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Detection of ionophore–cation complexes on phospholipid membranes

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SUMMARY

Cation complexation by valinomycin-type ionophores on dimyristoyl-α-lecithin and dipalmitoyl-α-lecithin membranes has been demonstrated. The complexation reaction is measured by the enhancement of the fluorescence of the anionic hydrophobic fluorescent probe, 1-anilino-8-naphthylsulfonate upon association with the positively charged ionophore–cation complexes. The behavior of the ionophores was dependent upon whether the experiment was carried out above or below the crystalline–liquid crystalline phase transition temperature (T_t) characteristic for the phospholipid membrane. For the condition $T < T_t$ cation complexation was observed for the ionophores valinomycin, monactin, enniatin B and 18-crown-6. For the condition $T > T_t$, complexation could be demonstrated only for the case of enniatin B. The dependence of these effects upon cation, cation concentration, lipid, ionophore and temperature are discussed, and the results are interpreted in terms of the mobile carrier theory of ionophore action.

The mobile carrier theory of action for valinomycin and related ionophores[1–3] has received support from studies of monovalent cation transport facilitated by these agents in model and biological membranes[2,4,5]. It has been suggested that the mechanism of action involves the formation of a cation–ionophore complex on the membrane surface[2] and that this complex can transform into a lipophilic complex in which the hydration shell of the cation is completely replaced by the oxygen atoms of the ionophore.

For the case of valinomycin and the other ionophores with large ring diameters[6], the interface complex could be considered to be an association between partially hydrated cations and the ionophore in a conformation in which the ring is partially folded (*cf.* ref. 7). For enniatin B and the other ionophores the interface complex could either be similar to the lipophilic complex or could be a dissimilar complex with partially hydrated cations. Indirect evidence for the interface complex has been found in studies of ionophore-induced cation transport in rat liver mitochondria[8].

Abbreviation: ANS^-, 1-anilino-8-naphthylsulfonic acid.

In the present study, ionophore–cation complexes have been detected in model membrane systems, using 1-anilino-8-naphthylsulfonic acid (ANS^-) as a fluorescent indicator for the charged complex *in* or *on* the membrane. It is quite generally accepted that the quantum yield of ANS^- bound to proteins[9] and membranes[10] gives a measure of the non-polarity of these binding sites. The quantum yield of ANS^- bound to dipalmitoyl-α-lecithin vesicles indicates that the probe is bound on the surface of the membrane[10].

Two types of model membrane systems have been used in the present study: (1) monolayer vesicles consisting of approx. 500 Å diameter spheres of organic solvent (dibutyl ether, chloroform) covered with a monolayer of phospholipid[11] and (2) suspensions of sonicated lipid ("bilayer vesicles" here). These two preparations have similar dye- and cation-binding properties. Dimyristoyl- and dipalmitoyl-α-lecithin vesicles have been shown to undergo phase transitions[12,10] at characteristic temperatures, T_t, (23° and 37°, respectively)[10]. For the condition $T > T_t$, the ANS^- and cation binding is increased, most probably as the result of a general loosening of the membrane structure associated with the formation of "kinks" in the otherwise straight hydrocarbon chains of the lipid molecules[13]. As is shown below, the ionophore–membrane interaction is influenced by this transition.

Monovalent cations serve to increase the extent of binding of the negatively charged ANS^- to model membranes[10]. This effect is enhanced in the presence of valinomycin and other ionophores for the condition $T < T_t$, as is shown in Fig. 1. The enhancement effect plotted as Curve 3, increases with increasing K^+ concentration, saturating at high concentrations. For dimyristoyl monolayer vesicles, the maximal value of this effect is proportional to the valinomycin concentration ($5 \cdot 10^{-6}$ to $2.5 \cdot 10^{-5}$ M),

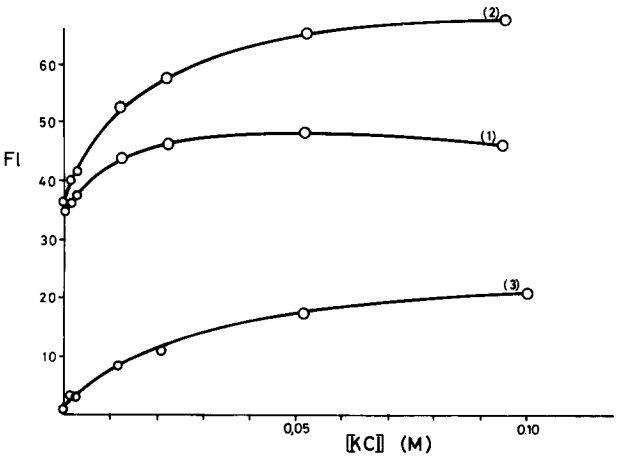

Fig. 1. Enhancement of membrane-bound ANS^- fluorescence by valinomycin, $T < T_t$. Fluorescent measurements were made on an Aminco Bowman Spectrophotofluorimeter with excitation and emission set at 375 nm and 470 nm, respectively. Curve 1, the KCl concentration dependence of ANS^- ($3 \cdot 10^{-5}$ M) fluorescence with monolayer vesicles[10] from dimyristoyl-α-lecithin (Koch Light Laboratories) (lipid concentration, $3.4 \cdot 10^{-4}$ M) in the presence of 1.0 mM Tris chloride, pH 7.0, at 15°; Curve 2, the above in the presence of $5 \cdot 10^{-6}$ M valinomycin, added from a stock solution, $5 \cdot 10^{-4}$ M in methanol; Curve 3, the enhancement effect, taken as the difference between Curves 2 and 1. The uncorrected emission maxima for ANS^- fluorescence in the absence and presence of valinomycin are 480 and 475 nm, respectively.

and is independent of both the lipid concentration ($1 \cdot 10^{-4}$ to $7 \cdot 10^{-4}$ M)* and the ANS^- concentration ($1.0 \cdot 10^{-5}$ to $1.0 \cdot 10^{-4}$ M). The effect is independent of the ratio of lipid to valinomycin for mole ratios of greater than 15. These observations are in agreement with the assumption of a 1:1 stoichiometry between a valinomycin–K^+ complex and additional ANS^- bound to the membrane. This stoichiometry would be expected for an association of the two oppositely charged species *in* or *on* the membrane. As is shown below, the order of cation specificity of this effect is $Rb^+ \geqslant K^+ > Cs^+ > Na^+$, as compared with the order $Na^+ \geqslant K^+ \geqslant Rb^+ \geqslant Cs^+$ for ANS^- fluorescence enhancement in these membrane systems in absence of ionophores[8,10]. This can be taken as further evidence that a direct cation–ionophore complex is involved.

The ratio of the enhancement value to valinomycin concentration in Fig. 1 corresponds to a quantum yield of approx. 0.05 for the ANS^- species associated with the complex, assuming that all of the ionophore is converted to the complexed form at high K^+ concentrations. If a close association is assumed between the complex and the ANS^-, then this low value of quantum yield indicates that the complex exists in the semi-polar environment of the membrane surface. The possibility that co-extraction of the valinomycin–K^+ complex and ANS^- (ref. 14) into the organic phase of the monolayer vesicles could have contributed to the observed enhancements can be eliminated by considerations of the following: (1) heterogeneous extraction experiments[2,8], (2) the wavelength of the emission maximum, (3) comparisons of the results for monolayer and bilayer vesicles, and (4) the dependence of the enhancement effect upon the phase transition.

ANS^- fluorescent enhancement effects have been found for the ionophores valinomycin, monactin, enniatin B and 18-crown-6 (*cf.* ref. 6) for each of the cations K^+, Cs^+, Rb^+, and Na^+ with dimyristoyl-α-lecithin monolayer and bilayer vesicles at 15°. For each ionophore, the apparent binding constant (taken as the reciprocal cation concentration giving half maximal effect) varies in the order $Rb^+ \geqslant K^+ > Cs^+ > Na^+$. The K^+/Na^+ specificity ratios of these effects are low (< 10) compared with the ratios for other systems[2,8]. This indicates that the complexes measured here may differ from the complexes found in non-polar solvents[2] or the complexes which traverse the hydrophobic regions of the membrane, particularly for the case of the large ionophore molecules. However, the ionophores also demonstrate low values of ion specificity based on the cation concentration dependence of their facilitation of cation transport in mitochondria[8] indicating that the complexes measured here may play an important role in the kinetics of transport.

The value of the enhancement effect at high cation concentration may be taken as a measure of the depth of binding of the ionophore–cation complex within the membrane if a close association between the complex and the ANS^- is assumed. The ratio of the enhancement effect at high cation concentration to the ionophore concentration varies in

*A K^+-dependent enhancement effect of valinomycin and related ionophores on ANS^- fluorescence in aqueous media in the absence of membranes or other hydrophobic binding sites has recently been demonstrated[14]. Since, under the present conditions, this effect is at most 10% of the effect obtained in the presence of vesicles, it does not affect the results or interpretations here. Considerations of valinomycin solubility, and measurements of 90° light scattering[8] indicate that the enhancement effect in the absence of membranes involves the formation of colloidal aggregates of ionophore molecules.

the order valinomycin ≈ monactin > enniatin B > 18-crown-6. The ion specificity of the maximal effect has the rank order $Rb^+ \geqslant K^+ > Cs^+ > Na^+$. These observations are in general agreement with the rank order based on transport for these ionophore–cation concentrations[2,8]. This is keeping with the notion that the transport efficiency of an ionophore–cation combination is related to the ease with which the interface complex can approach the more hydrophobic portions of the membrane.

Enhancement effects have also been observed in dipalmitoyl-α-lecithin vesicles for $T < T_t$. The quantum yields for the ANS^- species associated with the complex were smaller than for the case of dimyristoyl-α-lecithin vesicles, suggesting that the lipid with the longer chain length produces more rigid membranes which are less easily penetrated. For the case $T > T_t$, enhancement effects were observed only with enniatin B for the lipid and membrane preparations of this study. Negative effects were observed with the other ionophores, as shown for valinomycin in Fig. 2. That no substantial amount of K^+

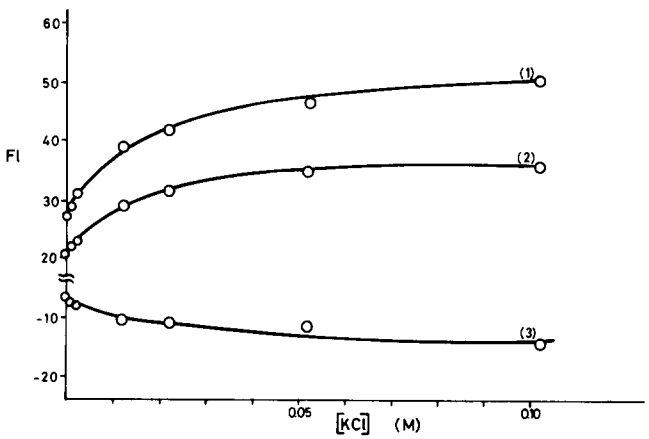

Fig. 2. Decrease of membrane-bound ANS^- fluorescence in the presence of valinomycin, $T > T_t$. The notation and conditions are identical to those in Fig. 1 except that $T = 30°$ and [valinomycin] = $1.5 \cdot 10^{-5}$ M.

complexation of the ionophore was involved, can be deduced from the similarities of the K^+ concentration dependence of all three curves. The negative effect can be explained by the assumption that valinomycin penetrates into the non-polar region of the membrane, disturbing the packing of the lipid molecules such that one valinomycin molecule can render six lipid molecules incapable of contributing to the ANS^- binding.

Grell *et al.*[15] have recently presented evidence based on circular dichroism for the complexation of enniatin B with K^+ *in* or *on* vesicle membranes produced from egg lecithin, These workers could find no evidence for valinomycin complexation with K^+ in this system but concluded from the spectra that the molecule was buried within the hydrophobic interior of the membrane. Since the phase transition temperature of egg lecithin is below 0° (ref. 12) the conclusions of Grell *et al.*[15] are appropriate for the condition $T > T_t$. There is thus good agreement between the results from the fluorescence and circular dichroism methods, and for the conclusions regarding the behavior of valinomycin and enniatin B

with membranes above their phase transition temperature.

The method of the present study has the advantage that it can report the complexation of the ionophores at low concentrations, leaving the bulk of the membrane structure relatively unperturbed. Evaluation of the ionophore–cation binding constants and the kinetics of the complexation reaction will be reported in a future communication.

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