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X537A: A Ca^{2+} Ionophore with a Polarity-Dependent and Complexation-Dependent Fluorescence Signal

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Summary. The Ca^{2+} ionophore X537A has the characteristics required of a hydrophobic fluorescent probe. The quantum yield of the uncomplexed anion varies from 0.021 in water to 1.0 in dioxane, increasing with decreasing solvent polarity. Complexation with K^+, Ca^{2+} or Ba^{2+} serves to increase the fluorescence signal in solvents of high polarity. In solvents of low polarity decreases in fluorescence upon complexation have been found. The ionophore $X537A^-$ binds to dimyristoyl-α-lecithin membranes with a quantum yield of 0.4, and evidence is given that the ionophore is situated on the membrane surface. The fluorescent signal of $X537A^-$ on the membrane increases with cation complexation and values are reported for the complexation constants of $X537A^-$ with several monovalent and divalent cations on the membrane. The use of the fluorescent signal of X537A in the study of the mechanism of cation transport facilitated by this ionophore is discussed.

Numerous studies [9–11, 14–16] have shown that the ionophorous agents of the valinomycin and nigericin types, which function as mobile carriers in model and biological membranes, are important tools for the study of membrane properties and monovalent cation transport. The discovery of the Ca^{2+} complexing activity of the ionophore X537A and its derivatives [4, 13] has extended the possibilities for permeability control to the divalent cations. The importance of this ionophore has recently been emphasized by the demonstration that it can produce leakage of Ca^{2+} from isolated sarcoplasmic reticula [4, 17] and can increase contractility in perfused heart [12] and can increase cardiac output in dogs (N. T. de Guzman & B. C. Pressman, *unpublished observation*).

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The present communication shows that the binding of X537A to phospholipid membranes and its complexation reactions at the membrane surface with monovalent and divalent cations are reported by the fluorescent signal of the ionophore. A knowledge of these reactions is necessary for the understanding of the elementary mechanism of action of X537A as a mobile carrier. The first evidence that X537A functions as a mobile carrier was the report of the extraction of monovalent [15] and divalent [13] cations from an aqueous phase into an oil phase by this ionophore.

Since these initial reports, X537A has been subjected to a fair amount of chemical and physical study. The molecule bears two ionizable groups, a carboxyl and a phenol, and is thus classed together with the negatively charged nigericin-type ionophores. X-ray structure analyses [8] have been performed on a Ba^{2+} complex, showing that the cation is sandwiched by two singly ionized X537A molecules. Isotope flux experiments in a lipid bilayer membrane indicate that the ionophore transports both monovalent and divalent cations predominantly in the neutral form [12]. The conformation of the complexes in solution has been studied by optical rotatory dispersion [12].

Previous attempts to study the complexation and conformation of ionophores have suffered from the lack of a sensitive spectral readout. The intrinsic fluorescence of X537A, on the other hand, is quite sensitive and gives direct measure of both the degree of binding of the ionophore to the membrane, and of the advancement of the complexation reaction. The complexation reactions of a number of valinomycin-type carriers on phospholipid membranes have been studied making use of the electrostatic effect of complexation on the binding of the negatively charged hydrophobic fluorescent probe 1-anilino-8-naphthalenesulfonate (ANS^-) [6]. This method gave information about the extent of the complexation reaction but the location and conformations of the ionophores and their complexes could only be inferred from the binding behavior [6]. The intrinsic fluorescence of X537A gives important information about the location of the ionophore in the membrane, about its freedom of motion and about the extent of complexation. Further, the method is eminently suitable for analysis using rapid reaction techniques [5] in membrane systems [7].

Materials and Methods

Fluorescence spectra were taken on a FICA 55 Spectrofluorimeter, which gives quantum-corrected excitation (220 to 550 nm) and emission (200 to 800 nm) spectra. The instrument is fitted with an additional rhodamine *b* quantum counter behind the sample cuvette, enabling sample transmission to be recorded simultaneously with

fluorescence on a Bryans 2600A3 y_1, y_2, x recorder. Inner filter effects at the excitation wavelengths were in most cases negligible. When necessary, corrections were made using the fluorescence/transmission characteristics of the cuvette (5 mm) and slit (1 mm) combination, as determined by use of standard fluorescent compounds. Additional absorption spectra were taken on a Cary 14 spectrophotometer.

All solvents were spectral grade. X537A was obtained as a gift of Hoffman La Roche. Dimyristoyl-α-lecithin was purchased from Koch-Light Laboratories. Monolayer vesicles were prepared with di-*n*-butylether as described by Träuble and Grell [19] with sonication at 30 °C. These structures have been characterized by electron-microscopy as ca. 500-Å diameter spheres of organic solvent, covered with a monolayer of phospholipid molecules [19]. Their equilibrium surface and phase transition properties are essentially the same as those of bilayer vesicles with the same lipid composition [6, 18].

Results and Discussion

Fluorescence in Solvents

The fluorescence signal of X537A arises from the salicylic acid residue of the ionophore. This group is probably directly involved in the complexation [8], and the fluorescence signal is thus expected to be sensitive to the degree of complexation and protonation of the ionophore. Fig. 1 compares the absorption and fluorescence excitation and emission spectra of the free acid in methanol. Excitation at the 310-nm peak and at the 244-nm shoulder results in a constant quantum yield (±10%). In the following experiments, the 310-nm peak was chosen for excitation.

Under the conditions of Fig. 1, the carboxyl group of the ionophore is dissociated. Titration experiments with HCl in methanol indicate that the

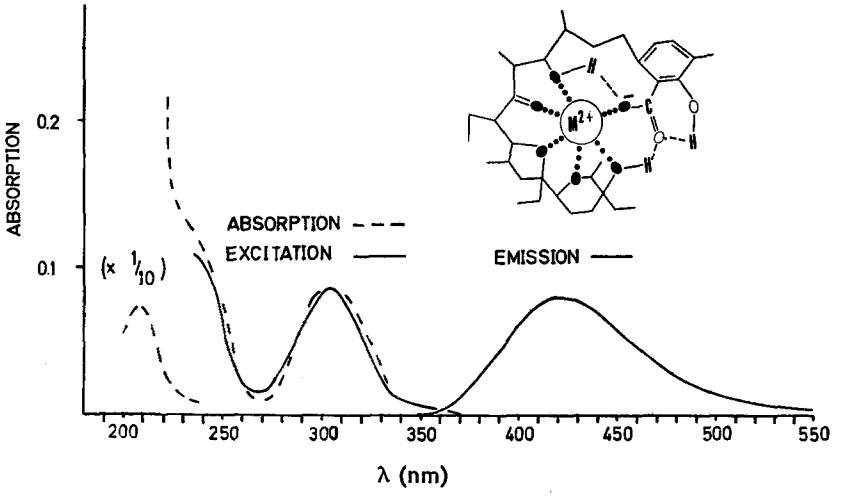

Fig. 1. Absorption and corrected excitation and emission spectra of 2.86×10^{-5} M X537A$^-$ in methanol at 25 °C. Fluorescence is in arbitrary units. The structure of the "unprimed" complex is given (*cf.* ref. [8])

carboxyl group has a pK_a of 4.35 ±0.15 (based on H^+ concentration), and that the quantum yield of the protonated form is less than 5% of that of the −1 charged form (assuming only the ground state to be affected by the HCl).

Weller [21] has shown that the excitation of salicylic acid results in a transfer of the internally hydrogen-bonded phenolic hydrogen to the carboxyl group, and that the fluorescence occurs from an excited state in which this transfer has occurred. These conclusions were based on comparisons of the differences in wave number between the emission peaks and the absorption peaks lying at the longest wavelength for a series of hydroxybenzoic acid derivatives. Comparison with the difference found here for $X537A^-$ (9,300 cm^{-1}) indicates that its fluorescence arises from a similar excited state (*cf.* ref. [21]). The absence of a blue shift in the fluorescence of X537A in methanol upon titration with tetramethylammonium hydroxide (TMAOH) showed that the phenolic OH remained undissociated for hydroxide ion concentrations up to 5 mM, as would be expected from the stabilizing effect of intramolecular hydrogen bond formation [1].

Table 1 shows that the fluorescent signal and the quantum yield of the X537A are very sensitive to the polarity of the solvent. A similar polarity dependence has been found for the fluorescence of A23187, a functionally related ionophore [4]. The quantum yield of $X537A^-$ decreases with increasing solvent polarity in a manner similar to that of the ANS^- derivatives which have enjoyed widespread use as probes of hydrophobic binding sites. Table 1 shows that the polarity dependence of the quantum yield of $X537A^-$ is comparable to that of 1.7 ANS^-.

Fig. 2 shows the correlation of the quantum yield of $X537A^-$ with solvent dielectric constant. A smooth line was drawn through the points representing the dioxane- and *n*-alcohol-water mixtures. The data for ethylene glycol (*E*), acetone (*A*) and DMSO (*D*) show deviations from this dependence, but similar deviations have been found for the last two solvents for 1.7 ANS^- in plots of Q against the Z polarity parameter [20]. Plotting the present data against this scale gave an approximately linear dependence between Q and Z for all points in Fig. 2, with the exception of these three solvents. For all solvents and solvent mixtures studied here, the quantum yield of the protonated form of X537A is only a fraction of the value found for the single negatively charged form.

It has recently been proposed that the quantum yield of 2-*p*-toludinylnaphthylene-6-sulfonate (2,6-TNS^-) is determined by the propensity of the solvent environment for dipole reorientation during the excited state of the probe [2]. Since the solvent dependence of the quantum yield of X537A is

Table 1. Polarity-dependence of the $X537A^-$ quantum yield[a]

Solvent	ε ($\times 10^{-3}$)	Q	$Q/Q_{(ANS)}$	Enhancement		
				K^+	Ca^{2+}	Ba^{2+}
H_2O, pH 7.3, 5 mM imidazole	2.5	0.021	2.95	0.34	1.15	15.3
Ethylene glycol	3.8	0.12	0.33	3.3[b]	1.0[b]	3.0[b]
30% Ethanol, 70% H_2O (v/v)	2.8	0.15	2.34	0.93	0.83	1.12
50% Ethanol, 50% H_2O (v/v)	2.9	0.27	1.50	1.22	0.79	1.38
Methanol	3.1	0.30	0.77	2.53	1.08	2.63
50% Dioxane, 50% H_2O (v/v)	2.9	0.40	1.33	1.24	0.75	1.40
Acetone	2.5	0.48	1.60	0.90	0.6[b]	0.2[b]
DMSO	3.1	0.60	1.30	1.03	0.42	0.99
Ethanol	3.3	0.79	1.41	1.22	0.81	1.03
1-Propanol	3.5	0.92	1.31	1.05	0.85	0.81
Dioxane	3.4	1.0	1.47	1.00	0.24	0.90

[a] Measurements were made at 25 °C on 6×10^{-6} solutions of the free acid titrated with TMAOH to a final concentration of 1 mM, when necessary to ensure full dissociation of the acid. The extinction coefficients, ε, ($M^{-1}\,cm^{-1}$) are for the 310-nm peak. Q was calculated from the total fluorescence emission, using fluoresceine in 0.1 M NaOH as a standard (*cf.* ref. [3]). For the solvent acetone, it was necessary to correct for inner filter effects at the exciting wavelength. Quantum yields for 1.7 ANS^- were taken from ref. [20]. The enhancement values are the ratios of the fluorescence yield of the ionophore after titration with the cation divided by the fluorescence yield of the ionophore in the presence of TMAOH before titration. The experimental uncertainty of all the measurements is about 5%.

[b] Values subject to large experimental variation.

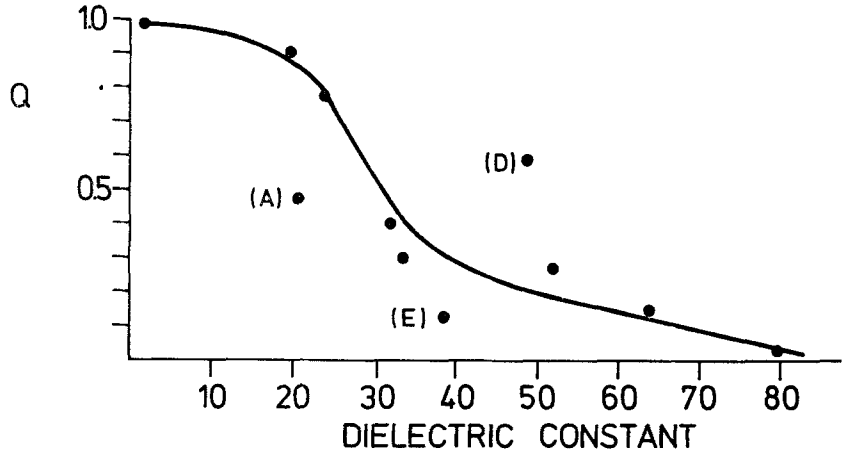

Fig. 2. Correlation of the quantum yield of $X537A^-$ with the solvent dielectric constant. The values of Q were taken from Table 1 and values of the dielectric constant were taken from ref. [20]. The symbols *E*, *A* and *D* indicate the data points for the solvents ethylene glycol, acetone and DMSO, respectively

similar to that of the ANS^- derivatives, it is possible that the major contribution solvent-dependent quenching of its excited state occurs by a similar mechanism.

The solvent dependence of Q for the K^+, Ca^{2+} and Ba^{2+} complexes of $X537A^-$ is somewhat less pronounced, indicating that in the complexed conformation, the salicylic acid group is somewhat more shielded from the solvent. Thus in polar solvents, complexation results in an enhancement of fluorescence, as shown in Table 1. In less polar solvents, complexation often results in a decrease in fluorescence. This is probably due to a small residual contribution of the cation or the complexed conformation to the quenching process. Differences in the enhancement values between cations can arise from differences in the influence of cations as electron acceptors or may result from differences in the complex conformations or their influence on the accessibility of the solvent to the salicylic acid group. The influence of the cation on the complexed conformation is being investigated by circular dichroism techniques, and will be reported in a future publication.

Fluorescence in Membranes

The binding of X537A to phospholipid membranes results in an increase in the fluorescent signal. The quantum yield of $X537A^-$ bound to dimyristoyl-α-lecithin vesicles at 30 °C, pH 7.3, is 0.41 ±0.06, a value close to that for the solvent methanol. Thus the "polarity" of the environment of the probe, or the propensity of the membrane environment for quenching via dipole reorientation or other mechanisms is similar to that of this semipolar solvent. At 15 °C, below the membrane phase transition temperature, the spectra and quantum yield (0.46 ±0.06) are similar. Taken together, these observations indicate that the chromaphoric group of the ionophore is situated at the membrane surface (*cf.* ref. [6]). The fluorescence polarization of the membrane-bound form is low (0.08), indicating fast rotation or fast segmental motion, with a correlation time of less than 10^{-9} sec.

In addition to reporting the location of the ionophore, the fluorescent signal can be used to study the binding equilibrium to the membrane and the protonation and complexation reactions in the membrane. These reactions alter the surface charge and the general dependence of the similar reactions of cation binding and ionophore complexation in the membrane have been formulated in a previous communication [7]. Total analysis of the X537A binding, protonation and complexation reactions using the Gouy-Chapman equations for membrane surface potential (*cf.* ref. [7]) in modified form in conjunction with a computer program will be reported

elsewhere. The salient points regarding the effects discussed below are the following: (a) Reactions introducing positive charge into the membrane are promoted by negative charge in the membrane and vice versa. (b) This effect is decreased by increasing electrolyte concentration in the medium. (c) Any charge-introducing reaction will have anticooperative character. The equilibrium data below will be expressed in terms of *apparent* equilibrium constants, evaluated at the point of half-extent of reaction.

The value of the apparent binding constant of the ionophore in the membrane, K_b, was evaluated as the reciprocal of the lipid concentration giving half-maximal X537A binding. For dimyristoyl-α-lecithin monolayer vesicles at pH 7.3 in the presence of 5 mM 1:1 electrolyte, K_b is 1.0×10^4 M^{-1}. The value increases with increasing electrolyte concentration. The statements given below concerning reactions on the membrane are based on data extrapolated to infinite lipid concentration. The fluorescence of the protonated form of X537A in the dimyristoyl-α-lecithin membrane is $6 \pm 2\%$ of that of the singly ionized species. Spectrofluorimetric titrations showed that the carboxyl group of X537A in the dimyristoyl-lecithin membrane has a pK_a between 6.7 and 6.9 and that the pK_a of the phenolic hydrogen is greater than 12. The corresponding values for salicylic acid in water are 3.0 and 13.8, respectively [1].

Cation complexation results in an increase in the fluorescent signal (enhancement) of up to 60% for membrane-bound X537A. Fig. 3 is a plot of the increase in fluorescence with Ca^{2+} concentration in double reciprocal form. Enhancement upon cation titration could not be detected below pH 6.5. Analysis at several lipid concentrations and over a range of pH values indicated that the enhancement is primarily due to cation complexation according to:

$$M^{n+}_{aq} + X537A^{-}_{memb} \xrightarrow{K_a} M{-}X537A^{(n-1)+}_{memb} \qquad (1)$$

where M represents the cation of charge n, and where aq and memb refer to the aqueous and membrane phases, respectively. K_a was determined experimentally as the reciprocal cation concentration giving a half-maximal increase in fluorescence. The constancy of K_a for pH values above 7.3 indicates that ionization of the phenolic hydrogen is not necessary for complexation on the membrane even for the divalent cations.

Experiments with phosphatidic acid/lecithin mixed membranes gave results expected from considerations of membrane charge. In general, the values of K_b were decreased, and the values of K_a were increased with increasing phosphatidic acid content of the membrane.

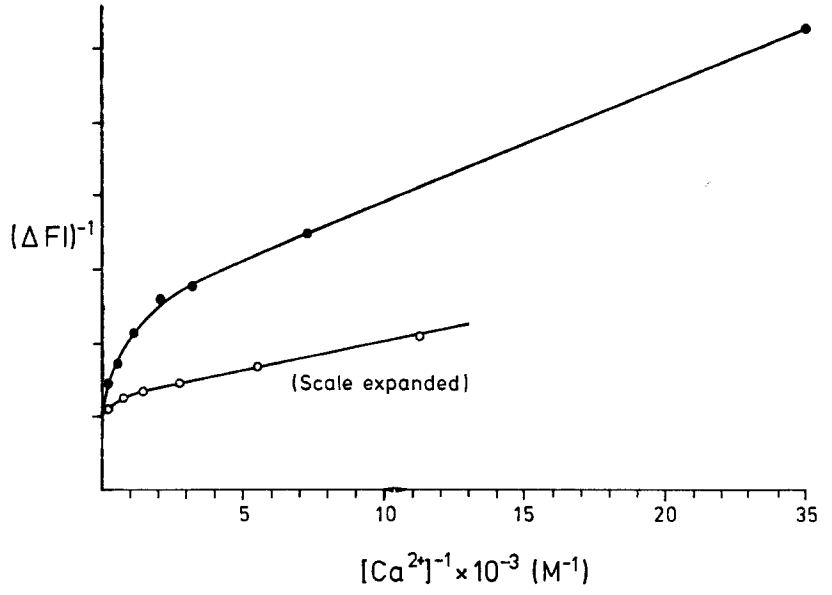

Fig. 3. Double reciprocal plot of the enhancement effect in dimyristoyl-α-lecithin monolayer vesicles. Δ Fl refers to the increase in fluorescence due to the addition of the cation chloride. The medium contained 1.8×10^{-5} M X537A, 5 mM imidazole, pH 7.3, 6.8×10^{-4} M lipid (T = 30 °C) and a variable $CaCl_2$ concentration. In the lower curve, the concentration scale is expanded 10 ×. The experimentally observed enhancement was 50 %

Fig. 4 shows the values of $\log(K_a)$ for various mono- and divalent cations as a function of their ionic radii. Experiments with La^{3+} and Mn^{2+} resulted in fluorescence decreases and paramagnetic quenching, respectively, and their complexation behavior will be discussed elsewhere. The values of K_a for the divalent cations are, with the exception of Sr^{2+}, at least an order of magnitude larger than those of the monovalent cations. Part of the divalent cation specificity found here arises from the effect of the $X537A^-$ binding to impart a negative surface charge to the membrane, thereby increasing the cation concentration near the membrane surface. Due to their higher charge, the divalent cations are influenced by this surface potential effect to a greater extent, and thus enjoy higher apparent binding constants. The "chemical" and electrostatic contribution to the complexation energy will be distinguished in a separate communication.

The specificity among monovalent and divalent cations can be discussed without reference to membrane electrostatics. In the membrane system, $X537A^-$ shows little monovalent cation specificity, with a small tendency to favor small cations with the exception of Li^+. This situation is reversed for the process of extraction of cations from an aqueous phase to an organic

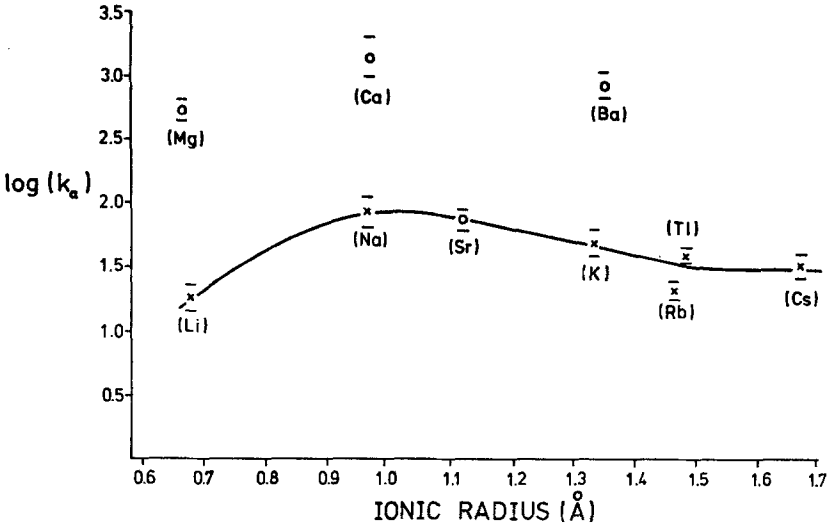

Fig. 4. K_a as a function of valency and ionic radius. The conditions were identical to those of Fig. 2. The measured enhancements varied from 30 to 60%. The anomalous behavior of Sr^{2+} will be discussed in a future publication

phase, with $Cs^+ \sim Rb^+ > K^+ \sim Na^+$ for the rank order of the heterogeneous complexation constants [12]. In the membrane system, there is a small specificity for Ca^{2+}, whereas the extraction system shows a large specificity with the rank order $Ba^{2+} \gg Sr^{2+} > Ca^{2+} > Mg^{2+}$ with selectivity ratios 2600:8.5:1.0:0.38 for the heterogeneous complexation constants normalized to that of Ca^{2+} [12].

This discrepancy in ion specificity and the above-mentioned lack of phase-transition dependence of the membrane complexation reaction lead us to believe that the membrane complexes reported here are fairly dissimilar to those involved in the rate-limiting step of X537A-facilitated cation transport; i.e., those which penetrate the hydrocarbon interior of the membrane. Evidence for the formation surface complexes of the neutral valinomycin-type ionophores on phospholipid membranes with an attendant decrease in ion selectivity has been presented previously [6]. We believe that the $X537A^-$ complexes detected here are of a "hydrophilic" type which have not achieved a "closed" or "hydrophobic" conformation, and that the propensity for transport is at least partially governed by the equilibrium constant for this transformation. The complexation reactions reported here can thus be seen as the first step of the X537A-mediated cation transport reaction. The role of these surface complexes in transport may be further elucidated by a study of the cation concentration dependence of the net

transport reaction, and it is planned to compare the K_a values reported here with K_m values for transport in future studies.

In conclusion, the use of the fluorescence of X537A offers a unique opportunity for direct study of the elementary steps in carrier-mediated cation transport and for the evaluation of the influence of membrane composition and membrane surface potential on these partial reactions. It is hoped that use of the techniques reported in this study together with a systematic investigation of the membrane-bound X537A with fluorescent lifetime and rapid kinetic techniques will enable a full interpretation of the ionophore reactions based on the fluorescent signal alone. This would facilitate the study of the pharmacological action of X537A in intact muscle and in heart by nonperturbing spectrofluorimetric techniques.

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