. We

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[1] Abbreviations: DBDS, 4,4′-dibenzamido-2,2′-stilbenedisulfonic acid; DNDS, 4,4′-dinitro-2,2′-stilbenedisulfonic acid; pCMBS, *p*-(chloromercuri)benzenesulfonate; RBC, red blood cell; SITS, 4-acetamido-4′-isothiocyano-2,2′-stilbenedisulfonic acid; TCA, trichloroacetic acid. The term *n*-alkanol is used to represent a straight-chain alkane with a primary hydroxy group.

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