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Acid-Base Properties of Ionophore A23187 in Methanol-Water Solutions and Bound to Unilamellar Vesicles of Dimyristoylphosphatidylcholine†

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ABSTRACT: The acid-base properties of ionophore A23187 in methanol-water solutions (0-95% w/w) and bound to unilamellar vesicles of dimyristoylphosphatidylcholine were examined by ultraviolet and fluorescence spectroscopy, and the spectral properties for the acidic and basic forms were defined under these conditions. Standard mixed-solvent buffers were employed to calibrate pH* measurement in the methanol-water solvents. In 65% methanol-water, two protonation equilibria were observed, the most basic of which displayed a value for the logarithm of the protonation constant ($\log K^*_H$) of 7.19 ± 0.05 at 25 °C and 0.05 M ionic strength. Instability of A23187 was encountered below pH* ~4; however, decomposition was slow enough to allow $\log K^*_H$ for the more acidic equilibrium to be estimated as 1.28. Comparison of these results to those obtained with the methyl ester of A23187 ($\log K^*_H$ = 1.32) and literature values for other model compounds allowed assignment of the more basic equilibrium to the carboxylic acid moiety and the more acidic one to the *N*-methylamino substituent of the benzoxazole ring. $\log K^*_H$ of the carboxylic acid increased from 5.69 ± 0.05 to 9.37 ± 0.05 over the range of solvent polarity encompassed by water to 95% methanol-water. Values for the ground state (absorption) and first excited state (fluorescence) were equal within experimental error. The logarithm of the protonation constant for the membrane-bound ionophore, measured under conditions where the surface potential generated by ionization did not significantly alter the equilibrium, was found to be 7.85 ± 0.05 at 25 °C and at ionic strength of 0.05 M in the aqueous phase. The value agrees with that observed in 80% methanol-water, as does the wavelength of maximum fluorescence emission for the membrane-bound free acid. An interfacial location for the monoprotonated form of the benzoxazolate moiety is proposed, both above and below the membrane phase transition temperature. The location of other regions of the A23187 molecule could not be assessed from these data.

Since the discovery that the polyether antibiotic A23187 is a calcium ionophore with high specificity for divalent cations (Reed & Lardy, 1972), thousands of reports have appeared describing the effects of this compound upon biological systems ranging from subcellular fractions to whole animals. These studies have provided evidence for the involvement of Ca^{2+} in a wide spectrum of biological control mechanisms including muscle contraction, stimulus-secretion coupling, mitosis, fertilization, gluconeogenesis, glycogenolysis, and many others [for reviews, see Rasmussen & Goodman (1977) and Kretsinger (1979)]. In addition to being diverse, the responses of tissues or cells to treatment with A23187 are often complex, depending upon such factors as concentrations of the ionophore, time of exposure, and ionic composition of the medium.

The physicochemical properties underlying the biological responses produced by A23187 are not sufficiently known to explain the biological effects in detail. The structure (see Figure 1) and some chemical properties of the free acid were reported by Chaney et al. (1974). Structures of complexes with a wide range of divalent cations are known in solution (Deber & Pfeiffer, 1976; Anteunis, 1977; Pfeiffer & Deber, 1979) and in the solid state (Chaney et al., 1976; Smith & Duax, 1976). The complex stoichiometries and binding affinity sequence have been studied in solution and by bulk phase extraction techniques (Reed & Lardy, 1972; Pfeiffer et al., 1974; Puskin & Gunter, 1975; Pfeiffer & Lardy, 1976; Young & Gomperts, 1977), and the transport sequence has been investigated in liposomes (Weissman et al., 1980; Pohl et al., 1980). It is generally concluded from these studies that complexes of stoichiometry A_2M[1] are responsible for divalent cation transport, with the overall electroneutral transport reaction arising from M^{2+} for $2H^+$ exchange. The binding affinity sequence for divalent cations arises from the isosteric nature of the complexes combined with the limited confor-

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[1] Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; *D*, dielectric constant; 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; HA, the free acid of A23187; A^-, the carboxylate anion of A23187; H_2A^+, the doubly protonated form of the carboxylate anion; A_2M, the 2:1 A23187/divalent cation complex; λ_{ex}, excitation wavelength of maximum fluorescence; λ_{em}, emission wavelength of maximum fluorescence; λ_{max}, wavelength of maximum absorbance.

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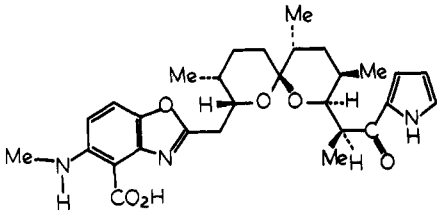

FIGURE 1: Structure of A23187. The structure was determined by Chaney et al. (1974). The methyl ester of A23187 utilized in the present study refers to the carboxylic acid methyl ester, with other structural features being the same as the free form shown in this figure.

mational mobility of the ligand. The transport sequence shows less selectivity than the binding sequence (Pohl et al., 1980) except for Mg^{2+} which can be transported at disproportionately slow rates under some circumstances (Debono et al., 1981). The specificity for divalent over monovalent cation transport is not complete and is probably, in part, a consequence of the ability to form monoprotonated species to obtain neutral 2:1 complexes with monovalent cations.

Beyond the above considerations, there are numerous areas where improved physicochemical understanding of this ionophore would facilitate interpretation of its biological effects, improve the prospects for the use of ionophores as pharmacological agents (Pressman & Guzman, 1975), and foster the use of the compound as a model transport system. More information is needed regarding the rate-limiting steps in the transport process and the relationships between complex stabilities, complexation kinetics, and the transport sequence. In addition, little is known regarding how the compound interacts with membranes and how these lipid–ionophore interactions influence its transport properties. These areas are poorly understood for the monocarboxylic polyether ionophores in general [for review, see Taylor et al. (1982)], although more detailed studies are beginning to appear (Haynes et al., 1980; Kolber, 1980; Painter & Pressman, 1980; Puskin et al., 1981).

A23187 is well suited for physicochemical studies of cation transport by electroneutral ionophores. Transport by the compound is predominantly electroneutral (Kafka & Holz, 1976; Wulf & Pohl, 1977), although at very high concentrations of Ca^{2+} a small fraction of apparent electrogenic transport, i.e., conductance, has been observed (Case et al., 1974). The specificity of A23187 for divalent over monovalent cations is greater than that observed for lasalocid A, which also binds and transports amines (Pressman, 1972; Williamson et al., 1974; Kafka & Holz, 1976; Holz, 1975). The isosteric relationship between divalent cation complexes with A23187 indicates that differences in transmembrane diffusion constants are not likely to account for the transport specificity (Pfeiffer et al., 1978; Pfeiffer & Deber, 1979). Finally, the spectroscopic properties of A23187 (absorption, fluorescence, and circular dichroism) have been shown to be sensitive indicators of protonation, complexation, conformation, and environment of the ionophore (Pfeiffer et al., 1974; Case et al., 1974; Pfeiffer & Deber, 1979; Puskin et al., 1981).

In the present paper we report the acid–base properties of A23187 in homogeneous solutions of different polarity and bound to DMPC vesicles. In addition, the absorption and fluorescence spectra of the ionophore in different protonation states and in different solvent environments are defined. The methanol–water solvents were chosen for this work because (1) operational pH scales and standard pH buffers are available for these mixed solvents, (2) an extensive data base is available on the properties of electrolytes in these solvents, and (3) the effective solvent polarity at aqueous–lipid interfaces is thought to fall within the polarity range encompassed by these solutions (Waggoner & Stryer, 1970; Shinitzky, 1974; Fernandez & Fromherz, 1977; Mukerjee et al., 1977; Vaz et al., 1978). Thus, data obtained in these solutions provide a reference for interpreting the spectral properties and protonation constant of the membrane-bound ionophore. This study is an initial step toward a molecular level understanding of cation transport by the compound. In a forthcoming paper these data are utilized to examine specific interactions of the free acid and anionic forms of A23187 with the phospholipid bilayer of DMPC vesicles. In addition, the findings provide the necessary background information for examining complexation kinetics and equilibria that are relevant to the ion transport mechanism.

Materials and Methods

L-α-Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma Chemical Co. (St. Louis, MO). The synthetic phospholipid migrated as a single spot when analyzed by thin-layer chromatography (chloroform–methanol–water, 65:25:4 v/v/v; silica gel H; 1 μmol of phospholipid). An unidentified spectral impurity (λ_{max} 258 nm) and potentially contaminating free fatty acids in the DMPC were removed by precipitation of the phospholipid in acetone–chloroform (9:1 v/v). The free fatty acid content of purified DMPC was determined after isolation by thin-layer chromatography (petroleum ether–diethyl ether–acetic acid, 80:20:1 v/v/v; silica gel H) and esterification with Meth-Prep I (Applied Science, State College, PA) by gas chromatography using internal standards. The free acid and methyl ester of A23187 were gifts from Robert Hamill of Eli Lilly Co. (Indianapolis, IN). Reagent grade methanol was distilled over zinc dust and potassium hydroxide. Distilled, deionized water was used in all solutions, and acid-washed glassware (sulfuric–nitric, 3:1 v/v) was used throughout. The specific resistance of the water was 13 MΩ cm or greater. Reagent grade perchloric acid (Fisher Scientific, Pittsburgh, PA) and tetraethylammonium hydroxide (Eastman Chemicals, Rochester, NY) were utilized without further purification. Tetraethylammonium perchlorate (Eastman Chemicals) was recrystallized 4 times from hot water before use. Triethylamine (Aldrich, Milwaukee, WI) was purified by distillation.

Measurement of Acidity in Methanol–Water Mixtures. All pH measurements were made on a digital pH meter (Fisher 144, Beckman 4500, or Orion 601) using a glass electrode (Sargent Welch S30050-15C, Beckman 41263 C7U, or Corning 476024) in combination with a polymer body reference electrode (Markson 1881). Acid–base titrations of A23187 (see below) were carried out in a series of methanol–water mixtures containing 0, 17.5, 35, 50, 65, 80, 90, and 95 wt % methanol. The operational pH* scales developed by de Ligny et al. (1960) and Gelsema et al. (1966, 1967) and extended by Bates et al. (1963) were used to determine the value of pH* in the various solvent mixtures. de Ligny has defined pa_{H^*} as the mixed-solvent equivalent of pa_H as

$$pH^* \simeq pa_{H^*} = -\log a_{H^*} \quad (1)$$

where a_{H^*} represents the molal activity of the solvated proton in a given solvent mixture, referred to the standard state in the same solvent composition.

Standard buffers of known pa_{H^*} were used to calibrate the pH meter in each solvent mixture. Under the conditions employed, the measured values of pH* read from the meter approximate pa_{H^*} in the same manner that aqueous pH approximates pa_H. A more detailed presentation of the practical and theoretical considerations of pH* measurement in methanol–water mixtures has been given by Rorabacher et al.

(1971) and references therein.

The methanol–water solvents contained 5 mM each of β,β'-dimethylglutaric acid (Sigma), Hepes (Sigma), and triethylamine to produce buffering over the pH* range of approximately 2–11. Tetraethylammonium perchlorate was added in sufficient amounts to bring the ionic strength to 0.05 M. EGTA (Sigma) was included at 0.10 mM to chelate trace amounts of divalent metals in the solutions. We found no spectroscopic evidence for interactions of these reagents with A23187 in the concentration ranges employed. The pH of the solutions was adjusted by the addition of either perchloric acid or tetraethylammonium hydroxide.

The solutions were maintained at the stated temperatures by the use of a jacketed beaker in conjunction with a temperature bath equipped with an external flow circuit. In addition, the solutions were swept with solvent-saturated nitrogen above neutral pH to prevent absorption of atmospheric carbon dioxide.

Vesicle Preparation. Unilamellar vesicles were prepared by the procedure of Huang & Thompson (1974) with modifications. Lipid dispersions were prepared by vigorously shaking 250–400 mg of DMPC in 8 mL of an aqueous solution containing 33 mM tetraethylammonium perchlorate, 5 mM each of β,β'-dimethylglutaric acid, Hepes, and Ches, and 0.1 mM each of EDTA and EGTA, pH 8.0 (adjusted with tetraethylammonium hydroxide), at approximately 35 °C. The lipid dispersion was sonicated for 2.5 h at 30 °C with a Heat Systems sonicator (no. W185), equipped with the cup horn attachment (no. 431A), at a setting of 6.5 (90 W). The bottom of the cylindrical glass vial (diameter 25 mm) was centered 1.5 cm above the radiating surface in order to maximize transfer of sonic energy (Barrow & Lentz, 1980). Thin-layer chromatography of DMPC after sonication revealed no decomposition products (cf. Hauser, 1971; Huang & Charlton, 1972).

The sonicated lipid solution was concentrated by ultrafiltration (Amicon 8MC, XM-100A filter) to a volume of 4 mL. Multilamellar species were separated from the unilamellar vesicles by passing the suspensions over a Sepharose CL-4B column (2.5 × 40 cm, 4 °C) previously equilibrated with the same aqueous solution described above (Huang, 1969). The turbidity of the effluent at 280 nm was monitored, and samples of the fractions were analyzed for lipid phosphate by the method of Bartlett (1959). Only those fractions characterized by a constant, low specific turbidity ($A_{280}/[P_i]$, millimolar concentration units; 1-cm path length) of approximately 0.04 were considered to be free of multilamellar vesicles or other undefined structures. Use of the cup horn attachment consistently resulted in high yields of unilamellar vesicles without the contamination by titanium that occurs with probe-type sonicators. The elution behavior, the value of the specific turbidity, and morphological examination of negatively stained preparations by electron microscopy indicated that these vesicles were similar to other well-characterized preparations of small unilamellar vesicles with an average diameter of 200–250 Å (Huang & Lee, 1973; Barenholz et al., 1977; Watts et al., 1978). The vesicles were used within 2 weeks after preparation even though, when stored at room temperature, the specific turbidity was essentially constant for 2 months.

Vesicles prepared by the above procedure at pH 4.0 (instead of pH 8.0) were unstable. At room temperature the specific turbidity of the unilamellar species increased slowly until the solutions became relatively opaque after 48 h. Vesicles prepared at this pH were therefore used immediately after preparation (specific turbidity <0.05).

A23187 was added to the liposomal suspension as the free acid in ethanol (final concentration of ethanol <0.1 vol %). At these low concentrations, ethanol does not significantly perturb lecithin vesicles (Jain & Wu, 1977).

The measurement of aqueous phase pH in the liposomal suspensions was carried out with the electrode combinations previously described. We noted that the addition of concentrated perchloric acid produced local areas of transient increased turbidity, suggesting destabilization of the liposomal structure. This did not occur with addition of concentrated base; therefore, pH titrations with lipid vesicles were always performed from acidic to basic pH values. Control experiments demonstrated that the pH extremes achieved during the acid–base titration did not result in decomposition of the DMPC (thin-layer chromatography analysis as described above).

Data Acquisition and Analysis. Absorption spectra were recorded with either a Cary Model 118 or an American Instrument Co. DW2a spectrophotometer. Data from these instruments were tabulated manually and fed into a microcomputer for analysis. Fluorescence spectra were obtained with an SLM 8000DS spectrofluorometer equipped with a double grating excitation monochromator and operated in the photon counting mode. This instrument was interfaced to an IMSAI 8080 microcomputer with dual disk drive for data manipulation and storage. Technical spectra were corrected by using factors obtained with a standard lamp.

The stepwise protonation of ionophore A23187 may be represented by

$$H^+ + A^- \rightleftharpoons HA \tag{2}$$

$$H^+ + HA \rightleftharpoons H_2A^+ \tag{3}$$

where A^-, HA, and H_2A^+ represent the anion, free acid, and a doubly protonated (see below) form of the ionophore, respectively. In solutions of methanol–water the corresponding mixed-mode protonation constants (units, m^{-1}) for these reactions are defined by

$$K^*_{H1} = \frac{[HA]}{a^*_{H^+}[A^-]} \tag{4}$$

$$K^*_{H2} = \frac{[H_2A^+]}{a^*_{H^+}[HA]} \tag{5}$$

Expressions of the same form were used to describe these equilibria when the ionophore was bound to DMPC vesicles. In that case, the equilibrium constants are designated as $K_H{}^b$ to reflect the bound state of the ionophore and the hydrogen ion activity term refers to the bulk aqueous phase.

The titration data were analyzed by using a nonlinear least-squares routine to obtain the best estimates of the protonation constants by minimizing the function

$$W = \sum(Y_i - Y_{calcd})^2 \tag{6}$$

For absorbance vs. pH* titrations, $Y_i = Abs_i$ (observed absorbance) and

$$Y_{calcd} = \frac{\epsilon_a K^*_H a^*_{H^+} + \epsilon_b}{1 + K^*_H a^*_{H^+}} [HA]_0 \tag{7}$$

where ϵ_a and ϵ_b represent the molar absorptivities of the acidic and basic forms of the ionophore in a given reaction and $[HA]_0$ is the total ionophore concentration. For the fluorescence titrations, $Y_i = F_i$ (observed fluorescence) and

$$Y_{calcd} = \frac{F_a K^*_H a^*_{H^+} + F_b}{1 + K^*_H a^*_{H^+}} \tag{8}$$

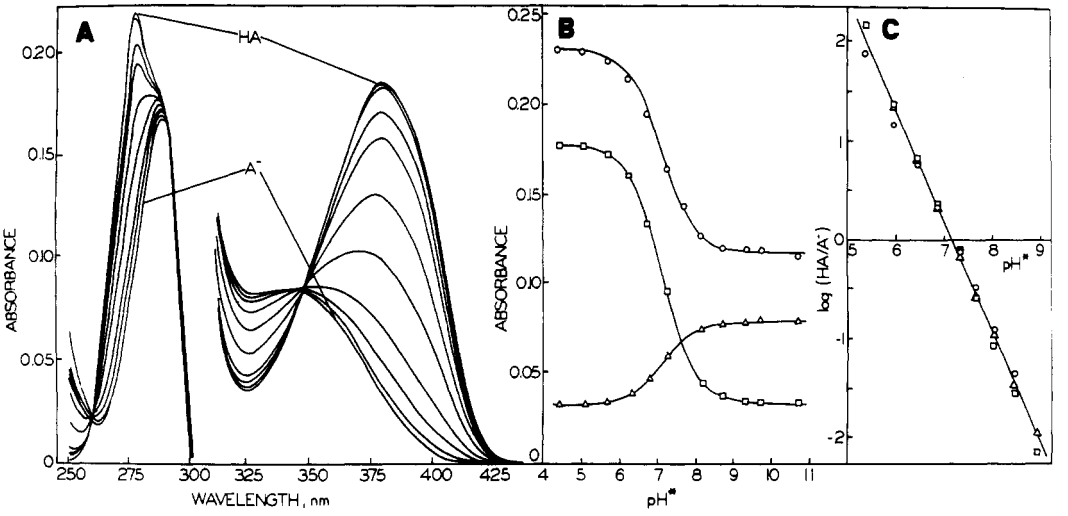

FIGURE 2: Spectrophotometric determination of log K^*_{H1} for A23187 in 65% methanol–water, 25 °C. The acid–base titration was carried out as described under Materials and Methods. The concentration of A23187 was 20 μM. The ionophore was absent from the reference solution which was maintained at a constant pH* of 4.0. (A) Absorption spectra were taken at pH* values between 4.41 and 10.70. The spectra of the free acid and monocarboxylate anion are labeled HA and A^- at the low and high pH*, respectively. The spectra are offset by an absorbance value of 0.2 between 250 and 300 nm. (B) The experimental absorbance values at 278 (○), 324 (△), and 380 nm (□) are plotted as a function of pH*. The solid lines represent the nonlinear least-squares fit of the data to eq 7. (C) The mole ratio HA/A^- was deduced from the spectral titrations at 278 (○), 324 (△), and 380 nm (□), and the results are plotted according to eq 9. A log K^*_{H1} of 7.19 was obtained from the intercept along the x axis. The slope of the line is −1.13 with a correlation coefficient of 0.9975.

where F_a and F_b are the fluorescence of the acidic and basic forms of the ionophore, and the other parameters are as previously defined.

A linear form of eq 4

$$\log K^*_{H1} = pH^* + \log \frac{[HA]}{[A^-]} \qquad (9)$$

was also used to determine values of K^*_{H1} when the spectrum of the acidic and basic forms could be obtained. For the second protonation step, the spectrum of the acidic form was not experimentally accessible. In this case K^*_{H2} could be obtained by use of (Benesi & Hildebrand, 1949)

$$\frac{1}{A - A_b} = \frac{1}{K^*_H \Delta\epsilon}\left(\frac{1}{a^*_{H^+}}\right) + \frac{1}{\Delta\epsilon} \qquad (10)$$

where A_b is the absorbance due to the base, A is the absorbance at a particular pH*, and $\Delta\epsilon$ is the difference between the extinction coefficients of the acid and the base at the wavelength being examined. This same equation was applicable to fluorescence titrations with appropriate changes in nomenclature.

Results

Spectroscopic Determination of K^*_{H1} and K^*_{H2} in Methanol–Water Solvents. The protonation constants of A23187 were determined by monitoring the effect of pH* on the absorption or fluorescence spectra. The spectrophotometric titration of A23187 in 65 wt % methanol–water over the pH* range 4–10 is shown in Figure 2A. Decreases in absorbance and shifts in λ_{max} occurred in both the 380- and 278-nm peaks as the pH* was increased. Reasonably well-defined isosbestic points were observed at 261 and 378 nm. The long wavelength absorption peak (λ_{max} 380) is due to the substituted benzoxazole chromophore since the α-ketopyrrole moiety is transparent in this region (Pfeiffer et al., 1974).

The spectral data were analyzed as described under Materials and Methods to yield a log K^*_H of 7.19 ± 0.05. This value represents the average of three determinations and was independent of wavelength. The agreement between the theoretical curves (eq 7) and the actual data at 278, 324, and 380 nm is shown in Figure 2, panel B. The excellent fit of the data to eq 7 and 9, the single set of isosbestic points, and the fact that log K^*_H was independent of wavelength demonstrated that a single equilibrium was responsible for this spectral transition.

At pH* values < 3.5, spectral changes indicated the presence of another species of the ionophore. An irreversible decrease of the absorbance at the 380-nm peak was observed in 65% methanol at pH* 1.5 (HCl or $HClO_4$) with a first-order half-life of approximately 1.7 h. Since the kinetics of decomposition were slow, the acid–base chemistry of A23187 at low pH* could be investigated. These studies revealed a second protonation equilibrium of the ionophore. Decreasing pH* below approximately 3.5 resulted in a reduced intensity of the 380-nm peak with little or no change in λ_{max} (Figure 3B). In addition, the 278-nm peak was red shifted to 289 nm, and isosbestic points were observed at 283 and 333 nm (Figure 3A). Analysis of the data using eq 10 yielded a log K^*_H value of 1.28 in 65 wt % methanol–water at 25 °C. The linear fit of the data to eq 10 and the fact that identical values of the protonation constant were obtained at 380 and 306 nm (Figure 3C) demonstrated that interference due to decomposition was minimal.

When the acid–base titration of the methyl ester of A23187 (MeA23187) was performed in 65% methanol, only the spectral transition at lower pH* was observed (data not shown). The log K^*_H value of MeA23187 was determined to be 1.32. This result showed that the acidic group in the parent compound with log K^*_{H1} = 7.19 in this solvent is undoubtedly the carboxyl group on the benzoxazole moiety. The *N*-methylamino group, also a substituent of the benzoxazole moiety, is very likely responsible for the protonation reaction with the log K^*_{H2} value of 1.28 since the model compound *N*-methylanthranilic acid was found to have a log K^*_{H2} value in the same range (data not shown). For the remainder of the work we have focused on the protonation equilibrium of the carboxyl group since the protonation constant of this substituent falls within the range of pH values relevant to most biological systems. We found no evidence for a solvent-dependent or membrane binding dependent increase in the

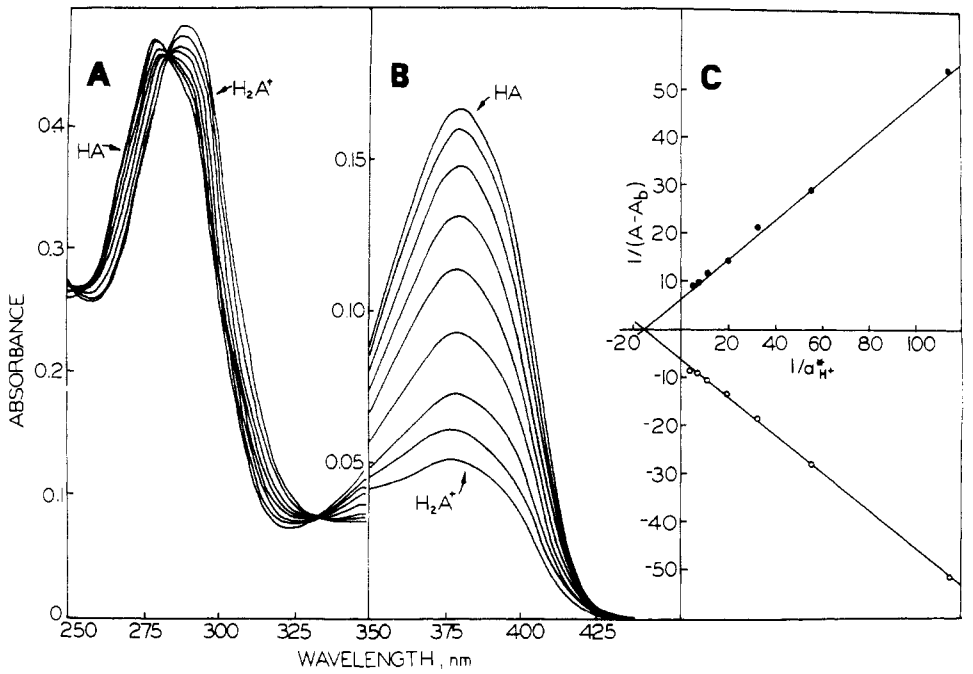

FIGURE 3: Spectrophotometric determination of log K^*_{H2} for A23187 in 65% methanol–water, 25 °C. The acid–base titration was performed as described under Materials and Methods. The concentration of A23187 was 20 μM. Absorption spectra were taken at pH* values ranging from 0.56 to 3.13. The spectra of the free acid and the doubly protonated species of A23187 are labeled as HA and H_2A^+ at the high and low pH*, respectively. Since completion of the titration was not possible due to technical problems associated with establishing pH* values below 1.0, the spectrum labeled H_2A^+ actually represents a mixture calculated to contain 84% H_2A^+ and 16% HA. A23187 was absent from the reference solution maintained at pH* 4.0. (A) Spectra were acquired between 250 and 350 nm. (B) Spectra were acquired between 350 and 435 nm. The sensitivity in this panel is 2.5 times that in (A). (C) The data at 306 (●) and 380 nm (○) are plotted according to eq 10.

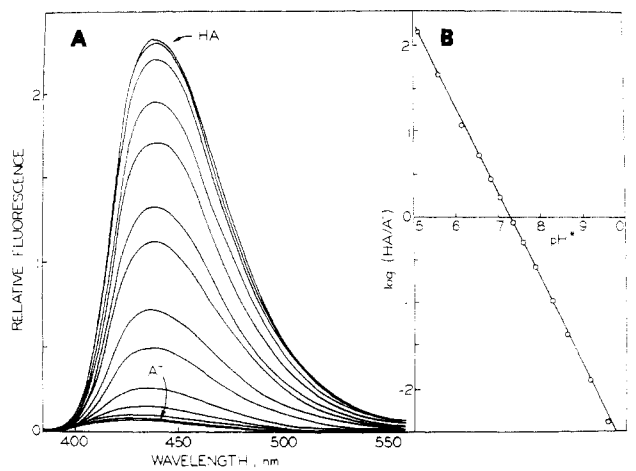

FIGURE 4: Fluorometric determination of log K^*_{H1} for A23187 in 65% methanol–water, 25 °C. The acid–base titration was carried out as described under Materials and Methods. The concentration of A23187 was 2.5 μM. (A) The corrected fluorescence emission spectra (λ_{ex} 381 nm) are displayed at a range of pH* values from 3.94 to 9.89. The spectra of the free acid and monocarboxylate anion are labeled HA and A^- at the low and high pH*, respectively. (B) The fluorescence data at 437 nm are plotted according to eq 9. A log K^*_{H1} of 7.24 was obtained from the intercept along the x axis. The slope of the line is −1.015, and the correlation coefficient is 0.99992.

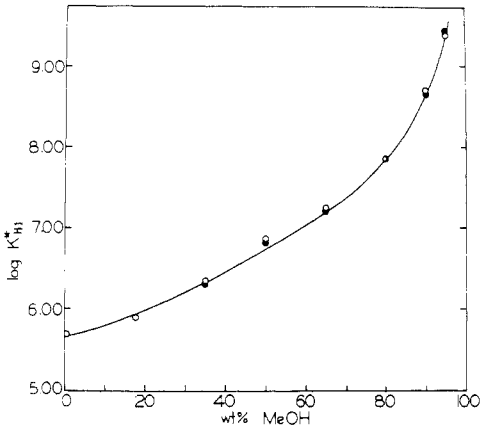

FIGURE 5: log K^*_{H1} of A23187 as a function of wt % methanol at 25 °C. The log K^*_{H1} of A23187 was determined from spectrophotometric (●) and fluorometric (○) titrations at various methanol–water compositions as described in Figures 2 and 4 and under Materials and Methods. The ionic strength of the solutions was maintained at 0.05, and the standard deviation in the log K^*_{H1} values is ±0.05.

protonation constant of the N-methylamino group sufficient to produce interference with studies of protonation equilibrium at the carboxyl group.

The fluorescence emission spectrum of A23187 is also sensitive to the protonation state of the carboxyl group. The fluorometric determination of log K^*_{H1} in 65% methanol–water is presented in Figure 4.

The fluorescence intensity of the compound is diminished along with a slight blue shift of the peak as the pH* is raised. The excited state log K^*_{H1} determined by fluorescence (7.24 ± 0.05) is equal within experimental error to the ground state value obtained from absorbance measurements; consequently, fluorometric data may be used to determine the extent of protonation of A23187 in the ground state.

The composition of the methanol–water mixtures was varied in order to observe the effect of solvent polarity and solvation effects upon the protonation constant of the carboxyl group, and the spectroscopic properties of the free acid and anion of A23187. The log K^*_{H1} of A23187 increased with increasing percentages of methanol (Figure 5). A similar dependence of log K^*_{H1} upon the percent methanol has been observed for a variety of simple carboxylic acids (Bates et al., 1963; Rorabacher et al., 1971).

The numerical values of log K^*_{H1} and the fluorescence properties for the free acid and anion of A23187 in metha-

Table I: $\log K^*_{H1}$ and Spectroscopic Parameters of the Free Acid and Anion of A23187 in Methanol–Water Solutions, 25 °C[a]

solvent (wt % MeOH)	D	$\log K^*_{H1}$ (±0.05) abs.	$\log K^*_{H1}$ (±0.05) fl.	HA λ_{max} (nm)	HA ϵ (10^{-3} cm^{-1} M^{-1})	HA λ_{em} (nm)	HA rel fluorescence	A^- λ_{ex} (nm)	A^- λ_{em} (nm)	A^- rel fluorescence
0	78.5		5.69	383	4.8	444	6.40	336	429	1.00
17.5	70.4		5.88	382		442	20.9	338	428	1.69
35	62.3	6.30	6.33	381	6.80	439	38.6	342	426	2.64
50	55.3	6.80	6.84	380	8.26	438	44.5	346	426	3.57
65	48.5	7.19	7.24	380	8.92	437	48.3	348	425	4.29
80	41.6	7.84	7.85	380	9.17	435	48.3	352	423	4.77
90	37.0	8.64	8.69	380	9.33	434	45.9	352	423	4.77
95	34.7	9.43	9.37	380	9.37	433	45.8	352	422	4.61

[a] The dielectric constants (D) were interpolated from the combined data of Akerlof (1932) and Albright & Gosting (1946). $\log K^*_{H1}$ values were determined from spectrophotometric (abs.) and fluorometric (fl.) titrations as described under Materials and Methods and in Figures 2 and 4. The extinction coefficient (ϵ) for the electronic transition of lowest energy for the free acid (benzoxazole moiety) was obtained at λ_{max} for that peak. Relative fluorescence values, normalized with respect to the fluorescence of the anion in H_2O (0 wt % MeOH), were obtained at excitation and emission wavelengths yielding maximal fluorescence (i.e., λ_{ex} and λ_{em}). The fluorescence parameters λ_{ex} and λ_{em} were obtained from corrected spectra of A23187 at pH* values corresponding to either the free acid (HA) or the carboxylate anion (A^-).

nol–water mixtures are presented in Table I. It is apparent that in aqueous solutions the fluorescence of both species is low, yet greater than zero. The fluorescence yield (defined as the amount of fluorescence, corrected for lamp intensity, when the excitation and emission wavelengths are λ_{ex} and λ_{em}, respectively) increased as the solvent polarity decreased between that of water and 65% methanol–water. Less polar solvents (including chloroform and hexane) had little further effect on fluorescence intensity. Both the free acid and the anion showed expected blue shifts in λ_{em} as solvent polarity was decreased; however, the changes were small. Although not shown in Table I, λ_{em} was significantly lower in nonpolar solvents which have polarity properties more comparable to that of the hydrocarbon interior of a membrane. For example, in chloroform λ_{em} was 423 nm, while in heptane the value was 413 nm.

$K_{H1}{}^b$ of A23187 Bound to Unilamellar Vesicles of DMPC. Complexation and decomplexation reactions associated with cation transport by ionophores are thought to take place at the lipid–water interface of membranes [see, however, Bennett et al. (1979)]. Thus, knowledge of the protonation state and location of A23187 associated with lipid bilayers is essential for understanding the transport mechanism. Accordingly, spectrophotometric and fluorometric determinations of $K_{H1}{}^b$ for the membrane-bound ionophore were carried out. Small, unilamellar vesicles of DMPC were used in this study because of the availability of extensive data on the physical properties of this model membrane system (e.g., Watts et al., 1978).

In studies of this type the generation of surface charge and resultant surface potential due to the formation of the negatively charged carboxylate anion can alter the protonation equilibrium. The effect of surface potential upon $K_{H1}{}^b$ is given by

$$\log K_{H1'}{}^b = \log K_{H1}{}^b - e\psi_s/(kT) \qquad (11)$$

where $K_{H1}{}^b$ is the actual protonation constant of a membrane-bound species, ψ_s is the surface potential in volts for the plane in which the dissociating acid is located, T is the temperature in kelvin, k is Boltzmann's constant, and e is the positive unit charge (Mukerjee & Banerjee, 1964). The surface potential, ψ_s, was calculated by means of the Gouy–Chapman equation [see Bangham (1972) for review]:

$$\psi_s = \frac{2kT}{e} \sinh^{-1}\left[\frac{\sigma}{(8kTn\epsilon_r\epsilon_0)^{1/2}}\right] \qquad (12)$$

where n is the concentration of the aqueous electrolyte in units of ions per meters cubed, ϵ_0 is the permitivity of free space (vacuum), and ϵ_r is the relative permitivity of the aqueous medium. Finally, the surface charge density (σ) may be approximated at any point along the titration by

$$\sigma = \frac{-\alpha(\text{A23187}^b)_e}{4\pi r^2} \qquad (13)$$

where A23187^b is the number of ionophore molecules bound per lipid vesicle, α is the degree of dissociation of the carboxylic acid, e is the positive unit charge, and r is the outer spherical radius of the vesicle. The physical parameters for DMPC vesicles reported by Watts et al. (1978) were employed for these calculations. The use of eq 13 assumes (1) that binding of A23187 to the membrane does not result in gross alterations in the membrane structure (a condition expected to be met at low mole ratios of ionophore to lipid), (2) that the charged ionophore species reside at the interface (see Discussion), and (3) that all of the ionophore is present on one side of the membrane. This latter assumption is probably not valid; nevertheless, the use of eq 13 leads to an upper limit of σ, and thus ψ_s. The acid–base titrations of A23187 bound to DMPC vesicles were carried out at mole ratios of A23187 to lipid ranging from 0.0013 to 0.010. Using the equations described above and assuming 2850 molecules of DMPC per phospholipid vesicle (Watts et al., 1978), we calculated a log $K_{H1}{}^b$ shift of 0.03 for the conditions normally used (4 μM A23187, 3 mM lipid phosphate, ionic strength = 50 mM, 25 °C). Since this value is an upper limit to ψ_s and is less than the experimental error for determination of log $K_{H1}{}^b$, the effects of surface charge generation may be ignored under these conditions. Analysis of the contaminating free fatty acids present in the vesicle suspensions (<0.0005 mole ratio) indicated that surface charge arising from the carboxylate form of these compounds could also be ignored.

Error in the determination of $K_{H1}{}^b$ would also result if a significant fraction of the compound were in solution rather than bound to vesicles, since the spectral data would represent a weighted average of the two components. The partitioning of the ionophore between the aqueous solution and the vesicles was determined by the enhancement of fluorescence upon association with the vesicles (Puskin et al., 1981). For the conditions normally employed the amounts of unbound free acid and anion were 2% and 6%, respectively, of the amount of ionophore utilized. These small unbound components would not significantly affect the observed protonation constants, particularly when determined fluorometrically since the

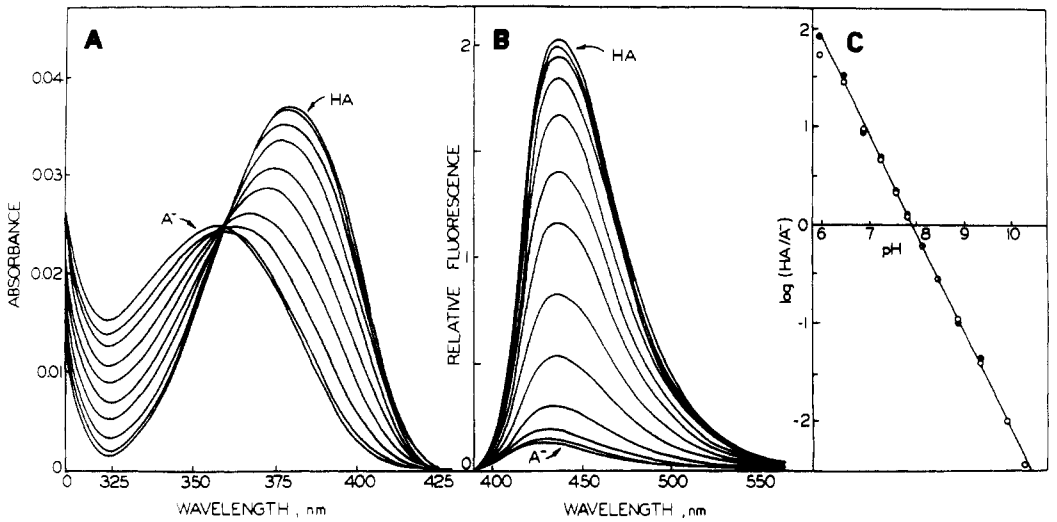

FIGURE 6: Spectrophotometric and fluorometric determinations of log $K_{H1}{}^{b}$ for A23187 bound to small unilamellar DMPC vesicles, 25 °C. Acid–base titrations were carried out as described under Materials and Methods. The concentration of A23187 was 4.0 μM, and the DMPC vesicles were present at a nominal concentration of 3.0 mM lipid phosphate. The spectra of the free acid and carboxylate anion are labeled as HA and A^-, respectively. (A) Absorption spectra were recorded at pH values between 5.41 (HA) and 9.31 (A^-). The reference solution contained the lipid vesicles at a constant pH (4.0) in the absence of ionophore. The upward shift in the base line at 445 nm as the pH was increased was subtracted from the spectra (see text). (B) The corrected fluorescence emission spectra of membrane-bound A23187 are presented at pH values between 5.41 (HA) and 10.76 (A^-). The excitation wavelength was 380 nm. (C) Data from panels A and B are plotted according to eq 9. The mole ratio HA/A^- was deduced from the spectral titrations at 380 nm for the absorbance data (●) and 435 nm for the fluorescence data (○). A log $K_{H1}{}^{b}$ of 7.85 was obtained from both the fluorescence and absorption data. By use of the combined data, the slope of the line is −0.995, and the correlation coefficient is 0.9990.

Table II: log $K_{H1}{}^{b}$ and Spectroscopic Parameters of the Free Acid and Anion of A23187 Bound to Unilamellar Vesicles of Dimyristoylphosphatidylcholine[a]

		HA				A^-			
temp (°C)	log $K_{H1}{}^{b}$ (±0.05)	λ_{max} (nm)	ϵ (10^{-3} cm^{-1} M^{-1})	λ_{em} (nm)	rel fluorescence	ϵ (10^{-3} cm^{-1} M^{-1})	λ_{ex} (nm)	λ_{em} (nm)	rel fluorescence
15	7.97	380		434		5.5	359	426	
25	7.85	380	9.1	435	44.7	5.4	359	427	4.63
35	7.67	380		436			359	427	

[a] log K^*_{H1} values were determined from fluorometric titrations of the membrane-bound ionophore as described in the legend to Figure 6 and under Materials and Methods. The extinction coefficients, ϵ, for the electronic transition of lowest energy (benzoxazole peak) were measured at λ_{max} for HA (380 nm) and A^- (359 nm). Relative fluorescence values were obtained as described in footnote *a* to Table I. The fluorescence parameters λ_{ex} and λ_{em} were taken from corrected spectra of membrane-bound A23187 at pH values corresponding to either the free acid (HA) or the carboxylate anion (A^-).

fluorescence yields in an aqueous environment are low (Table I).

During the spectrophotometric titrations the base line shifted upward as tetraethylammonium hydroxide was added to adjust the pH. This effect was attributed to changes in the osmotic strength of the medium during the titration, leading to a contraction of the vesicles and a change in light scattering. The isosbestic points at lower wavelengths (265 and 285 nm) were more poorly defined than the one at 357 nm, which is consistent with this interpretation since light scattering is proportional to λ^{-4}. Subtracting the base-line shift at 445 nm, a wavelength at which A23187 is transparent, resulted in a reasonably well-defined isosbestic point at 357 nm but not at the shorter wavelengths. Therefore, only data from the 380-nm peak, corrected for base-line shift at 445 nm, were utilized. The fluorescence emission titrations were not subject to these light-scattering artifacts since the use of a double grating monochromator and narrow slit widths (2 nm) effectively eliminated Rayleigh scattering from the emission spectrum.

The spectrophotometric and fluorometric acid–base titrations of A23187 bound to the vesicles at 25 °C are presented in Figure 6, panels A and B, respectively. The spectral changes observed in the case of the membrane-bound ionophore are similar to those observed in methanol–water solutions. At 25 °C the log $K_{H1}{}^{b}$ was 7.85 ± 0.05. The excellent fit of the titration data for membrane-bound A23187 to eq 9 (Figure 6, panel C) confirmed that the generation of surface potential due to deprotonation of A23187 did not affect the log $K_{H1}{}^{b}$ under these conditions.

As observed in homogeneous solutions, log $K_{H1}{}^{b}$ values of A23187 bound to vesicles measured by absorption and fluorescence were equal within experimental error (Figure 6, panel C). The protonation constant did not change within experimental error when the concentration of A23187 was varied between 2.5 and 10 μM or when the nominal concentration of phospholipid was varied between 1 and 3 mM. The value was also the same when the liposomes were prepared at pH 4.0 instead of pH 8.0. When EDTA was absent the apparent log $K_{H1}{}^{b}$ was considerably lower (approximately 7.0), indicating the presence of contaminating divalent metal ions in the solutions (see below).

The log $K_{H1}{}^{b}$ data and relevant fluorescence and absorbance parameters for the free acid and anion bound to the membrane are summarized in Table II. The extinction coefficient for the membrane-bound free acid (9.1×10^3 cm^{-1} M^{-1}) was approximately equal to the value observed in 80% methanol. The spectral properties of the acid and anionic forms of the bound ionophore are nearly identical when compared at 15, 25, and 35 °C. These findings suggest that the microenvironments experienced by the two species are not greatly af-

fected by passing through the phase transition temperature at approximately 23 °C (Lentz et al., 1976; Watts et al., 1978). This indication is supported by the fact that for these three temperatures a nearly linear relationship (correlation coefficient = 0.979) exists between log $K_{H1}{}^{b}$ and $1/T$.

Discussion

The similarity of the log K^*_{H2} values for A23187 and MeA23187 at low pH* indicates that the same functional group is involved in each case and that the group is not the carboxyl moiety. The functional group involved in this equilibrium is most likely the *N*-methylamino substituent on the benzoxazole chromophore. The assignment is supported by comparison with the model compounds, *N*-methylanthranilic acid and 1-amino-2-carboxynaphthalene, which have log K_{H2} values of 2.01 and 1.01, respectively, in water (Martell & Smith, 1974). Although the log K_H values of *N*-methylanthranilic acid are only 2.8 log units apart in water, the log K^*_{H2} values tend to decrease slightly up to 80% methanol–water (D. R. Pfeiffer and R. W. Taylor, unpublished observations). For A23187 the log K^*_H values in 65% methanol–water are separated by approximately 5.9 log units. These findings indicate that the doubly protonated form of A23187 is unlikely to be involved in the ion transport reactions at physiological pH.

When the techniques of de Ligny et al. (1960) and Bates et al. (1963) were utilized for the measurement of pH* in methanol–water mixtures, reliable values for the carboxylate protonation constant (K^*_{H1}), referable to a standard state in that solvent composition, were obtained in solvents ranging from water to 95% methanol–water mixtures (Figure 5). The dependence of the log K^*_{H1} values upon solvent composition compared to literature data for simple carboxylic acids (cf. Bates et al., 1963; Rorabacher et al., 1971) and the absence of similar values for MeA23187 demonstrated that the carboxylic acid group of A23187 is responsible for the spectral transitions in the pH* range 4–11. The values reported here for A23187 are significantly higher than those reported previously (Pfeiffer et al., 1978). We attribute this difference to the present use of chelators to remove contaminating divalent cations which can cause an apparent decrease in log K^*_{H1} by competitively displacing hydrogen ion. The excited state protonation constant (i.e., K^*_{H1} of the lowest energy excited state singlet) determined by fluorescence was shown to be equal to the ground state value measured by absorption. Demonstration that the values determined by the two methods are the same is a prerequisite for the direct use of fluorescence to monitor protonation or complexation of the ionophore.

The value of the log K_{H1} in aqueous solution is somewhat lower than the value of approximately 6.3 reported by Puskin et al. (1981). However, the values are not directly comparable since the latter studies were done at ~21 °C in the presence of 100 mM NaCl and at higher A23187 concentrations (14.3 μM). The value of log K^*_{H1} for A23187 in 95% methanol–water solvent (9.40) may be compared with log K^*_{H1} values of 10.30 and 7.6 obtained in methanol for the ionophores monensin (Hoogerheide & Popov, 1979) and X-537A (Degani & Friedman, 1974), respectively. A more revealing comparison can be made by comparing the log K^*_{H1} values of A23187 with simple monocarboxylic acids (CA), such as acetic or benzoic acid, across the solvent range studied. At methanol percentages less than 50 the ratio of protonation constant values, K^*_{H1} (A23187):K^*_{H1} (CA), is essentially constant. As the methanol content is raised to 95% the ratio progressively increases by approximately 10-fold. When the ratio of protonation constants is examined as just described, electrostatic effects and proton solvation effects on the trend of log K^*_{H1} should be canceled to a large extent. Therefore, the increase of K^*_{H1} for A23187 relative to simple carboxylic acids reflects a stabilization of the free acid form of A23187 or conversely an increase in the basicity of the anionic form of A23187 relative to the model compounds. The solvent basicity of methanol–water mixtures is thought to pass through a maximum in the region from 65% to 80% methanol and decrease sharply with increasing methanol content (Rorabacher et al., 1971; Popovych, 1970). Thus, the formation of internal hydrogen bonds in A23187 (Pfeiffer & Deber, 1979) is facilitated as competition from the solvent decreases at high methanol percentages. The acid form of A23187 may be stabilized by formation of a hydrogen bond between the benzoxazole ring nitrogen and the carboxyl proton. Similar intramolecular hydrogen bonding effects have been noted for the antibiotic X-537A (Degani & Friedman, 1974).

The log of the protonation constant of A23187 bound to small unilamellar DMPC vesicles was found to be 7.85 ± 0.05 and was again the same whether determined by fluorescence or absorbance (Figure 6). As with the solution data, divalent metal contamination necessitated the presence of chelators. In the absence of chelators a lower log $K_{H1}{}^{b}$ (approximately 7.0) was observed, and at high pH λ_{ex} was 370 nm instead of 359 nm (data not shown). Analysis by atomic absorption revealed that approximately 20 μM Mg^{2+} was present in the vesicle solution along with lower concentrations of other divalent cations. A previously reported value of 6.7 ± 0.2 for A23187 bound to egg lecithin vesicles (Wulf & Pohl, 1977) may have been low because of divalent cation contamination; however, other conditions of that determination were also different from those employed here. The log apparent K^*_{H1} of the membrane-bound ionophore was not sensitive to the pH at which the vesicles were prepared. This indicated either that the permeability of these liposomes to H^+ and/or OH^- is high enough to prevent the occurrence of a significant pH gradient during the acid–base titration or that the ionophore partitioned only into the outer monolayer of the bilayer. Consistent with the former hypothesis is the finding that large unilamellar vesicles have significant permeability to protons (Nichols & Deamer, 1980; Nichols et al., 1980; cf. Prigent et al., 1980).

A comparison of the spectral and acid–base properties of A23187 in solution and bound to DMPC vesicles provides some insight into the environment and interactions of the membrane-bound ionophore, particularly of the benzoxazole moiety which bears the carboxylic acid function. Fluorescence emission of probes has been used as an indicator of effective polarity of microenvironments within bilayer membranes (e.g., Waggoner & Stryer, 1970). Although shifts in λ_{em} were observed for both HA and A^- as the percent methanol was varied, the magnitude of the shifts was relatively small (Table I). A further complication related to this type of analysis is that at the time of emission, solvent relaxation about the chromophore in the excited state may not be complete or may not occur to the same extent in the membrane as it does in methanolic solutions. With these limitations in mind the emission maximum for HA bound to the vesicles indicates a microenvironment with an effective polarity similar to that of 80% methanol–water. The microenvironment of the bound molecule, when expressed as an effective dielectric constant, would have a value of approximately 37 which is in the range expected for a molecule bound at the membrane–water interface (Shinitzky, 1974; Vaz et al., 1978). The use of λ_{em} as an indicator of effective polarity for A^- within the DMPC membrane is not justified with the data at hand since λ_{ex} for

this species falls outside the range observed in the reference solvents. The anomalous value of λ_{ex} for A^- may be due to a different ionophore conformation or specific interactions with the membrane that do not occur in the methanol–water solutions.

The log $K_{H1}{}^b$ is approximately 2 log units higher than the value observed in aqueous solution. Since the net surface charge at the DMPC interface is 0 and the surface potential generated by deprotonation of HA was negligible (see Results), this shift in log $K_{H1}{}^b$ indicates a decrease in effective polarity of the environment experienced by the benzoxazole group upon binding to the vesicle. The log K_{H1} for the membrane-bound ionophore is also nearly equal to the value observed in 80% methanol–water. Thus both the spectral parameters for HA and the log K_{H1} data suggest a predominantly interfacial location for the benzoxazole chromophore. The data in Table II further indicate that the environment of this portion of the molecule is not greatly affected by the liquid–gel phase state of the membrane interior.

On the basis of the ability of nitroxide spin probes located at different depths in the bilayer structure to quench the fluorescence of A23187, Puskin et al. (1981) have concluded that the free acid form of the ionophore is distributed in both the head group and hydrocarbon regions of vesicle membranes. Thus, the present data are not in complete agreement with the earlier study. A potential problem with the fluorescence quenching technique is that the membrane packing defects introduced by a relatively bulky nitroxide spin probe could alter the distribution of the ionophore between the interface and the membrane interior, favoring an increased partition into the hydrocarbon region. On the other hand, it is undoubtedly an oversimplification to assess the polarity of the ionophore's environment in the membrane solely by comparing spectral properties and the protonation constant with values obtained in the methanol–water solutions. The validity of the second approach depends upon how well properties of the membrane (polarity, solvation effects, basicity, etc.) are mimicked by the reference solvents. As pointed out earlier for the species A^-, the ionophore may experience specific interactions with structural components of the phospholipid bilayer. Interactions between the membrane and other chemical or conformational forms of the bound ionophore may produce relatively large effects on the protonation equilibrium which are not accounted for in comparisons to methanol–water solutions. Other factors not considered in this study include the possible effects of surface curvature on log $K_{H1}{}^b$ and the "primary medium effect" which arises from the fact that the membrane-bound ionophore and the proton lost upon ionization experience different solvent environments in the vesicle aqueous phase system, whereas all species in the equilibrium have an identical environment in the reference methanol–water solutions. For a comparison of the protonation constant of the membrane-bound compound to a value in a methanol–water solvent, the energy to transfer the proton from an aqueous phase to the reference solvent should be accounted for (Fernandez & Fromherz, 1977). Since the values of these energetic terms are not yet agreed upon, we have not applied correction of this type in the present study. If this effect were taken into account, agreement between the solvent environments indicated by the emission maximum and the log $K_{H1}{}^b$ values might not be as good. In addition, we observed a much lower value of log $K_{H1}{}^b$ (7.01) when the ionophore was bound to Triton X-100 micelles (data not shown), structures possessing an interfacial polarity similar to that of DMPC vesicles (Cordes & Gitler, 1973; Fernandez & Fromherz, 1977). This observation strengthens the concept that solvation effects and potentially other ionophore–lipid interactions must also be important in establishing the acid–base properties of this ionophore. Thus, it is clear that additional work will be necessary before the ionophore–membrane interactions of the various chemical forms of A23187 are fully understood.

A log $K_{H1}{}^b$ between 7 and 8 is probably desirable for ion transport by A23187 in biological systems at or near physiological pH. Under these conditions a significant amount of both HA and A^- would be associated with the membrane. The presence of both species is very likely necessary since A^- is a reactant in the complexation reaction with divalent cations and the transmembrane diffusion of HA probably completes the electroneutral transport reaction sequence [see Pfeiffer et al. (1978)]. Thus, while log $K_{H1}{}^b$ may vary with membrane composition, surface charge, etc., a value significantly outside the range 7–8 might result in much lower rates of ion transport, depending on the relative magnitudes of the rate constants for the component reactions of the transport cycle.

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