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Ion Association Reactions with Biological Membranes, Studied with the Fluorescent Dye 1-Anilino-8-naphthalenesulfonate

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Summary. (1) When salts are added to buffered suspensions of membrane fragments containing the fluorochrome 1-anilino-8-naphthalenesulfonate (ANS), there is an increased fluorescence. This is caused by increased binding of the fluorochrome; the intrinsic fluorescence characteristics of the bound dye remain unaltered. These properties make ANS a sensitive and versatile indicator of ion association equilibria with membranes. (2) Alkali metal and alkylammonium cations bind to membranes in a unique manner. Cs^+ binds most strongly to rat brain microsomal material, with the other alkali metals in the order $Cs^+ > Rb^+ > K^+ > Na^+ > Li^+$. The reaction is endothermic and entropy driven. Monovalent cations are displaced by other monovalent cations. Divalent cations and some drugs (e. g., cocaine) displace monovalent cations more strongly. (3) Divalent cations bind to membranes (and to lecithin micelles) at four distinct sites, having apparent association constants between 50 and 0.2 mM^{-1}. The characteristics of the titration suggest that only one species of binding site is present at any one time, and open the possibility that structural transitions of the unassociated coordination sites may be induced by divalent cation binding. Divalent cation binding at the weakest site (like monovalent cation binding) is endothermic and entropy driven. At the next stronger site, the reaction is exothermic. Monovalent cations affect divalent cation binding by reducing the activity coefficient: they do not appear to displace divalent cations from their binding sites.

The fluorescent dye 1-anilino-8-naphthalenesulfonate (ANS) has been used as a probe for the detection of conformational changes in a number of enzymes [3, 8]. ANS has also been implicated as a possible probe of

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conformational change in biological membranes [2, 24]. In all these reports the rationale underlying the description of ANS as a probe of conformationa change is the observation [23, 27] that ANS, which exhibits a very smal fluorescence in aqueous solution, emitting at 520 nm, fluoresces increasingly on raising the mole fraction of nonpolar solvent in water-solvent mixtures The increased fluorescence is accompanied by a shift to shorter-emissior wavelengths and a narrowing of the band width. In proteins and membranes the fluorescence characteristics of bound ANS may be used to monito the polarity of its micro-environment.

At the outset of the present work, we hoped that ANS fluorescence changes might be used to detect conformational changes at the active site of the Na^+-K^+-activated ATPase of rat brain microsomal membranes and of human red cell ghosts. Although our search for the required synergism between Na^+ and K^+ additions and for an effect of ouabain on the fluorescence was negative, it was immediately apparent that ANS fluorescence in these membranes was responsive to the salt concentration which determined the extent of ANS binding, leaving its fluorescence characteristics unchanged. This paper describes how ANS fluorescence may be used as a very simple and sensitive indicator of ion binding by biological membranes and lecithin micelles. Elementary accounts of early work by us [14, 22] and by other workers [13, 20, 26] have been published.

Materials and Methods

Microsomal membranes were prepared from accumulated frozen rat brains. Standard methods were used to sediment dense material from a 1:10 homogenate of brains in 0.25 M sucrose and 10 mM Tris-ethylendiaminetetraacetate (EDTA), pH 7.4. The light membrane suspension was adjusted to pH 6.8 by the addition of acetic acid, and the microsomal material was harvested by centrifugation at $78{,}000 \times g$ (avg) for 30 min (Spinco centrifuge, 30 rotor). The membranes were washed repeatedly according to the following schedule in order to remove bound cations: (1) resuspend in 10 mM Tris-EDTA, pH 7.4, and sediment at $105{,}000 \times g$ (avg); (2) resuspend in water and sediment at $105{,}000 \times g$ (avg); (3) resuspend in water, bring to pH 4 with acetic acid, and sediment at $10{,}000 \times g$; (4) resuspend in water, bring to pH 7.4 with tetraethylammonium hydroxide and sediment at $105{,}000 \times g$ (avg); (5) resuspend in water and sediment at $105{,}000 \times g$ (avg); and (6) resuspend in water at a concentration of approximately 4 mg protein per ml and store frozen in 1-ml lots. The membranes were thawed and homogenized in a Dounce-type hand homogenizer before use.

This procedure produces material having only half the activity of a normal preparation with respect to the K^+-activated phosphatase reaction (Gomperts, *unpublished observation*) and contains less than 0.3×10^{-9} moles of K^+ per mg protein.

Red cell membranes were used in a few early experiments. They were prepared by standard methods [9]. No special precautions were taken to remove bound cations. The membranes were stored at 5 °C and used within a few days.

Lecithin (Applied Science Laboratories, State College, Pa.) from beef brain was used to prepare micelle suspensions. First, 50 mg lipid in organic solvent was taken to dryness under a stream of nitrogen. Then 50 ml of water was added and the mixture shaken briefly. After 30-min standing, the mixture was centrifuged (7,000 $\times g$, 10 min). The supernatant material was stored in the cold and used over a period of about a week.

ANS, ammonium salt (K and K Laboratories, Inc., Plainview, N.Y.) was twice recrystallized as the magnesium salt. It was prepared as a 16 mM solution and treated with activated carbon. Before use, it was passed through a column of Dowex 50 resin in the choline form to remove Mg^{++}. For most titrations with cations, a standard suspension of microsomal membranes (about 0.1 mg protein/ml) in Tris-Cl, 20 mM, pH 7.4, containing ANS, 60 μM was used.

For most fluorescence measurements, a differential fluorometer was used. Two cuvettes, both containing the standard suspension, were placed in a holder which vibrates at 10 cycles/sec, so that each cuvette was alterately in and out of the monochromatic excitation beam. Small volumes of concentrated solutions of the titrated salts were added to one cuvette only. After the system attained equilibrium (about 1 min), the signals from the two cuvettes were compared, giving the fluorescence increment Δf. This fluorometer has been described previously [1, 5]. Certain titrations, in particular the measurements of temperature dependence, were made with a Perkin-Elmer Hitachi MPF-2A instrument.

For pH titrations, fluorescence measurements and pH measurements with a glass electrode were made simultaneously. A suspension of microsomal membranes in 100 μM ANS (unbuffered) was placed in a 5-ml beaker and constantly stirred. Hydrochloric acid or tetraethylammonium hydroxide was added to adjust the pH. Fluorescence measurements were made with a compensated fluorometer. Excitation was from the 366-nm line of a water-cooled 1-kw mercury arc lamp; photomultipliers were provided so that differential measurements could be made between the intensity of lamp emission and fluorescence emission.

Unbound Ca^{++} and Mn^{++} were measured by following the optical density change of the dye murexide at 450 nm in an Aminco-Chance dual-wavelength spectrophotometer. The reference wavelength was 510 nm. Ca^{++} or Mn^{++} was first titrated into a suspension of microsomes in a solution of murexide (16 μM) in 20 mM Tris-Cl, pH 7.4, and optical density changes were recorded. This was followed by a similar titration in which the microsome suspension was omitted in order to provide a calibration of optical density change against free divalent cation concentration. Also, free Mn^{++} was measured by E.P.R. techniques on a Varian V 14 500A spectrometer at 9.5×10^9 cycles/sec. Once again, the instrumental signal was measured as a function of cation concentration in the presence and absence of microsomes. Measurements were made in 20 mM Tris-Cl, pH 7.4.

All titrations were performed at room temperature unless otherwise indicated.

Results

Monovalent Cations

When NaCl is titrated into a buffered suspension of membranes containing ANS, the fluorescence increases according to a hyperbolic function of cation concentration. This is most conveniently illustrated as a double reciprocal plot of fluorescence increment against cation concentration, as in the lowest line in Fig. 1. Since the double reciprocal plot is linear, we

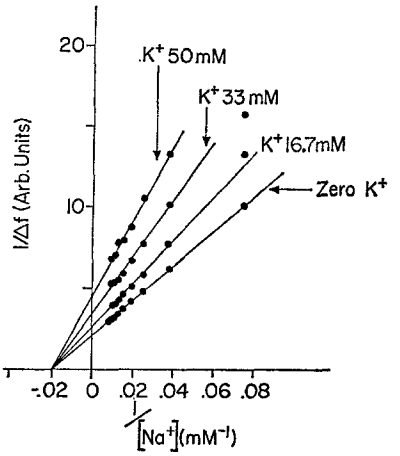


Fig. 1. Graphs of reciprocal fluorescence change vs. reciprocal NaCl concentration. A suspension of rat brain microsomal membranes (about 0.1 mg protein/ml) was used in 20 mM Tris-Cl, pH 7.4, containing ANS (60 μM). The upper three lines show the effect of different fixed concentrations of KCl

conclude that the fluorescence change is due to interaction of the titrated cation with the membranes in a purely statistical manner [16], and it is therefore possible to read the apparent binding constant K' for the salt onto those regions of the membrane which are sensitive to ANS, directly from the negative intercept on the abscissa.

If an alternative salt such as KCl is added to the buffered ANS microsome suspension before the titration, the initial fluorescence is increased, but the fluorescence change during the NaCl titration is less (as in the upper lines of Fig. 1). The double reciprocal plots all converge at the same point on the abscissa, indicating that the association constant for the titrated salt is unaffected by the presence of K^+. We conclude that the ordinate scale of reciprocal fluorescence change is a linear function of the amount of titrated cation bound to the membranes at ANS-sensitive sites and that the intercept of the extrapolated line at this axis is a function of σ^0, the number of ions bound at infinite concentration of titrated cation. From this it is easy to see that the second cation occupies sites on the membrane which would otherwise be available to the titrated cation. Much lower concentrations of divalent cations are needed to displace titrated monovalent cations from the membrane.

ANS Binding

Measurement of ANS bound to microsomes which had been isolated at room temperature from ANS solutions containing known concentrations

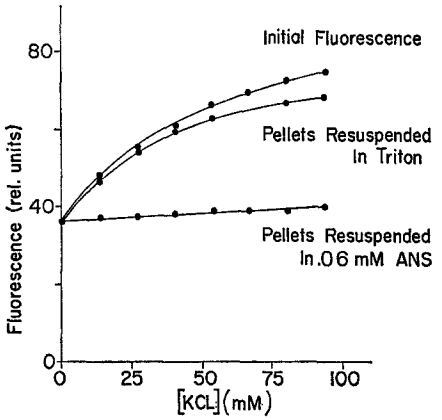


Fig. 2. The effect of salt concentration on the binding of ANS to human red cell ghosts. Fluorescence was first measured in the standard membrane-buffer-ANS mixture at different salt concentrations (top curve). The suspensions were then divided into two parts, and the membranes collected by centrifugation. One set of membranes was homogenized in a solution of Triton x-100 and the fluorescence measured (middle curve). This gives a measure of the amount of ANS bound to the membranes. The other set of membranes was resuspended in buffered ANS solution containing no added salt (lowest line). The experiment shows that fluorescence enhancement is caused by binding of ANS and that this is rerversed when the salt is removed

of salt showed that the fluorescence change during salt titrations is accompanied by increased binding of the dye (Fig. 2). Further, if microsomes isolated from ANS solutions containing different cation concentrations were resuspended in buffered ANS solutions containing no added salt, the fluorescence of all samples was the same, indicating that ANS binding is reversed when the salt concentration is reduced.

By altering the protein concentration only, in solutions containing fixed concentrations of various salts and ANS, it was possible to construct a graph of reciprocal protein concentration vs. reciprocal fluorescence change (Fig. 3). The intercept at the ordinate of the extrapolated line is indicative of the fluorescence of the dye bound to an infinite amount of protein, i.e., under conditions in which all the dye present is bound to the membranes. Since the intercept does not change with altered amounts of salt in the suspension, we conclude that salt concentration has no effect on the quantum efficiency of the bound dye and that the quality of the microenvironment of the bound dye is independent of the solution salt concentration.

When ANS was titrated into membrane suspensions and the salt concentration changed for each titration, we found (Fig. 4) that the association constant of the membrane-dye complex is constant (for rat brain microsomes, $K'_{ANS} = 11 \pm 2\ mM^{-1}$ at 25 °C) and independent of the salt concentration,

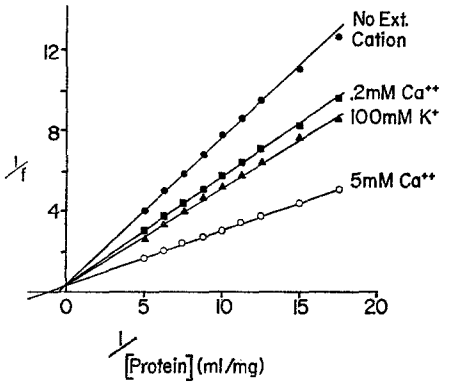


Fig. 3. Graphs of reciprocal fluorescence change vs. reciprocal protein concentration at different concentrations of KCl and $Ca(NO_3)_2$

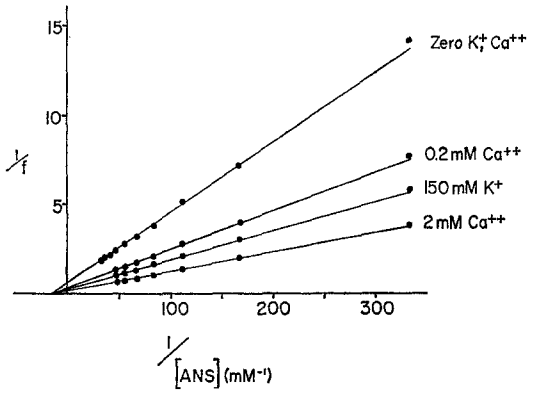


Fig. 4. Graphs of reciprocal fluorescence change vs. reciprocal ANS concentration at different concentrations of KCl and $Ca(NO_3)_2$

but that the amount of dye bound at infinite dye concentration (Δf_{max}) is dependent on salt concentration. We conclude that the fluorescence change during salt titration is solely caused by increased binding of the dye. The effect of adding salt is to create new binding sites for ANS, in agreement with previously reported results (13, 14, 20).

ANS binding increases with increase in H^+ concentration, and the pH titration curve for both microsomes and for red cell ghosts suggests the involvement of a group having a pK of 4.25 at low ionic strength (*see* Fig. 5). A similar titration carried out at higher ionic strength (I=0.15) indicated a pK of 3.2 to 3.5 for red cell ghosts [13]. The decrease of pK with increasing ionic strength is indicative of the ionization of a neutral acid, $AH \rightleftharpoons A^- + H^+$. The behavior of red cell ghosts on electrophoresis has been shown to be caused by the negatively charged

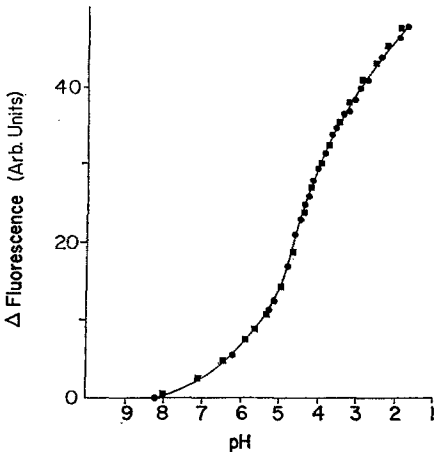


Fig. 5. pH titrations of rat brain microsomes using ANS as an indicator. ▪ microsomes treated with neuraminidase, • untreated microsomes

carboxyl groups of sialic acid [12]. However, treatment of red cell ghosts and microsomes with the enzyme neuraminidase, which cleaves sialic acid, was without effect on either the pH (*cf.* Fig. 5) or the monovalent and the divalent metal salt titrations, using ANS as an indicator.

The experiments described so far allow us to conclude that salt-induced enhancement of ANS fluorescence in our systems is caused by reversible binding of the anionic fluorochrome. In a general way, it may be added that binding of ANS occurs when individual charges or the diffuse charge of the membranes are neutralized by ions present in solution.

Effects of Individual Alkali Metal Ions

The group of alkali metal chlorides together with NH_4Cl were titrated in the standard buffered microsome-ANS mixture (*see* Fig. 6). The lines of the double reciprocal plots are all approximately parallel (i. e., $K' \cdot \Delta f_{max} =$ constant). The ANS binding capacities at infinite cation concentration decrease in the order $NH_4^+ > Li^+ > Na^+ > K^+ > Rb^+ > Cs^+$. At any concentration of monovalent cation, Li^+ enhances the binding of ANS more than Na^+ and so on, in that order, which is held right up to infinite salt concentrations. The association constants increase in this order (*see* Table 1). We may conclude that either there is a systematic variation in the stoichiometry between ANS binding and the binding of various cations, or there is a systematic variation in the limiting occupancy of the membrane by the cations. Either way, it appears that the rank order is dictated by the size of the nonhydrated ion or a related parameter.

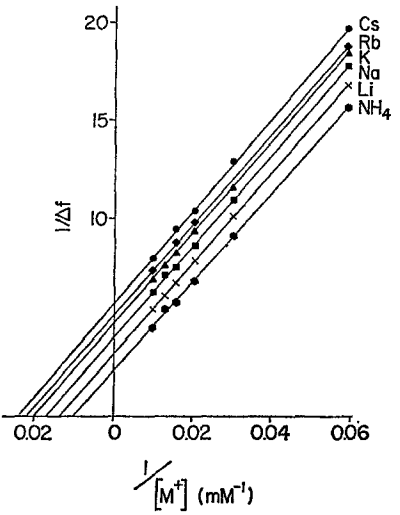


Fig. 6. Graphs of reciprocal fluorescence change vs. reciprocal salt concentration for the series of alkali metal chlorides and NH_4Cl. Measurements made at 13.5 °C

Table 1. *Apparent association constant K′ at different temperatures for monovalent cations* (K mM^{-1})

Cation	K'		
	25 °C	31 °C	38 °C
NH_4^+	0.014	0.017	0.022
Li^+	0.015	0.018	0.024
Na^+	0.020	0.023	0.027
K^+	0.020	0.024	0.028
Rb^+	0.022	0.026	0.029
Cs^+	0.024	0.028	0.030

That some displacement of water molecules from the hydration shells of the alkali metal cations is involved in the process of binding to membranes is suggested by observed sequence of association constants. The association constant for Li^+ is smaller than that of Na^+, indicating that more work is needed to transfer Li^+ from the aqueous phase to the membrane than Na^+. Sequences of the type observed by us are characteristic for synthetic sulfonate ion exchange resins and carboxylic ion exchange resins under conditions in which most of the carboxylate groups are unionized [15, 19] and for glass electrodes of the low field strength type [15]. Of the 5! possible selectivity orders for alkali metal cations, 11 sequences or rank orders have been predicted on the basis of the anionic field strength

of the exchanger phase and variation of the overlapping of fields of neighboring fixed groups [11, *see* also 17]. Of these, the affinity order we observe corresponds to sequence 1, indicating a low anionic field strength. The comparison of biological membranes with synthetic ion exchangers of defined structure offers a possibility of clarifying the structure of membrane ion exchange sites.

Titrations with Alkylammonium Chlorides

The effects of the homologous series of methyl and ethyl ammonium chlorides on ANS binding support the conclusion that ionic size is of importance. Fig. 7 shows the series of double reciprocal plots for the ethyl series. If the parameter $K' \cdot \Delta f_{max}$, which is about the same for all the alkali metal cations, may be taken as characteristic of an alkali metal type interaction with the membrane under the stated conditions, then the methyl and ethyl ammonium ions, for which this parameter is the same, may be said to behave similarly (except the tetraalkyl ammonium ions). By plotting graphs of Δf_{max} for the different alkylammonium compounds as a function of molecular weight, we find that the projected ANS binding at infinite cation concentration varies linearly with the molecular weight of the cation, and therefore crudely with the molecular size. Clearly, with the methyl and ethyl ammonium cations, we are observing a series of cations with a large range of size, which might progressively limit the occupancy of membrane exchanger sites.

The apparent association constants for the methyl and ethyl ammonium cations increase linearly with increasing molecular weight in each series.

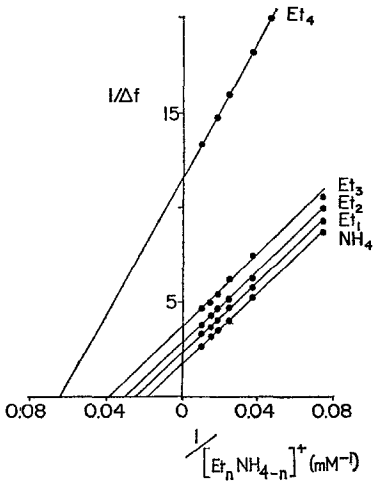


Fig. 7. Graphs of reciprocal fluorescence change vs. reciprocal concentration for the series of ethylammonium chlorides and NH_4Cl

Within each series, addition of a further alkyl substituent produces a constant increment of association constant, an increment which is greater for ethyl than for methyl.

Local Anaesthetics

In view of the recently reported effects of some local anaesthetics on the ANS responses in mitochondria [4], it was of interest to study their effect on ion binding to simpler membranes. A number of the readily available local anaesthetic compounds, such as butacaine, dibucaine and tetracaine, were found to be unsuitable for the present studies because they formed highly fluorescent precipitates with ANS, when added in the concentration required. The studies were therefore restricted to cocaine and procaine, both as hydrochlorides. The results of direct titrations of these substances are shown in Table 2 where the parameters Δf_{max} and $K' \cdot \Delta f_{max}$ are compared with those for K^+. As for the larger alkylammonium compounds,

Table 2. *Binding parameters of local anaesthetics and K^+ with rat brain microsomal membranes*

Substance titrated	K' (mM^{-1})	$\frac{\Delta f_{max}}{\Delta f_{max(K^+)}}$	$\frac{K' \cdot \Delta f_{max}}{K' \cdot \Delta f_{max(K^+)}}$
K^+	0.023	1	1
Procaine	0.05	1.7	3.7
Cocaine	0.08	3.0	10.5

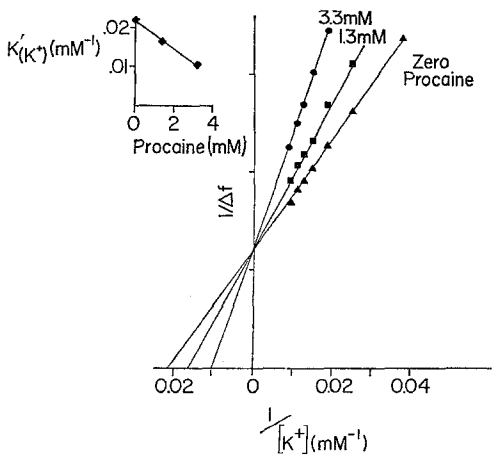


Fig. 8. Graphs of reciprocal fluorescence change vs. reciprocal KCl concentration at different concentrations of procaine. Inset shows linear decrease of $K'_{(K^+)}$ with procaine concentration

Δf_{max} is higher and the association constant higher for the local anaesthetics than for K^+.

In their effects as inhibitors of K^+ binding, cocaine and procaine are very different. Cocaine appears to exclude K^+ from microsomal membranes by a simple displacement mechanism. Procaine, which has two separated charge centers, provides the only instance in this study of a systematic shift in the association constant of a monovalent cation (*see* Fig. 8). Procaine does not appear to displace K^+ from binding sites by direct replacement, and the association constant of K^+ varies inversely with procaine concentration.

Effect of Temperature on ANS-Sensitive Monovalent Cation Binding

For all the monovalent cations, there is a systematic increase in association constant with increase in temperature (*see* Table 1). At the same time, the fluorescence decreases.

We observe that the rank order of constants is maintained throughout the range of temperatures studied, but that the selectivity of the membranes for the various cations decreases with increase of temperature. From the variation of association constants with temperature, we have calculated the thermodynamic constants ΔH^0, ΔG^0 and ΔS^0 for cation binding to microsomal membranes at 25 °C (Table 3). Cation binding to membranes is characterized by a large positive entropy change. This is contrary to the behavior expected of elementary association phenomena, in which the disappearance of particles from the system would be reflected by a decrease of entropy. The binding of monovalent cations is endothermic, and so the reactions may be said to be driven by the very favorable entropy change.

The positive entropy change in cation binding is most probably related to the breakdown of the ordered structure of the coordinated water molecules in the solvated cation hydration shells. A large cation such as Cs^+

Table 3. *Thermodynamic data for membrane monovalent cation association*

Cation	ΔH^0 kcal/mole (25–38 °C)	ΔG^0 kcal/mole (25 °C)	ΔS^0 eu (25–38 °C)
NH_4^+	6.0	−1.56	25
Li^+	5.5	−1.62	24
Na^+	3.9	−1.77	19
K^+	4.0	−1.78	19
Rb^+	3.4	−1.82	16
Cs^+	2.5	−1.89	15

which has a comparatively high aqueous entropy value, and which is not so extensively hydrated, produces a smaller entropy change than the smaller cations.

The endothermic character of the reaction is most probably caused by the large aqueous solvation energies of the cations. ΔH decreases with crystal radius. The amount of dye bound for small cations is greater than for large ones, but the more extensive modification of the hydration shell has the effect of lowering the association constants. These contrary trends may serve as a qualitative explanation of the apparent constancy of the parameter $K'\Delta f_{max}$ among the alkali metal and smaller alkylammonium cations.

Divalent Cations

In addition to conducting titrations of ANS response with divalent cations, we attempted to determine their binding characteristics with microsomal membranes by direct measurement of free cation concentration in solution. This was done with the dye murexide for both Ca^{++} and Mn^{++}, and by electron spin resonance techniques for Mn^{++}. Both these techniques provide signals proportional to the amount of free divalent cation in solution. The amount of bound divalent cation could then be deduced from knowledge of the total amount added to the system. From the data obtained, Scatchard plots [21] were constructed (*see* Fig. 9). For both Ca^{++} and Mn^{++}, it

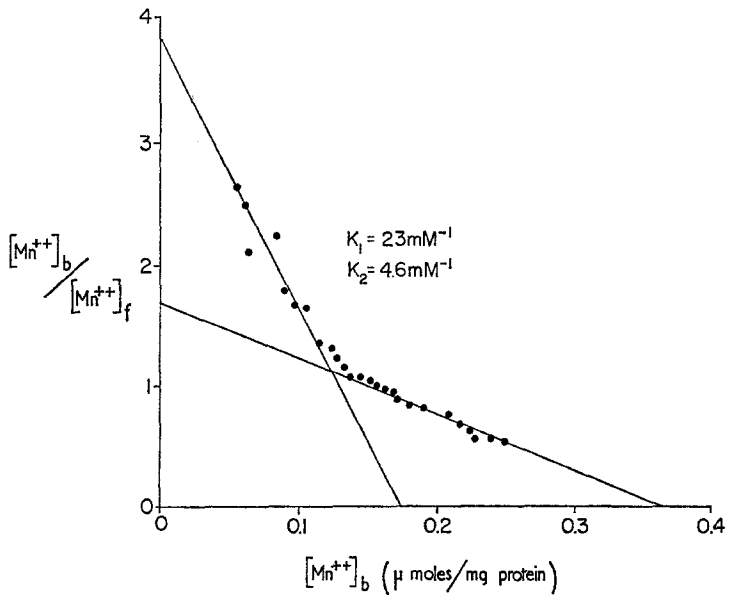


Fig. 9. Scatchard plot for $MnCl_2$ binding to rat brain microsomal membranes, measured with murexide indicator

was apparent that a number of distinct binding "sites" could be detected, whereas only one site for monovalent cations was identified. The electron spin resonance technique is more sensitive at high concentrations and leads to the tentative identification of four different site species (though the two weakest binding sites were only detectable with difficulty, using high concentrations of microsomes to amplify the vanishingly small difference between total and free Mn^{++}). Direct determination of free cation enabled us to construct tables of free cation concentration in the presence of any known amount of protein after the addition of any (total) amount of cation. These tables could then be used to correct the reciprocal cation concentration scales in the ANS titrations which become erroneous at very low concentrations, when it is assumed that the free cation concentration is equal to the total amount added. (Such assumptions were tenable for monovalent cations and for the higher concentrations of divalent cations.) Further, the Scatchard plots extrapolated to infinite cation concentration $\left(\text{i. e., } \frac{M^{++}\text{bound}}{M^{++}\text{free}}=0\right)$ enabled us to derive the amount of each species of binding site per mg protein.

Titrations of Mn^{++} and Ca^{++} in the presence of ANS confirmed the existence of four species of binding sites (Fig. 10). The parameters of the four binding sites as determined by the three techniques are shown in Table 4 for Mn^{++} and Ca^{++}. It will be seen that the constants for ion association agree rather well. We always observed a very abrupt transition from site to site on the double reciprocal presentation of ANS fluorescence.

Comparison of the extrapolated specific fluorescence enhancements for the individual sites (i. e., the amount of ANS bound due to the saturation of that site, noting that the quantum yield remains constant) with the information on cation binding from Mn^{++} titrations by the EPR or murexide methods shows that the stoichiometry varies from site to site qualitatively as shown in Table 5.

A series of titrations with alkaline earth nitrates was made, using the concentration range of site 3. As in the case of the monovalent cations, the lines are again nearly parallel (except for Be^{++}), with the product $K' \cdot \Delta f_{max}$ again about constant but different from that of the monovalent cations (*see* Table 6).

The behavior of Be^{++} is clearly different from the other divalent alkaline earth cations and Be^{++} titrations were complicated by the fact that the microsomal suspension agglutinates at low concentrations (at 1 mM Be^{++}, the microsome suspension is converted to a few large clumps of material). A mixed-rank order for the other four alkaline earth cations was observed

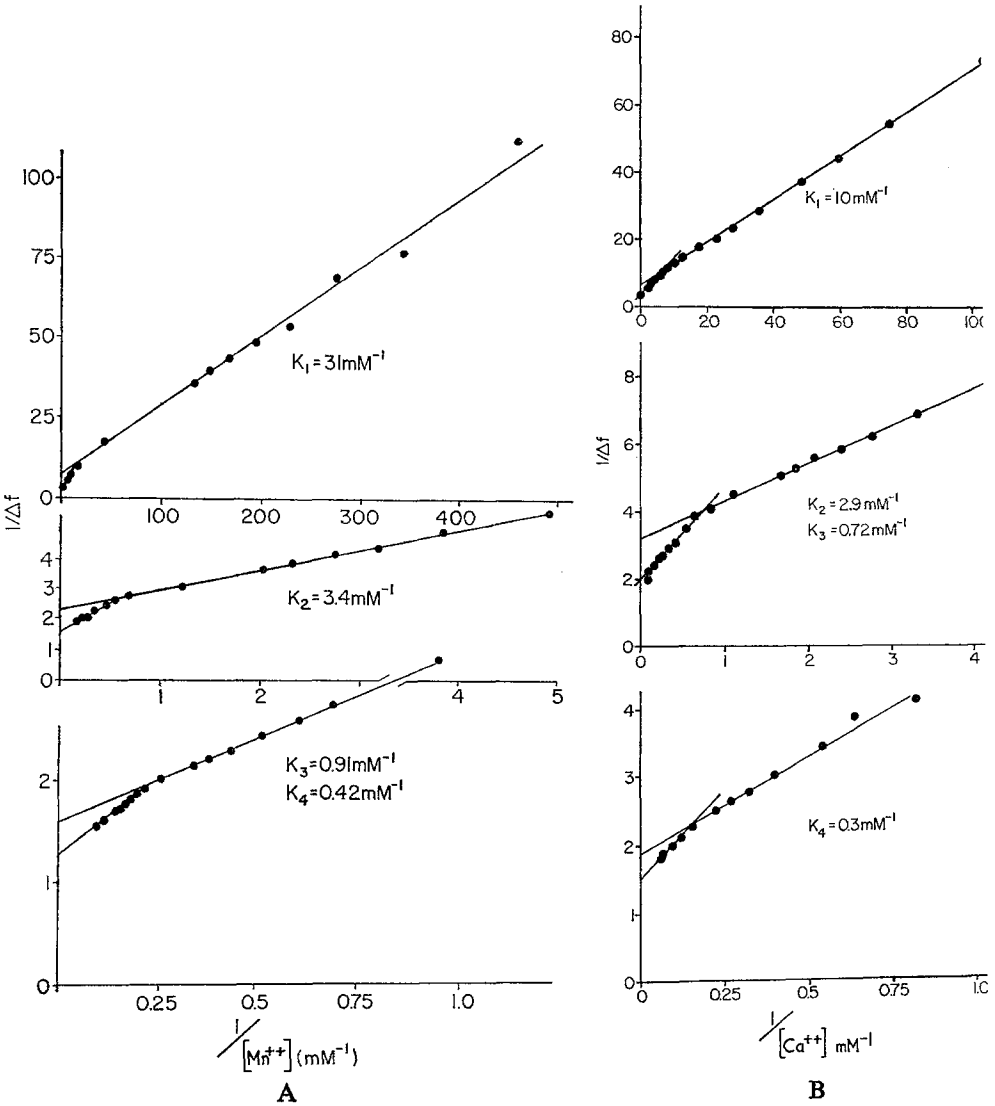


Fig. 10. Graphs of reciprocal fluorescence change vs. reciprocal concentration of fre $MnCl_2$ (A) and free $CaCl_2$ (B), over a concentration range of about three decades. Th cation concentration scales (in the low concentration regions) have been corrected usin data from absolute measurements using solvent phase indicators

(at site 3) with Mg^{++}, Sr^{++} and Ba^{++} bound more strongly than Ca^{++} The binding parameters of Mg^{++}, Sr^{++} and Ba^{++} were very close to on another. Studies on the binding characteristics of divalent cations on syn thetic ion exchange materials are much less advanced than those wit monovalent cations [19], but it has been shown that rank orders are dictate

Table 4. *Binding data for $MnCl_2$ and $Ca(NO_3)_2$ with rat brain microsomal membranes. Comparison of different techniques*

Cation	Site	EPR		Murexide		ANS		
		K'(mM^{-1})	σ^0(nmoles/mg protein)	K'(mM^{-1})	σ^0(nmoles/mg protein)	K'(mM^{-1})	$\Delta f'_{max}$ (arbitrary units at 60 μM ANS)	nmoles ANS/mg protein estimated at infinite concentration of ANS
Mn^{++}	1	23	170	23	170	31	130±30	8
	2	4.6	190	5.2	160	3.4	310±50	19
	3	–	–	–	–	0.9	185±30	11
	4	–	–	–	–	0.42	145±50	9
Ca^{++}	1	–	–	12.5	80	10	160±50	10
	2	–	–	2.5	80	2.9	160±50	10
	3	–	–	–	–	0.71	180±50	11
	4	–	–	–	–	0.3	140±50	8.5

by considerations similar to those which are pertinent for monovalent cations. On this basis, 7 rank orders have been predicted out of the 24 possible arrangements of the 4 alkaline earth cations [25], and the order we observe for microsomal-alkaline earth cation interaction corresponds approximately to rank order V.

Table 5. *Relative number of bound Mn^{++} ions leading to the same fluorescence incremen*

Site no.	1	2	3	4
Relative no. of ions / Fluorescence increment	3	2	5	6

Table 6. "*Site* 3" *binding parameters of alkaline earth cations compared with* K^+

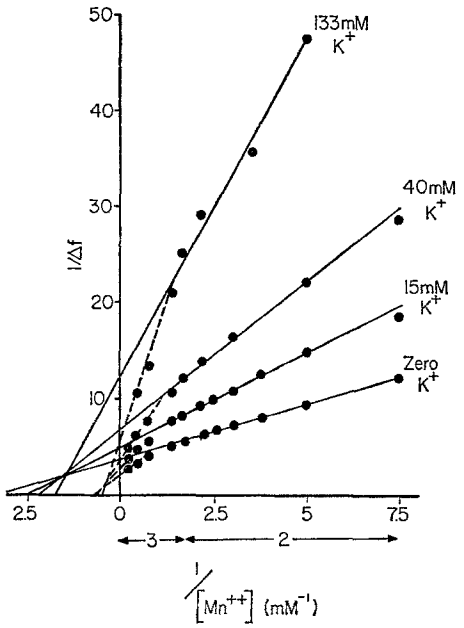
Parameter	Cation					
	K^+	Be^{++}	Mg^{++}	Ca^{++}	Sr^{++}	Ba^{++}
$K'(\text{mM}^{-1})$ (at site 3)	0.017	0.05	0.52	0.46	0.52	0.52
$\Delta f_{max}/\Delta f_{max}(K^+)$	1.0	1.36	1.42	1.76	1.42	1.42
$\frac{K' \cdot \Delta f_{max}}{K' \cdot \Delta f_{max}(K^+)}$	1.0	4.0	42.0	47.0	42.0	42.0

Competition Between Monovalent and Divalent Cations

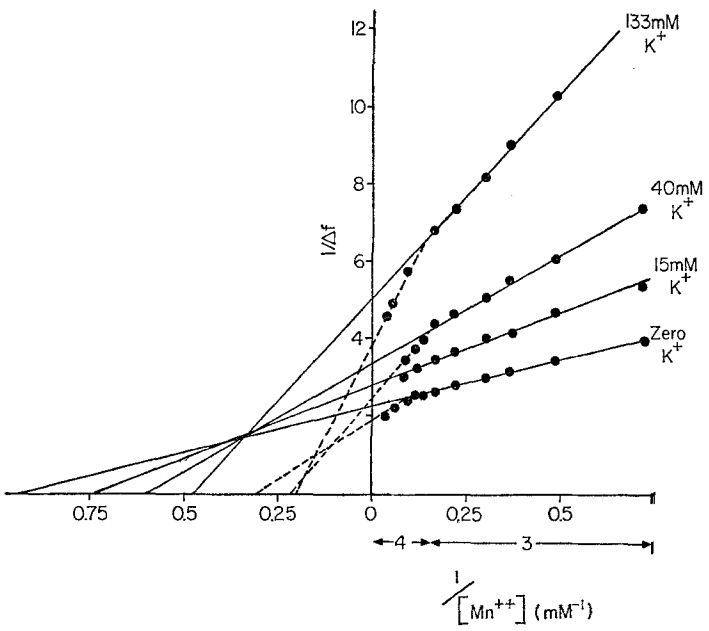
Competition studies with monovalent and divalent cations were made using K^+ and Mn^{++} as alternative representative cations. Qualitatively, the displacement of monovalent cations from binding sites by divalent cations is similar to the displacement by alternative monovalent cations, (*see* Fig. 1) except that only about 0.7 mM $MnCl_2$ is needed to halve Δf_{max} in a K^+ titration, compared with about 45 mM of an alternative monovalent cation: K'_{M^+} remains constant. When divalent cations are titrated in the presence of different fixed concentrations of monovalent cation, we observe a systematic decrease of both Δf_{max} and K' (*see* Fig. 11). This behavior is characteristic of the first three Mn^{++} binding sites, and was observed regularly with the other divalent cations. In comparing the effect of monovalent cations on the series of divalent cation binding sites, we find that Δf_{max} is reduced to about 30% of its normal site 1 value by 133 mM KCl, whereas the site 4 value is not significantly altered. The effect of KCl on Δf_{max} for sites 2 and 3 is roughly intermediate between these extremes.

Effect of Temperature on Divalent Cation Binding. Thermodynamic Characterization of Sites 3 and 4

We studied the effect of temperature on the two weakest binding sites (sites 3 and 4) for Ca^{++} and Mn^{++}. For both cations, the value of K'_4 increases with temperature in a similar manner to the monovalent cations.



A



B

Fig. 11. Graphs of reciprocal fluorescence change vs. reciprocal concentration of free $MnCl_2$ over the ranges of sites 2 and 3 (A), and sites 3 and 4 (B) at different concentrations of KCl

Table 7. *Thermodynamic data for membrane divalent cation association reactions*

Cation	Site	ΔH^0 kcal/mole (25–38 °C)	ΔG^0 kcal/mole (25 °C)	ΔS^0 eu (25–38 °C)
Ca^{++}	3	−2.3	−4.1	6
	4	+2.6	−3.4	20
Mn^{++}	3	−3.1	−4.3	4
	4	+1.5	−3.6	17

The value of K_3' decreases. The values of ΔH^0, ΔG^0 and ΔS^0 for sites 3 and 4 were calculated by standard procedures from the association constants. The values of these parameters are shown in Table 7. The thermodynamic parameters for site 4 binding, with ΔH^0 small and positive and ΔS^0 large and positive, resemble those for monovalent cations, although the values of ΔH^0 are smaller than for monovalent cations.

Site 4 is the weakest and last occupied of the binding sites, and the general similarity of the thermodynamic description of site 4 binding and that of monovalent cations suggests that site 4 binding is probably best described as a Coulombic interaction between single isolated fixed-point charges and the divalent cations.

The interaction between membrane and divalent cations is stronger at site 3 than at site 4, and we observe negative values of ΔH^0, an exothermic reaction. The values of ΔS^0 are less at site 3 than at site 4, and this is probably indicative of a more organized association between membrane and cations since the process of removing cations from solution will be the same over the entire concentration range.

Cation and ANS Binding Sites

We attempted to identify the nature of the anionic sites responsible for the ANS-sensitive binding of divalent cations. Extraction of microsomes with acetone or with chloroform-methanol (3:1) resulted in total abolition of the ANS response on titration with cations, but boiling for 5 min or treatment with urea (5 M) had only a limited effect in this respect. A suspension of lecithin micelles (about 0.1 mg lipid/ml) in buffered ANS solution was titrated with Mn^{++} and found to have four binding sites with the following association constants: $K_1' = 21.7\ mM^{-1}$, $K_2' = 4.0\ mM^{-1}$, $K_3' = 0.74\ mM^{-1}$, K_4' present but not determined. As with microsomal membranes, the titrations of lecithin with Mn^{++} show sharp transitions between the sites. Because of the rather close identity of the constants obtained

for the pure lipid suspension and for biological material, we conclude that the binding of cations at ANS-responsive sites is to the polar groups of phospholipid.

It is relevant to remark that the relative quantum efficiency of bound ANS remains constant at all four Mn^{++} binding sites. Similarly, the degree of depolarization of a plane-polarized excitation beam remains constant at $p = 0.1 \pm 0.02$. Whatever the nature of the Mn^{++} binding site, the environment of the bound ANS is the same, insofar as this is reflected by its fluorescence properties.

Titrations with Cations of Higher Valency

Titrations of microsomal suspensions in buffered solutions of ANS were made with La^{3+} and Th^{4+}. The characteristics of the interactions of these ions with the membranes are qualitatively quite different from those of the monovalent and divalent cations. The maximum fluorescence at high La^{3+} concentration is two times higher than for divalent cations, and with Th^{4+} it is higher still. This is probably because individual membranes achieve a net positive charge as they approach saturation with polyvalent cations. The titrations are complicated by the tendency of the microsomes to agglutinate in the presence of about 0.2 mM La^{3+} and 0.02 mM Th^{4+}. This may be caused by the formation of a population of oppositely charged regions on the membranes. We observed, for both La^{3+} and Th^{4+}, a sigmoidal response of ANS fluorescence with total cation concentration. This is illustrated in Fig. 12 for La^{3+}. No attempt was made to identify the various binding sites. By titrating ANS in the presence of different fixed concentrations of La^{3+}, we were able to show that the sigmoidal response arises from an increase in the affinity of the membranes for the fluorochrome in the presence of La^{3+} (*see* Fig. 13). This is quite at variance with our observations for mono- and divalent cations, as described earlier. The quantum yield remains unchanged.

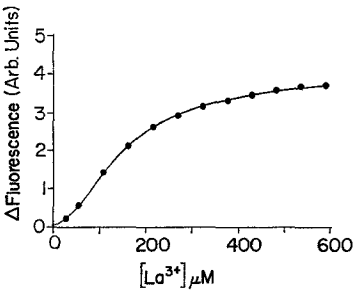


Fig. 12. Graph of fluorescence change vs. concentration of $LaCl_3$

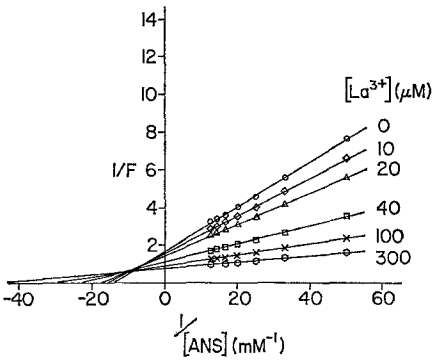


Fig. 13. Graph of reciprocal fluorescence change vs. reciprocal ANS concentration at different concentrations of $LaCl_3$

Discussion

A Model for Competition between Monovalent and Divalent Cations

It will be noted that the energy differences between the binding of solvated divalent cations at the different apparent sites on biological membranes are small. In such circumstances, special care must be taken in the determination of the parameters of the association phenomena [7]. We offer some general remarks about the significance of our observations especially in terms of competition between monovalent and divalent cations, and ask, in particular, the question, "What is the fluorescence change Δf when a given amount of ion B is added to a suspension of microsomes in buffered ANS solution containing a fixed concentration, C_A, of ion A?"

Consider two ions, A and B, that bind to a membrane. Our experiments suggest that there may be an interaction between the total number σ_A of available sites for A, and the number of B ions bound:

$$[E] \cdot \sigma_A = [E] \cdot \sigma_A^0 - p_A \cdot [C_B]_b \tag{1}$$

and similarly for σ_B:

$$[E] \cdot \sigma_B = [E] \cdot \sigma_B^0 - p_B \cdot [C_A]_b \tag{2}$$

where σ_A^0 and σ_B^0 represent the total number of available sites for the individual ions when the other ion is absent from the system; $[C_A]_b$ and $[C_B]_b$ represent the concentrations of the bound cation A and B; p_A and p_B are proportionality constants of the interaction; $[E]$ is the concentration of membrane particles.

If the binding of one ion of A or B leads to a fluorescence increment Q_A or Q_B, then the fluorescence increment Δf_B in a titration of B in the

presence of a fixed amount of A is

$$\Delta f_B=(Q_B\cdot\sigma_B\cdot x_B+Q_A\cdot\sigma_A\cdot x_A-Q_A\cdot\sigma_A^0\cdot x_A) \tag{3}$$

where

$$x_A=\frac{1}{1+\dfrac{1}{K_A\cdot\gamma_A\cdot[C_A]}} \quad \text{and} \quad x_B=\frac{1}{1+\dfrac{1}{K_B\cdot\gamma_B\cdot[C_B]}};$$

γ_A and γ_B represent activity coefficients; K_A and K_B represent binding constants.

Expressed statistically, x_A and x_B represent the average occupation of binding sites by cations A and B (i. e., of binding sites not occupied by the alternative cation).

$$x_A=\frac{[C_A]_b}{[E]\cdot\sigma_A} \quad \text{and} \quad x_B=\frac{[C_B]_b}{[E]\cdot\sigma_B}.$$

Combining these relationships with Eqs. (1) and (2), we obtain:

$$[C_A]_b=\frac{[E]\cdot x_A\cdot(\sigma_A^0-p_A\cdot\sigma_B^0\cdot x_B)}{1-p_A\cdot p_B\cdot x_A\cdot x_B} \tag{4}$$

and

$$[C_B]_b=\frac{[E]\cdot x_B\cdot(\sigma_B^0-p_B\cdot\sigma_A^0\cdot x_A)}{1-p_A\cdot p_B\cdot x_A\cdot x_B}.$$

We now rewrite Eq. (3)

$$\Delta f_B=[E]\frac{(Q_B-Q_A\cdot p_A\cdot x_A)(\sigma_B^0-p_B\cdot\sigma_A^0\cdot x_A)}{\dfrac{1}{x_B}-p_A\cdot p_B\cdot x_A},$$

and by replacing x_B from its definition, we obtain

$$\Delta f_B=[E]\frac{(Q_B-Q_A\cdot p_A\cdot x_A)(\sigma_B^0-p_B\cdot\sigma_A^0\cdot x_A)(1-p_A\cdot p_B\cdot x_A)^{-1}}{1+\{(1-p_A\cdot p_B\cdot x_A)K_B\cdot\gamma_B\cdot[C_B]\}^{-1}}. \tag{5}$$

Note that in our example x_A is constant.

In Eq. (5), the numerator represents the apparent limiting fluorescence, $\Delta f_{\max_B}$, in a B titration; we see that if the concentration of A is zero, i. e., $x_A\to 0$, the limiting fluorescence expression reduces to $Q_B\cdot\sigma_B^0$. The apparent association constant is $K_B'=(1-p_Ap_Bx_A)\gamma_BK_B$.

We may now apply this general Eq. (5) to the specific case of competition between K^+ and Mn^{++}.

(1) Titration of K^+ in the presence of a fixed concentration of Mn^{++} (notation: $A=Mn^{++}$, $B=K^+$). The apparent association constant of K^+ is not affected by the presence of Mn^{++}; there is only a diminution of the total number of available sites. The constancy of the association is well explained by the observation that monovalent cations bind weakly and cannot therefore interact strongly with the Mn^{++} sites available so that $p_{Mn^{++}} \to 0$ in Eq. (1).

We have $K'_{K^+} = \gamma_{K^+} K_{K^+}$.

In the concentration range of the titration (12 to 120 mM), γ_{K^+} varies by less than 10%, and it is not possible to detect the influence of such a variation on the observed association constant K'_{K^+}, which remains constant at all concentrations of divalent cation. By putting $p_{Mn^{++}} = 0$ in Eq. (5), we obtain

$$\Delta f_{K^+} = \frac{[E] \cdot Q_{K^+} (\sigma^0_{K^+} - p_{K^+} \sigma^0_{Mn^{++}} \cdot x_{Mn^{++}})}{1 + \frac{1}{K'_{K^+}[C_{K^+}]}}. \tag{6}$$

The total number of sites available to monovalent cations decreases with increase in the concentration of Mn^{++} following Eq. (2).

$$[E] \cdot \sigma_{K^+} = [E] \cdot \sigma^0_{K^+} - p_{K^+} [C_{Mn^{++}}]_b .$$

Assuming as before that the Mn^{++} binding is unaffected by the presence of monovalent cations ($p_{Mn^{++}} = 0$), so that all Mn^{++} sites are available for Mn^{++}, we may put $\sigma_{Mn^{++}} = \sigma^0_{Mn^{++}}$ in Eq. (2):

$$[E] \cdot \sigma_{K^+} = [E] \cdot \sigma^0_{K^+} - p_{K^+} \cdot \sigma^0_{Mn^{++}} \cdot x_{Mn^{++}} .$$

This agrees with the numerator of Eq. (6) and offers an explanation of the decrease of sites available to monovalent cations when divalent cations are present.

(2) Titration of Mn^{++} in the presence of a fixed concentration of K^+ (notation: $A=K^+$, $B=Mn^{++}$).

Eq. (5) gives (with the condition $p_{Mn^{++}} = 0$)

$$\Delta f_{Mn^{++}} = \frac{[E] \cdot (Q_{Mn^{++}} - Q_{K^+} \cdot p_{K^+} \cdot x_{K^+}) \sigma^0_{Mn^{++}}}{1 + \frac{1}{K_{Mn^{++}} \cdot \gamma_{Mn^{++}} \cdot [C_{Mn^{++}}]}} \tag{7}$$

We may now ask, if monovalent cations have no effect at all on the binding of divalent cations why do we observe an apparent decrease in

the total number of sites available to divalent cations when monovalent cations are present? The answer to this is to be found in the numerator of the expression for $\Delta f_{Mn^{++}}$ above, which contains a term in x_{K^+}, the average occupation of monovalent cation binding sites. During the titration of divalent cations in the presence of monovalent cations, there will be a decrease of the contribution of the monovalent cation to the fluorescence because $[C_{K^+}]_b$ will decrease according to Eq. (4)

$$[C_{K^+}]_b = [E] \cdot x_{K^+} (\sigma^0_{K^+} - p_{K^+} \cdot \sigma_{Mn^{++}} \cdot x_{Mn^{++}})$$

in accord with the observations.

Note that the effect of monovalent cations is greater on the ANS fluorescence due to divalent cations at the strongest binding sites.

From Eq. (7) we get the inequality:

$$\left(1 - \frac{p_{K^+}}{Q_{Mn^{++}}} \cdot Q_{K^+} \cdot x_{K^+}\right)_1 < \left(1 - \frac{p_{K^+}}{Q_{Mn^{++}}} \cdot Q_{K^+} \cdot x_{K^+}\right)_2 < \left(1 - \frac{p_{K^+}}{Q_{Mn^{++}}} \cdot Q_{K^+} \cdot x_{K^+}\right)_3 < \left(1 - \frac{p_{K^+}}{Q_{Mn^{++}}} \cdot Q_{K^+} \cdot x_{K^+}\right)_4 .$$

Q_{K^+} and x_{K^+} are constant for the different divalent cation binding sites at constant concentration of monovalent cation, so

$$\left(\frac{p_{K^+}}{Q_{Mn^{++}}}\right)_1 > \left(\frac{p_{K^+}}{Q_{Mn^{++}}}\right)_2 > \left(\frac{p_{K^+}}{Q_{Mn^{++}}}\right)_3 > \left(\frac{p_{K^+}}{Q_{Mn^{++}}}\right)_4 .$$

Earlier we found that $(Q_{Mn^{++}})_1 \simeq (Q_{Mn^{++}})_2 > (Q_{Mn^{++}})_3 > (Q_{Mn^{++}})_4$ (*see* Table 5), and thus $(p_{K^+})_1 > (p_{K^+})_2 > (p_{K^+})_3 > (p_{K^+})_4$. This means that when Mn^{++} is bound at the tighter binding sites, there is a considerable interaction with the simple charged sites of the membrane resulting in extensive ejection of K^+. The tight binding and the strong displacement of monovalent cations when Mn^{++} is bound to the membranes is in agreement with the proposal [18] that stable coordination complexes may be formed with carbonyl and amino groups and phosphate.

When divalent cations are titrated in the presence of monovalent cations, there is a great change in the ionic strength between the individual titrations, because of the high concentration of monovalent cation required to produce significant changes in the divalent cation ANS response. As a result, the activity coefficient of the divalent cation will decrease [6], and this probably explains the change in the observed association constant, $K'_{Mn^{++}}$ (N.B. $K'_{Mn^{++}} = \gamma_{Mn^{++}} \cdot K_{Mn^{++}}$), of the tight binding sites of Mn^{++}. For the weaker sites, the

changes in the observed association constants are greater than these considerations would predict. The continuing shift of the association constants of the weaker binding sites on addition of monovalent cations may be explained by the formation of chloride manganese complexes $MnCl_n$. At strong sites, the difference between the energy of stripping of chloride or aquo groups from the Mn^{++} ion is probably very small compared with the energy released on binding the ion to the site. At weak sites, only the aquo cation contributes to the ion exchange process, and so the Cl^- ion concentration affects the concentration of Mn^{++} available to the ion exchange process.

The Concept of Multiple Binding Sites for Divalent Cations

Following our observations that (1) monovalent cations bind weakly, and in a unique manner to membranes (over the range 10^{-4} to 10^{-1} M), (2) divalent cations bind strongly and at a number of apparent "sites", and (3) the binding constants of divalent cations to biological membranes and to lecithin micelles are similar, we wish to consider the concept of the term "sites" as applied to the binding of divalent cations to membranes. There are a number of possibilities. (1) The polar groups are organized into discrete arrangements of four types at which divalent cations can bind. We observe the successive occupation of all four types. (2) Before divalent cations are added, the polar groups on the surface of the membrane or micelle are all indistinguishable from one another. The first divalent cations added take up the most stable configuration possible. The point comes (site 1 saturation) when, because of the occupation of polar groupings in a site 1 configuration, succeeding occupation has to be in a less favorable configuration. (3) Once again as in (2), the polar groups are initially indistinguishable. The point is reached when the addition of further Mn^{++} causes a transformation in the arrangement of the polar groups of the membrane. The divalent cation association reaction thereafter is less favorable. The third concept is favored by the observation that the transition between sites for Mn^{++} (and also for other divalent cations) is of a very abrupt nature. Addition of divalent cations to different species of preexisting sites (1), or in different configurations in a single ordered arrangement (2) should lead to a continuous curve of the form

$$\Delta f = \sum_{i=1}^{i=4} \left(\frac{\Delta f_{\max_i}}{1 + \frac{1}{K_i'[C]}} \right).$$

The abrupt transitions lead us to suspect that only one species of unoccupied "site" is present on the membrane at any single time during the titration. Whatever the nature of the transition may be, it is not detectable in terms of the fluorescence characteristics of the ANS.

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