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The Detection of Ionophorous Antibiotic-Cation Complexes in Water with Fluorescent Probes

(alkali cations/alamethicin/nigericin/trinactin/valinomycin/relative affinities)

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ABSTRACT **The binding of alkali cations by the ionophorous antibiotics valinomycin, nigericin, alamethicin, and the macrotetralide actins has been shown to occur, in aqueous media, by the use of the fluorescent probes 1-anilino-8-naphthalene sulfonate and 2-*p*-toluidinyl-6-naphthalene sulfonate. The interaction of the ionophore–cation complexes with the fluorescent dyes produced enhanced fluorescence emission, increased lifetime and polarization, and a significant blue-shift of the emission maxima of the fluorescence spectrum. At constant antibiotic and fluorophore concentrations in water, the intensity of the fluorescence emission was found to be a function of the cation concentration. This permitted relative cation affinities to be determined for alamethicin ($Na^+ \cong K^+$), valinomycin ($Rb^+ > K^+ > Cs^+$), nigericin ($K^+ > Rb^+ > Na^+ > Cs^+$) and trinactin ($NH_4^+ > K^+ > Rb^+ > Cs^+$).**

Certain microbial metabolites, termed ionophorous antibiotics, have been observed to produce profound effects upon passive ion transport in erythrocytes, bacteria, lipid bilayer membranes, and energy-dependent ion transport in mitochondria (1–3). These phenomena are all markedly dependent upon the alkali-cation composition of the medium. This dependency has been accounted for by the discovery of the remarkably specific alkali-cation binding properties of these antibiotics (1, 4, 5). Among the ionophores are such groups as: (*a*) the cyclodepsipeptides (i.e., valinomycin and the enniatin antibiotics) and the nactin cyclodepsides, which form positively charged complexes with alkali-cations, (*b*) nigericin and monensin, linear chains of oxygen-containing heterocyclic rings that cyclize by hydrogen bonding between carbonyl and hydroxyl groups at opposite ends of the molecule, and whose cation complexes are electrically neutral, and (*c*) alamethicin, a cyclic polypeptide with a free carboxyl group that can form neutral or net positively charged complexes. Cation selectivity in organic solvents or model membrane systems varies from high K^+/Na^+ affinity for valinomycin, macrolide actins, and nigericin, and poor discrimination between these two cations for alamethicin, to Na^+ preference for monensin (1, 4–6, 26). A common property of all these ionophores is the ability to carry alkali-cations across lipid barriers, or into solvents of low polarity (1, 4, 5). X-ray crystallography, proton magnetic resonance, infrared spectroscopy, and optical rotatory dispersion have revealed that the cations are associated through induced dipole interactions with carbonyl, ether, or hydroxyl oxygen atoms of the ionophore. Conformational changes occur upon reaction with the cation, with the result that the cation (stripped of its hydration shell) resides within a cavity formed by the polar oxygen groups. The latter are in turn enclosed within the lipophilic portions of the ionophore, which comprise the exterior of the complex (7–10). The shielded cation can be solubilized in media of low polarity, or carried across the hydrocarbon interior of a membrane as a mobile complex. Formation of several cation–ionophore complexes in organic solvents has been demonstrated by various physicochemical methods (8, 9, 11, 12), but stability of the complexes is drastically reduced by addition of water to the solvent (8, 9) so that complex formation in purely aqueous media cannot be detected (8). Pressman (13) developed a method to quantitate complex formation by utilizing a two-phase system of water and butanol–toluene. Double reciprocal plots of alkali-cation concentration in the aqueous phase versus concentration of radioactive cation extracted into the organic phase as a complex with the ionophore were used to obtain a hybrid water–lipid phase dissociation constant $K_D^{W/L}$. The $K_D^{W/L}$ values for K^+ complexes of valinomycin and nigericin were 49 and 0.09 mM, respectively. In an attempt to study the possibility of complex formation in water, we have employed two fluorescent dyes, 1-anilino-8-naphthalene sulfonate (ANS) and 2-*p*-toluidinyl-6-naphthalene sulfonate (TNS), which have been widely used as probes for hydrophobic regions of proteins and cellular membranes (14–19).

METHODS AND MATERIALS

Fluorescence emission, excitation, and polarization were measured at 25 ± 0.2°C in 1.0-cm quartz cells. A Perkin-Elmer MPF-2A ratio recording spectrophotofluorometer was used. When required, fluorescence spectra were corrected for nonlinearity of the xenon source, monochromator efficiency, and detector response by the method of Melhuish (20). The absorbance of solutions analyzed for fluorescence was maintained, as far as practicable, below 0.1. Solutions were made up to 2.0 ml and buffered at pH 7.4 by 10 mM Tris·HCl (Mann). Light scattering was minimized, when necessary to increase sensitivity and accuracy, by positioning a Polacoat 105 UV, quartz polarizing filter, oriented horizontally, in the path of the excitation beam (21). Polarization (p) of fluorescence was measured with Polacoat 105 UV filters in both excitation and emission paths, the equation: $p = I_{vv} - GI_{vh}/I_{vv} + GI_{vh}$. I is the observed fluorescence intensity, the subscripts refer to the axial orientation of the polarizer and

Abbreviations: ANS, 1-anilino-8-naphthalene sulfonate; TNS, 2-*p*-toluidinyl-6-naphthalene sulfonate.

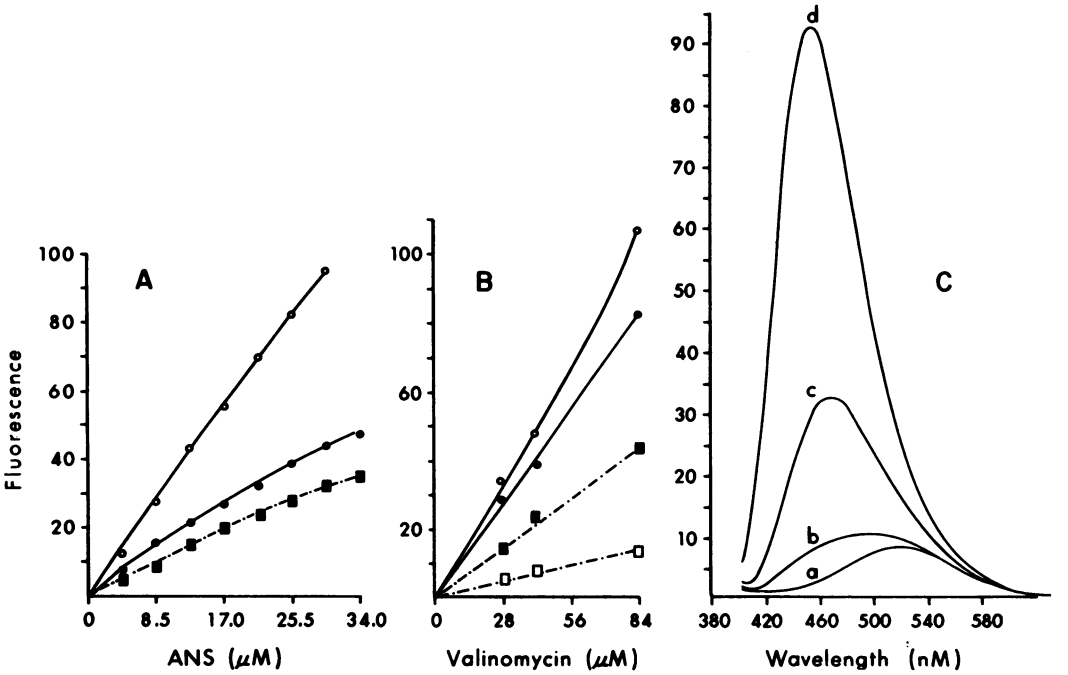

FIG. 1. Fluorescence emission as a function of valinomycin, cation, and ANS concentration. Excitation at 380 nm, slits at 10-nm bandwidth. Fluorescence expressed in arbitrary units. *A*: KCl 0.225 M, valinomycin 28 μM (■), 47.5 μM (●), 85 μM (○). *B*: KCl 0.225 M, ANS 4.25 μM (□), 12.75 μM (■), 25.5 μM (●), 34 μM (○). *C*: Fluorescence emission spectra (uncorrected). *a*, Valinomycin 2.8 μM + ANS 84 μM, titration with KCl; *b*, 0.05 M; *c*, 0.25 M; *d*, 1.0 M.

analyzer, respectively, and G is the grating correction factor I_{hv}/I_{hh} (22). Fluorescence lifetimes were measured with a TRW 75A decay time fluorometer coupled to an oscilloscope. Absorption spectra were measured with a Gilford 4000 recording spectrophotometer. The ionophorous antibiotics were determined colorimetrically (23). A 0.1% solution of tetrabromphenolphthalein ethyl ester (TBEE) in chloroform was shaken with an equal volume of water containing 1.0 M KCl and the ionophore. The molar absorbancy index of the K^+–valinomycin complex with TBEE in $CHCl_3$ at 616 nm was 1.49×10^5. Magnesium ANS (Polysciences) was purified by repeated (3×) crystallization from hot water and filtration through Norit. The potassium salt of TNS (Sigma) and tetrabromphenolphthalein ethyl ester (Eastman) were used without further purification. Organic solvents were spectroquality grade (MC&B, Eastman). Valinomycin was obtained from Calbiochem. Various macrotetralide actins were gifts from Drs. C. G. Smith and D. M. O'Brian (Squibb), Prof. V. Prelog (Zurich), and Dr. H. Bickel (Ciba); nigericin and monensin from Drs. J. M. McGuire, H. Higgins, A. Agtarap, and J. W. Chamberlin (Eli Lilly); alamethicin from Dr. G. B. Whitfield Jr. (Upjohn); and various crown polyethers from Dr. H. K. Frensdorff (DuPont).

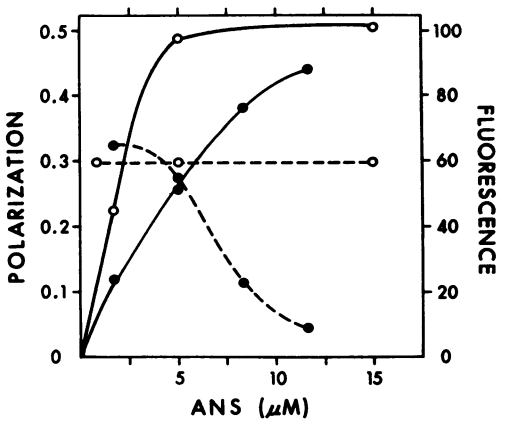

FIG. 2. Fluorescence intensity (–) and polarization (– –) of K^+ complexes of valinomycin (●) and nigericin (○), as a function of ANS concentration. Valinomycin 28 μM, nigericin 17 μM, and KCl 1.0 M.

RESULTS AND DISCUSSION

In the absence of alkali-cations, none of the ionophores studied, with the exception of alamethicin, affected the fluorescence of ANS or TNS in aqueous* solution. When ANS solutions were titrated with alamethicin, fluorescence increased and the peak-emission wavelength (uncorrected) shifted from 520 to 485 nm. Subsequent addition of Na^+ or K^+ salts further increased fluorescence and shifted the emission peak to 470 nm. No significant selectivity between Na^+ and K^+ was observed. With valinomycin, macrotetralide actins, or nigericin, the addition of alkali-cations produced striking enhancement of ANS or TNS fluorescence, with substantial shifts in peak-emission wavelength†. The formation of fluorescent complexes is dependent upon the reaction between the ionophore and cation, and the subsequent interaction with the fluorophore.

* Antibiotic stock solutions were made with acetone or ethanol. The organic solvent concentration in the fluorescence assays did not exceed 2.5%.

† Fluorescence changes were not observed upon the addition of alkali-cations to solutions of monensin or several synthetic "crown" polyethers.

The magnitude of the fluorescence emission depends on each of the components of the reaction, as shown for valinomycin in Fig. 1. In the presence of a constant concentration of K^+–valinomycin complex, the fluorescence is a function of the fluorophore concentration and similarly, the fluorescence varies with antibiotic concentration when the dye and cation concentrations are fixed. The corrected emission maximum for the ANS–valinomycin–cation complexes is 460 nm (455 uncorrected). The sensitivity of the method is apparent from the considerable increase in the fluorescence signal over background, at valinomycin concentrations as low as 2.8×10^{-6} M (Fig. 1,*C*).

The fluorescence of the dye molecules bound to the cation–ionophore complexes is rather highly polarized and, in the case of valinomycin complexes, the polarization decreases substantially as saturation of the binding sites with the fluorophore increases (Fig. 2). Depolarization could result from energy transfer between fluorophore molecules bound within about 3.5 nm of each other to individual ionophore molecules in a micelle. No depolarization was seen upon titration of K^+–nigericin complexes with ANS, probably because few dye molecules are bound (see below) so that distances between fluorophore molecules are too great to permit intermolecular energy transfer. The possibility that some of the above-mentioned effects are attributable to dye interaction with aggregates of cation–ionophore complexes requires further investigation.

The binding affinity of ANS for K^+–valinomycin was measured by titration of the latter with the fluorescent probe, according to the method of McClure and Edelman (24), for a protein–fluorophore binding system affected by a small modifier molecule. Titrations conducted at two concentrations of KCl gave linear plots of fluorescence versus fluorescence/ANS concentration, with the same slopes, from which the apparent dissociation constant was calculated to be 5×10^{-5} M, a value similar to that reported for ANS binding to apohemoglobin (14), myosin (25), and beef heart mitochondria (16). By the same method the dissociation constant for the ANS complex with K^+–nigericin was calculated to be 8.5×10^{-6} M.

ANS association with the ionophore–cation complexes was also shown by studies of the partition of the fluorophore in a two-phase system of chloroform and water. Very little ANS, measured spectrophotometrically, entered the organic phase in such a system if the aqueous phase contained either valinomycin or KCl. However, in the presence of valinomycin *plus* KCl, a marked partition of ANS in favor of the chloroform phase occurred. Valinomycin was detected only in the organic phase. By titration of K^+–valinomycin or K^+–trinactin with increasing concentrations of ANS in water, and subsequent extraction with $CHCl_3$, it was determined that a 0.9:1 molar ratio of ANS to ionophore could be obtained in the organic phase, which indicates that the complexes probably exist as neutral ion-pairs in this solvent. The K^+–nigericin complex in chloroform bound only 1 mol of ANS per 60–70 mol of nigericin. The maximum of the fluorescence emission spectrum of ANS bound to K^+–valinomycin complex in $CHCl_3$ was shifted to a lower wavelength, as compared to free ANS in chloroform (Fig. 3, *B*). Thin-layer chromatography of the chloroform phase (after equilibration with an aqueous phase containing K^+, valinomycin, and ANS) on silica gel showed two fluorescent bands, one with a yellow fluorescence (Fig. 3, *A*1), and a second faster-moving, bright-blue fluorescent band (Fig. 3, *A*2). The fluorescence spectra of the material from these bands in aqueous media were characteristic of free ANS and the ANS complex with K^+–valinomycin, respectively (Fig. 3,*C*). A third, nonfluorescent band was demonstrated at the solvent front by charring with sulfuric acid (Fig. 3,*A*3). The tetrabromphenolphthalein ethyl ester test for valinomycin was positive only for the bands at the solvent front and the fast-moving blue fluorescent band. The blue fluorescent band was therefore identified as the K^+–valinomycin–ANS complex, whereas the nonfluorescent band at the solvent front was free valinomycin. Fluorescent K^+–trinactin and K^+–nigericin complexes with ANS were similarly separated from unbound ANS and ionophore, in several solvent systems.

Cation dependence of antibiotic–fluorophore interactions

The fluorescence of ANS or TNS complexes with valinomycin, macrotetralide actins, and nigericin was found to be dependent upon the *species* of the alkali-cation present in the solution. This dependency was the basis for the determination of their relative cation affinities in aqueous media. Fluorescence of valinomycin–Cs^+ and –Rb^+ complexes with ANS are shown in Fig. 4. It can be seen that fluorescence was enhanced at much lower concentrations of Rb^+ than Cs^+. Plots of the reciprocal of fluorescence intensity of valinomycin complexes

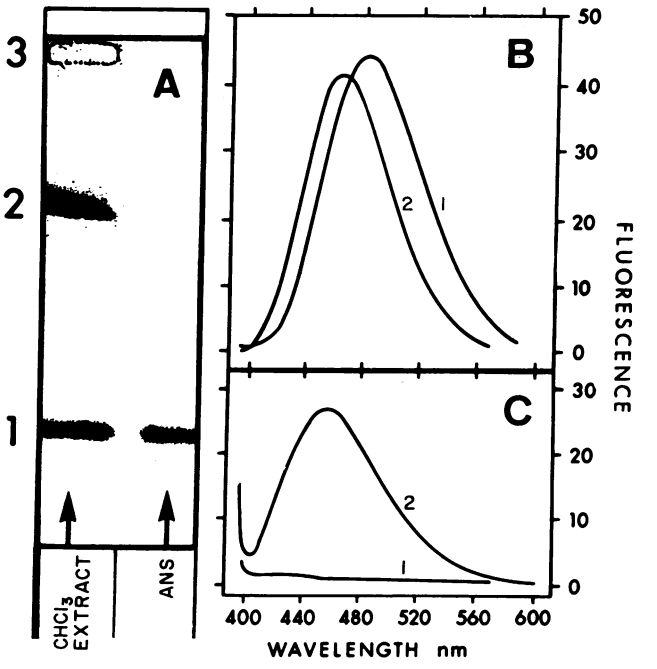

FIG. 3. *A*: Thin-layer chromatogram showing fluorescent bands. *Left*, chloroform phase after equilibration with aqueous phase containing 1.0 M KCl, 560 μM valinomycin, and 500 μM ANS. *Right*, ANS in chloroform. Dotted area at position 3 on the left represents the position of the band that appeared after charring with 15% aqueous sulfuric acid at 100°C for 5 min. The silica-gel G plate was activated at 110°C for 30 min and the developing solvent was chloroform–methanol–water 65:25:4. *B*: Fluorescence emission spectrum of *1*, ANS in chloroform, and *2*, the chloroform phase containing K^+–valinomycin plus ANS. Excitation at 380 nm, slit bandwidths 6 nm. *C*: Fluorescence emission spectra of *1*, the band in position 1 and *2*, the band from position 2 of the thin-layer chromatogram of the $CHCl_3$ phase. The solvent is water containing 1.0 M KCl. Other conditions as in *B*. The spectra are direct traces, uncorrected.

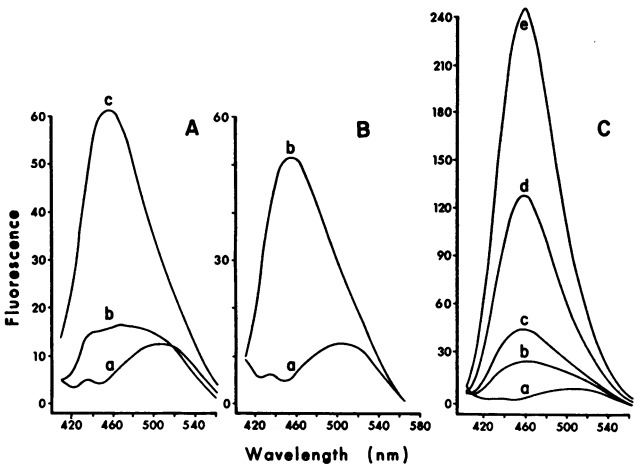

FIG. 4. Titrations of valinomycin + ANS with cesium and rubidium chlorides. *A*: (*a*) Valinomycin 11.25 μM + ANS 21.25 μM; *b*, as in *a*, +0.075 M CsCl; *c*, +0.490 M CsCl. *B*: *a*, Valinomycin + ANS; *b*, as in *a* +0.025 M RbCl. *C*: Titrations of valinomycin + ANS with 0.0125, 0.025, 0.075, 0.183 M RbCl, *b–e*. Other conditions the same as in Fig. 1.

versus the reciprocal of the cation concentration (Fig. 5) are essentially linear over the range studied, so that the binding of cation to valinomycin appears to follow a simple Langmuir adsorption isotherm. The apparent dissociation constants, determined from the slopes, are 0.17, 0.43, and 8.38 M, respectively, for the Rb^+, K^+, and Cs^+ complexes of valinomycin. As expected, the affinities of valinomycin for cations in aqueous media is much less than in organic solvents (12). No fluorescence was detected when valinomycin was titrated with NaCl in the presence of ANS or TNS. The extrapolated maximum fluorescence intensity value was the same for each of the complexes with valinomycin, which suggests that the quantum yields are the same for each, and that the configuration of the various complexes are quite similar.

Titrations of nigericin with alkali-cations (i.e., Fig. 6,*A*), in which ANS or TNS was utilized as the fluorophore, reveal a different order of cation selectivity, $K^+ > Rb^+ > Na^+ > Cs^+$. Also the K^+ affinity for nigericin was 10–20 times greater

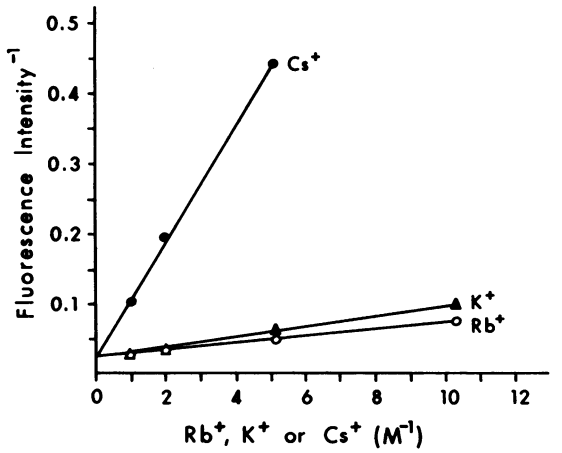

FIG. 5. Double-reciprocal plot of fluorescence against cation concentration for valinomycin complexes. Valinomycin concentration is 11.25 μM, ANS 21.25 μM. Excitation at 380 nm, emission recorded at 455 nm.

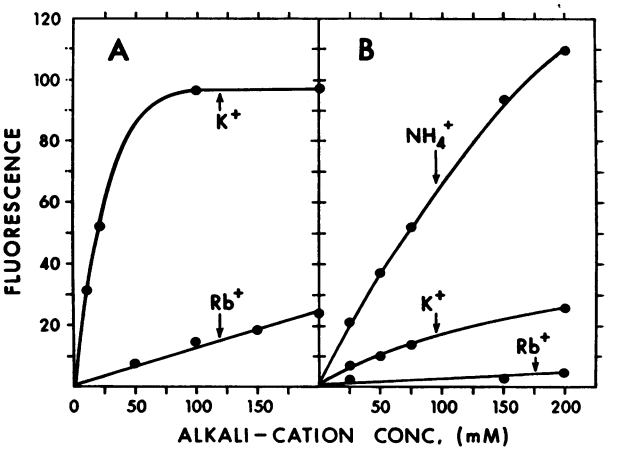

FIG. 6. Fluorescence titrations of nigericin (*A*) and trinactin (*B*) with alkali cations. ANS 42 μM, trinactin 6.4 μM, nigericin 17 μM. Other conditions as in Fig. 1.

than that for valinomycin. The various actins also bound K^+ better than Rb^+. However, as previously found in studies using organic solvents and model membrane systems (26), the actins have the greatest affinity for NH_4^+ (Fig. 6,*B*). With trinactin and ANS, half-maximal fluorescence was obtained at ~0.75 M NH_4^+, and NH_4^+ affinity was about three times that of K^+. The order of affinity of macrotetralide actins for K^+, as measured by this fluorescence method, was; trinactin > dinactin > monactin. This is in accord with the prediction of Eisenman and associates (26) that the principal effect of $-CH_3$ group addition in this series would be on the aqueous dissociation constant of the cation–ionophore complexes.

The fluorescence lifetime for ANS increased from ~0.5 nsec in water to 8.9–9.6 nsec for K^+ or Rb^+ complexes with valinomycin. The quantum yield of fluorescence (ϕ) therefore was calculated to be 0.40–0.44, from the relationship: $\phi = r/r_0$, where r_0, the intrinsic fluorescence lifetime for ANS, has been calculated to be 22 nsec (25, 27). The corrected emission maximum of ANS bound to cation–valinomycin complexes is 460 nm, a value lower than that for ANS in dioxane (29) or acetone. Data for fluorescence of TNS bound to several cation–ionophore complexes, and in various solvents of decreasing polarity (as determined from Kosower's Z-scale, ref.

TABLE 1. *Peak emission wavelength, and wavenumber* (ω), *for TNS in solvents and bound to ionophore complexes*

	Z*	ω_{max} ($cm^{-1} \times 10^{-4}$)	λ_{max} (nm)
Water	94.6	2.00	500
Methanol	83.6	2.24	443
Ethanol	79.6	2.33	429
n-Butyl alcohol	77.7	2.36	423
Dioxane	(75)†	2.42	413
K^+–nigericin	—	2.35	425
K^+–trinactin	—	2.37	422
K^+–valinomycin	—	2.42	413

* Z scale is an empirical measure of solvent polarity (28).

† Z value for pure dioxane, calculated from extrapolation of Kosower's data for 70–90% dioxane. Excitation at 366 nm, bandwidths 4 nm. Spectra are corrected.

28), is shown in Table 1. The spectral shifts observed for both fluorophores when bound to cation–ionophore complexes may result from binding to sites of very low polarity (15, 29), or from restricted solvent molecule relaxation (30) in the region of the binding sites. The fluorescent reactions described may be applicable to the study of the reaction kinetics of cation–antibiotic interactions in water, which utilize rapid reaction or relaxation (31) methods, and to the study of the cation binding reactions of the ionophores in biological and model membrane systems.

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