Physiology of ionophore transport of potassium and sodium ions across cell membranes: valinomycin and 18-crown-6 ether

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Abstract: The processes involved in transport of K^+ and Na^+ by the carrier ionophores valinomycin and 18-crown-6 ether across cell membranes have been elucidated using quantum mechanical modelling: 1. Formation of the {ionophore-M^+} complex: desolvation (ΔG_{desolv}) of the central cavity of the ionophore, change in configurational energy $T\Delta S$, desolvation of the $M(H_2O)_{6-7}^+$. 2. Desolvation of the {ionophore-M^+} complex prior to entering the membrane environment. 3. Permeation through the lipophilic environment of the membrane, which is dependent on the lipophilicity (ΔG_{lipo}), dipole moment μ, and molecular volume of the {ionophore-M^+} complex. 4. Release of the M^+ on the intracellular side, and diffusion of the free ionophore back towards the extracellular side to restart the process. Results from this study show that it is possible to design molecular structures to enhance the ability of crown ethers to selectively transport alkali metal ions across lipid membranes.

Keywords: ionophore; valinomycin; crown ether; potassium ion; sodium ion; cell membrane transport; quantum mechanical modelling.

1 Introduction

Transport across cell membranes can be broadly divided into passive transport where molecules are transported down a high to low concentration gradient, or active transport where energy needs to be supplied to move molecules against a low to high concentration gradient. Passive transport of physiologically essential species and drugs across cell membranes can be classified in six major categories:

1 lipid dissolution and diffusion

2 facilitated diffusion

3 ligand gated channels

4   voltage gated channels

5   diffusion mediated carrier ionophores (such as valinomycin, crown ethers)

6   pore forming ionophores (such as gramicidin).

This study will focus on category (5).

Carrier ionophores include valinomycin (K^+), lasalocid acid, an antibacterial agent and a coccidiostat, which transport Na^+, K^+, Ca^{2+}, Mg^{2+} across membranes, (Brasseur and Ryusschaert, 1986; Antonenko and Yaguzhinsky, 1988) nigericin, an antibiotic (K^+, H^+, Pb^{2+}), and the ionophore carrier for Ni transport in the cyanobacterium Anabaena cylindrica, which is thought to transport Mg^{2+} into the cell. Jarrell and Sprott (1982) Ionophores that preferentially transport Li^+ (compared to Na^+, K^+ etc) across lipid bilayers are also known (Zeevi and Margalit, 1985). Many synthetic ionophores are based on crown ethers, cryptands, and calixarenes. Ionophores disrupt the membrane potential by conducting ions through a lipid membrane in the absence of a protein pore, and often have cytotoxic properties. They are produced naturally by a variety of microbes and act as a defence against competing microbes. Synthetic ionophores (and biologically occurring ionophores) open up the possibility of designing drugs that disrupt the cellular membrane potential, thereby altering the permeability of the membrane as well as having specific cellular impacts. The chemo-therapeutic properties of common carrier ionophores are well known, but less is known about their ability to permeate cell membranes, partly because the mechanisms of the passive, facilitated and active transport of drugs across cell membranes is equivocal (Fong, 2014a, 2014b, 2015b).

Where ions are being transported, an electrochemical potential which depend on the concentration of ions on either side of the membrane, is a driving force, as well as the chemical potential which depends on the concentration of solutes on either side of the membrane (Feher, 2012; Voet and Voet, 2010). When a carrier ionophore such as valinomycin is transporting K^+ and Na^+, the {valinomycin-K^+} complex, and free valinomycin and ions are present. A pH gradient across the membrane also involves an additional driving force, the proton motive force (PMF). The movement of any molecule or ion down, or up a concentration gradient involves a change in free energy, ΔG. When energy is released, ΔG is negative, or if energy is consumed or required to move the species, ΔG is positive.

$$\Delta G = RT \ln \left(\left[X_{\text{inside}} \right] / \left[X_{\text{outside}} \right] \right) + \left(z \right) \left(F \right) \left(V_m \right), \tag{1}$$

where R is the gas constant, T is the absolute temperature, $[X_{\text{inside}}]$ is the solute concentration inside the cell, and $[X_{\text{outside}}]$ is the concentration outside the cell. The second term $(z)(F)(V_m)$ is the free energy of the electrochemical potential for ionic species, where z is the charge on the ion, F is Faraday's constant 23.06 kcal/mol of charge released moving down a voltage gradient of 1 volt, and V_m = the membrane potential (typically rest values are about –70 in mammalian cells, –10 to –14 human red blood cells, bacterial cells –130 to –140, E.coli –110 mV to –140 mV).

The total electrical potential of a membrane is made up of the membrane potential (V_m) due to gradients in ion concentrations across the membrane, the surface potential due to lipids with charged zwitterionic head groups, and the dipole potential (V_D) which arises due to the alignment of dipolar lipid head groups and water dipoles in the interface region between the hydrophobic membrane interior and the aqueous phase. The typical

resting value of V_m 70 mV corresponds to the electric field strength about 10^7 V/m inside a 5 nm thick membrane. In contrast, the dipole potential V_D changes sharply across the head group area resulting in much stronger electric fields, on the order of 10^9 V/m (Warshaviak et al., 2011; Peterson et al., 2002).

If there is a pH difference inside the cell compared to outside the cell, an additional driving force across the membrane is the proton motive force (PMF).

$$\text{PMF} = V_M - \left[\left(2.303RT \right) / F \right] \Delta pH, \tag{2}$$

where $\Delta pH = pH$ difference across membrane and $(2.303RT)/F = 58.8$ mV at room temperature. At acid conditions, the PMF is dominated by ΔpH, at alkaline conditions, PMF $\sim V_M$. Typical PMF values for E.coli are –120 mV to –160 mV.

Valinomycin is a naturally occurring cyclic dodecadepsipeptide antibiotic, which has been widely studied (Feher, 2012; Voet and Voet, 2010). Valinomycin is highly selective for potassium ions over sodium ions within the cell membrane, acting as a potassium-specific transporter and facilitates the movement of potassium ions through lipid membranes 'down' an electrochemical potential gradient. The stability constant K for the {valinomycin-K^+} complex is 10^6 and for the {valinomycin-Na^+} complex only 10, resulting in a 10,000 times selectivity for K^+ over Na^+. The conformations adopted by the molecule are dependent on the solvent, with six of the 12 carbonyl groups being responsible for binding to metal ions and interactions with polar solvents, and the isopropyl and methyl groups being dominant in interacting with non-polar solvents. In experimental salt extraction equilibrium measurements (Eisenman et al., 1991), the $Na^+ \rightarrow K^+$ ion replacement showed that valinomycin prefers binding K^+ to Na^+ by –5.4 kcal/mol. Other experimental studies of the permeability ratio in lipid membranes (Eisenman and Alvarez, 1992) show that valinomycin selects K^+ rather than Na^+ with a selectivity of about –6 kcal/mol.

Valinomycin raises the permeability ratio (P_K/P_{Cl}) of human blood erythrocytes in net flux experiments by ca. 20 fold at very low [K^+] concentrations on one side of the membrane, and low [Cl^-] extracellularly, corresponding to a V_M of ca. 50 mV. By comparison the pore forming ionophore gramicidin had a ca. 100 fold increase in K^+ permeability (Frohlich and King, 1988).

The X-ray structures of the {valinomycin-K^+} shows that valinomycin binds to K^+ through 6 *ester* carbonyl oxygens in a quasi-octahedral arrangement (av bond length 2.8Å)such that the K^+ is centrally located within the cyclic valinomycin. The structure of {valinomycin-Na^+} is quite different, with the Na^+ 'external' to the valinomycin and bonded to three carbonyl ester oxygens (av bond length 2.7Å), a water molecule, and interacting with the (picrate) anion. The Na^+ is displaced in the complex 2.3Å away from the position occupied by K^+ in the analogous complex, where a water molecule resides (Steinrauf et al., 1982; Hamilton et al., 1981; Neupert-Laves and Dobler, 1975). The difference between the sodium and potassium complexes is attributed to the smaller atomic radius of Na^+ compared to K^+, and possibly due to co-ordination chemical interaction differences or cavity size constraints that physically prevent valinomycin collapsing onto the smaller Na^+ (Varma et al., 2008, 2011). The structures were thought to reflect the large differences in valinomycin selectivity towards K^+ over Na^+.

The structures of {valinomycin-K^+} and {valinomycin-Na^+} in solution may be quite different from those in the crystal, where lattice packing forces can induce changes from solution, and particularly where water molecules are hydrogen bonded to the complex,

and conformational barriers of the valinomycin are low. It is known that valinomycin undergoes conformational changes in different solvents, so crystallisation from different solvents might result in conformationally different complexes in the crystalline form (Simon et al., 1978). It is also highly likely that several conformationally different complexes exist in solution, if the conformational barriers amongst species are low. It has been shown that the structure of {valinomycin-K^+} in the crystal and solution are almost the same (Huang and Williams, 1981). However the structure of {valinomycin-Na^+} in solution is not known. There have been several detailed molecular dynamic computations of these complexes, and a likely solution structure for {valinomycin-Na^+} has 4 ester carbonyl oxygens coordinated to Na^+ (av bond length 2.65Å) (Varma et al., 2008, 2011; Kholmurodov et al., 2010) which is similar to the crystal structure which has 3 bonds to the carbonyl oxygens and one water molecule hydrogen bonded to the Na^+. There are several likely conformations of similar energy for {valinomycin-Na^+} in solution that are similar to the crystal structure, and water molecules hydrogen bonded to the Na^+ ion need to be considered.

The X-ray structure of valinomycin itself shows that the ring structure folds into a shape similar to the seam on a tennis ball, forming a large barrel with hydrophobic external surface and a large hydrophilic cavity inside the barrel with 12 carbonyls and 6 ether oxygens. When the hydrophilic cavity has a small volume, the cavity is more spherical, and contains two water molecules. It is also possible for the cavity to swell to include 12 water molecules, then the ring structure takes on an elongated ellipsoidal shape (Hašek et al., 2009). This configurational flexibility of valinomycin allows it to complex with larger metal ions with ease (Feher, 2012; Voet and Voet, 2010).

Valinomycin is an antibiotic which kills microbial cells by disrupting the essential role of ion gradients (by collapsing ion gradients across cellular membranes) in active secondary transport and energy conservation. It is not used clinically, but is routinely used experimentally where there is a need to remove cellular membrane ion gradients. Valinomycin is also used as a non-metallic isoforming agent in potassium selective electrodes.

Valinomycin displays pH-dependent activity against selected Gram-positive bacteria, including *Staphylococcus aureus*, *Listeria innocua*, *Listeria monocytogenes*, *Bacillus subtilis*, and *Bacillus cereus* ATCC 10987. Antimicrobial activity bacteria was highest at alkaline pH values, where the membrane potential V_M is the main component of the proton motive force PMF (equation (2)) (Tempelaars et al., 2011). It is known that valinomycin and other ionophores are not effective against Gram-negative bacteria which are protected by the presence of an outer membrane that prevents access of these compounds to the inner membrane, thereby acting as a selective permeability barrier between the cytoplasm and the outside environment.

Valinomycin has recently been reported to be a potent agent at concentrations between 3.3 μM and 10 μM against severe acute respiratory-syndrome corona virus (SARS-CoV) in infected Vero E6 cells (Wen et al., 2007; Wu et al., 2004). Valinomycin treatment induced mitochondrial swelling and minor nuclear changes in cell lines (BV-2, C6, HEK 293), and in primary mouse microglia and astrocytes by perturbing cellular K^+ homeostasis. Mitochondrial swelling and autophagy are common features of valinomycin-exposed cells. Valinomycin promotes an autophagic cell death mode, but not apoptosis (Klein et al., 2011). Valinomycin is highly cytotoxic to tumour cells only under hypoglycemic conditions, acting as a GRP78 downregulator (Ryoo et al., 2006).

Crown ethers also act as carrier ionophores for K^+ and Na^+, and have been found to be toxic to E.coli (Tso and Fung, 1980; Tso et al., 1981). At a sub-lethal dosage, the crown ether affects the three phases in the bacterial growth curve as evidenced by an appearance of a lag period, an occasional decrease in the stationary phase at a lower microbial population. Potassium ion but not sodium ion can reduce the lag induced by the presence of 18-crown-6. Crown ethers are generally thought to transport K^+ faster than Na^+ ions across membranes (Eisenman et al., 1972, 1973) but it has been found that in the presence of higher alkanoic acids, such as stearic acid, 18-crown-6 ether can selectively transport K^+ ions but not Na^+ (Inokuma et al., 1984). The structures of the {18-crown-6 ether $-K^+$} and {18-crown-6 ether $-Na^+$} have been determined (Kobrsi et al., 2006; Steed et al., 2003). The {18-crown-6 ether $-Na^+$} structure shows two methanol molecules complexed to the Na^+ above and below the Na^+-crown ether plane (Na-O 2.4 Å), and with three shorter Na-O bonds 2.5Å and two longer Na-O bonds 2.75Å and one non-complexed O atom. The structural difference between the K^+ and Na^+ crown ether complexes is similar to that found with the valinomycin complexes, and reflects the smaller ionic radius of the Na^+ ion. The restricted conformational variability of the crown ether ring may give insights into the energetics behind why the {valinomycin-Na^+} complex behaves so differently from the physiology of the {valinomycin-K^+} and which may involve large conformational changes in the valinomycin during complexation to Na^+.

A comprehensive model of how carrier ionophores are transported across cell membranes would be useful in designing new drugs that can counter diseases such as SARS or be cytotoxic to tumour cells under hypoglycemic conditions.

2   Results and discussion

In membrane transport studies of K^+ and Na^+ with and without the addition of ionophores such as valinomycin or 18-crown-6 ether, the solvation energies and any required conformational changes to the ionophore of these species in water need to be determined. The relevant processes are:

1   Solvated M^+ + Solvated Ionophore $\rightarrow$ Desolvated M^+ + "Desolvated Ionophore" (where the ionophore undergoes a partial desolvation when binding to M^+ occurs, and conformational/configurational changes to allow binding to M^+) *Extracellular/Interface.*

2   Desolvated M^+ + "Desolvated Ionophore" $\rightarrow$ Solvated {Ionophore-M^+} *Extracellular/Interface.*

3   Solvated {Ionophore-M^+} $\rightarrow$ Desolvated {Ionophore-M^+} *Interface/ Membrane diffusion.*

4   {Ionophore-M^+} $\rightarrow$ Ionphore + M^+ *Intracellular.*

5   Ionophore re-enters membrane to permeate back into extracellular environment to restart transport process (1).

The experimental water solvation energies of Na^+ and K^+ ions have been determined as -87.2 kcal/mol and -71.0 kcal/mol (Marcus, 1994; Rizzo et al., 2006). The solvation energies using the SMD model employed in this study for the species $Na(H_2O)_7^+$ and

$K(H_2O)_6^+$ are -86.2 kcal/mol and -72.3 kcal/mol, respectively, which are in good agreement with the experimental values. So the necessary desolvation energy for these ions are very high, but particularly the difference between Na^+ and K^+ is 13.9 kcal/mol, which is a significant contributor towards the observed differences in selectivity between the {valinomycin-M^+} complexes, along with difference in binding energy between valinomycin and M^+, and conformational or configurational changes required by valinomycin during complexation (Rose and Henkens, 1974) The structural characteristics of the Na^+ and K^+ in water have been determined by large angle x-ray scattering and double difference infrared spectroscopy. The literature results suggest that the hydration shells vary from 4-8 for Na^+ and 6-8 for K^+. The $Na(H_2O)_6^+$ and $K(H_2O)_7^+$ species have M^+- O bond distances of 2.42Å and 2.81Å (Mahler and Persson, 2012).

The dominant conformation of valinomycin in the free state in water is taken to be the computationally optimised geometry and the conformation in the bound state is taken to be the optimised geometry of the {valinomycin-K^+} complex without the K^+. The geometry of {valinomycin-K^+} is essentially the same as the crystal X-ray structure. The changes in valinomycin conformation from the free state to the bound state can be quantified in terms of configurational energy (TΔS) (Chang et al., 2007; Kar et al., 2013) and desolvation energy ΔG_{desolv} changes. The configurational and desolvation energy changes in water for free and bound valinomycin configurations are -3.2 kcal/mol and 7.5 kcal/mol and the changes from the bound state of valinomycin to the {valinomycin-K^+} complex are 2.4 kcal/mol and -0.3 kcal/mol, respectively (this study). Overall, it can be seen that the desolvation effect dominates (change of 7.2 kcal/mol) as valinomycin in the free state is transformed into the {valinomycin-K^+} complex.

The {valinomycin-Na^+} complex, as per the X-ray structure, has a greater ΔG_{desolv} than the {valinomycin-K^+} by 11.0 kcal/mol, which is consistent with the difference in X-ray structures where the Na^+ is 'external' to the valinomycin ring, and more exposed to water solvation. The configurational and desolvation energy changes in water for free and bound valinomycin configurations are 0 kcal/mol and -3.1 kcal/mol and the changes from the bound state of valinomycin to the {valinomycin-Na^+} complex are 1.6 kcal/mol and 26.9 kcal/mol, respectively. Overall, it can be seen that the desolvation effect (change of 30.0 kcal/mol) dominates as valinomycin in the free state is transformed into the {valinomycin-Na^+} complex.

However, there is a likelihood that solution structure of {valinomycin-Na^+} complex with four ester carbonyl oxygens bonded to the external Na^+ is different from the X-ray structure which has three ester carbonyl oxygens and a water molecule bonded to the Na^+. (Varma et al., 2008) The ΔG_{desolv} of the former structure (dipole moment μ, 9.9D in water) is 12 kcal/mol lower than that of the latter (μ 16D), consistent with a less polar structure (lower μ) that is less accessible to water molecules.

The calculated difference in vacuo ΔG for {valinomycin-K^+} (geometry with X-ray structure) and {valinomycin-Na^+} (solution geometry with Na^+ bonded to 4 ester carbonyl oxygens) is about 7.4 kcal/mol. Since the $\Delta G_{bind} = \Delta G_{complex} - \{\Delta G_{valin} + \Delta G_{ion}\}$, the difference between ΔG_{bind} between K^+ and Na^+ binding to valinomycin allowing for solvation effects, and configurational changes required of valinomycin to allow binding, can be calculated. The difference between desolvation of $K(H_2O)_6^+$ and $Na(H_2O)_7^+$ is 13.9 kcal/mol, the difference in desolvation of valinomycin in the configuration

that binds to K^+ and the configuration that binds to Na^+ is 15.2 kcal/mol, and the {valinomycin-K^+} has a lower solvation energy than {valinomycin-Na^+} by 1.0 kcal/mol The difference between K^+ and Na^+ is –0.5 kcal/mol. The difference in configurational energy between the free unconstrained valinomycin and the configuration that is required for binding to the metal ions is 2.2 kcal/mol (the configurational energy for Na^+ is higher). The difference in ΔG for binding between {valinomycin-K^+} and {valinomycin-Na^+} in water is *estimated* to be –6.0 kcal/mol. This estimate compares with an experimental binding selectivity difference between the complexes of –5.4 to –6 kcal/mol (Eisenman et al., 1991; Eisenman and Alvarez, 1992). The overall thermodynamic balance for binding in water is largely driven by the differences on the desolvation energies of the $M(H_2O)_x^+$ species and offsetting larger solvation energy for the formation of the {valinomycin-Na^+} requirement compared to the {valinomycin-K^+} complex. These data indicate that the structures used in the calculations closely approximate those in solutions. Since the structure of {valinomycin-K^+} in solution is the same as that in the crystal (Huang and Williams, 1981), this data is consistent with the solution structure of the {valinomycin-Na^+} having Na^+ bonded to 4 ester carbonyl oxygens rather than the X-ray structure which has three ester carbonyl oxygens bound to the Na^+ as well as a water of crystallisation (this structure can be dismissed from being a significant contributor in solution based on the calculated ΔG and solvation values).

It is clear that from the solvation and thermochemical data above for the {valinomycin-K^+} and {valinomycin-Na^+} complexes that the configurational changes required for the free valinomycin molecule to complex with Na^+ are far greater than the corresponding changes required for complexing with K^+. This can be more clearly seen by comparing the '{valinomycin} free unconstrained' configuration of valinomycin in Table 1 which was obtained by optimising the free configuration (and ensuring there were minimal steric clashes occurring), and comparing this configuration with the "{valinomycin} free configurations" which were obtained by removing the M^+ ion from the {valinomycin-M^+} complexes, leaving behind the valinomycin, then optimising the resultant structures. (*This procedure was used as the X-ray structures are known, particularly the $M^+ – O$ bond lengths of the {valinomycin-M^+} complexes, but there are many possible conformations of valinomycin of similar energy, and it is not computationally feasible to identify all possible configurations or conformations.*) The '{valinomycin} free unconstrained' configuration should be a reasonable approximation of the range of lowest energy configurations of the free valinomycin that can easily complex with K^+ or Na^+ (Hašek et al., 2009). It is clear that the molecular properties of the '{valinomycin} free configurations' obtained from the {valinomycin-K^+} complex more closely resemble those of the '{valinomycin} free unconstrained `configuration'} than those from the {valinomycin-Na^+} complex. The configurational entropy (TΔS) for the configurational changes required for the valinomycin to form {valinomycin-K^+} is 2.7 kcal/mol compared to 4.9 for the required changes for the formation of the {valinomycin-Na^+} complex. This is consistent with the view that the smaller ionic radius of Na^+ does not allow easy insertion into the central cavity of the valinomycin, whereas the larger K^+ radius more easily fits into the central cavity of valinomycin and requires far less distortion of the valinomycin ring structure (Feher, 2012; Voet and Voet, 2010; Varma et al., 2008).

It has been widely assumed that the {valinomycin-K$^+$} complex more easily diffuses through the lipid bilayer of the cell membrane than K$^+$ ions because of the lipophilic or hydrophobic shell that valinomycin confers around the K$^+$. A model of transport through cell membranes has been recently developed that applies to passive and some active transport processes (Fong, 2014a, 2015b):

$$\text{Membrane transport} = \Delta G_{\text{desolvation}} + \Delta G_{\text{lipophilicity}} + \text{Dipole Moment} + \text{Molecular Volume.} \tag{3}$$

The ΔG_{desolv} for the {valinomycin-K$^+$} is 73.5 kcal/mol compared to {valinomycin-Na$^+$} 72.5 kcal/mol. The ΔG_{desolv} for the free valinomycin varies between 54.5 to 66.3 kcal/mol, depending on whether the metal ion is released from {valinomycin-Na$^+$} or the {valinomycin-K$^+$} complex.

The solvation energy in n-octane has been shown to be a good proxy for the lipophilicity or hydrophobicity (Fong, 2014a, 2014b, 2015b). The ΔG_{lipo} for the free valinomycin (–28.9 kcal/mol) compared to the {valinomycin-K$^+$} complex (–39.7) decreases by –11.0 kcal/mol, a significant increase in lipophilicity for the complex, mainly due to decreased exposure of the polar ester groups and amide groups *inside* the valinomycin ring structure. The external oriented isopropyl and methyl groups do not significantly alter their orientations in the complex compared to the free valinomycin.

The ΔG_{lipo} for the {valinomycin-Na$^+$} complex (-38.1 kcal/mol) is less lipophilic than the {valinomycin-K$^+$} by 1.6 kcal/mol. By comparison it is noted that the ΔG_{lipo} values for K(H$_2$O)$_6^+$ and Na(H$_2$O)$_7^+$ are –26.6 kcal/mol and –28.9 kcal/mol indicating far lower hydrophobicities for these species as expected. Water has a value of –2.0 kcal/mol on this scale, and n-octanol –5.9 kcal/mol. The ΔG_{lipo} values for the free valinomycin varies between –26.5 and –28.9 depending on whether the metal ion is 'released' from the {valinomycin-Na$^+$} or the {valinomycin-K$^+$} configuration to produce the geometries used in the calculations.

Table 1   Properties on ionophores in free and bound configuration, {ionophore-M$^+$} complexes in water

	$\Delta Gdesolv$ (kcal/mol)	$\Delta Glipo$ (kcal/mol)	Dipole moment (μ) D	Molecular volume (cm^3/mol)	Charge M^+ AU	Change ΔG (kcal/mol)	Change $T\Delta S$ (kcal/mol)
{valinomycin-K$^+$}	73.5	–39.7	3.9	847	0.848	11.5	2.5
{valinomycin} bound configuration	73.8	–27.5	3.0	747		11.5	3.2
{valinomycin} free configuration	66.3	–28.9	3.4	842		0 ref pt	0 ref pt
{valinomycin} free unconstrained configuration	63.3	–29.7	14.3	696		–2.3	2.7
{valinomycin-Na$^+$}	72.5	–38.1	9.9	834	0.973	4.1	1.6
{valinomycin} bound configuration	57.6	–25.8	3.3	686		4.7	0
{valinomycin} free configuration	54.5	–26.5	4.7	751		0 ref pt	0 ref pt

Table 1   Properties on ionophores in free and bound configuration, {ionophore-M$^+$} complexes in water (continued)

	ΔGdesolv (kcal/mol)	ΔGlipo (kcal/mol)	Dipole moment (μ) D	Molecular volume (cm^3/mol)	Charge M^+ AU	Change ΔG (kcal/mol)	Change $T\Delta S$ (kcal/mol)
{valinomycin} free unconstrained configuration	63.3	−29.7	14.3	696		−8.3	4.9
{valinomycin-Na$^+$} X-ray structure	84.5	−40.3	16.0	827	0.927	20.0	3.9
{18-crown-6-K$^+$}	58.8	−26.2	1.0	249	1.027	−44.8	17.7
{18-crown-6} bound configuration	44.8	−14.9	1.4	238		−42.6	13.7
{18-crown-6} free configuration	25.4	−9.6	0	224		0 ref pt	0 ref pt
{18-crown-6-Na$^+$}	49.0	−27.0	1.5	227	0.888	−14.2	9.5
{18-crown-6} bound configuration	41.3	−11.7	0.4	202		−12.9	6.2
{18-crown-6} free configuration	25.4	−9.6	0	224		0 ref pt	0 ref pt
{18-crown-6-Na$^+$.(H$_2$O)$_2$}	57.5	−27.	0.6	243	0.916	−11.7	15.3
{18-crown-6-Na$^+$.(H$_2$O)$_2$} H-bonded*	46.6	−25.6	1.6	227	0.687	−19.4	18.1
						Binding energy[#]	
{18-crown-6-K$^+$-C$_9$H$_{19}$CO$_2$−}	18.3	−15.0	7.8	411	0.813	−15.7	
{18-crown-6-Na$^+$-C$_9$H$_{19}$CO$_2$−}	19.2	−14.7	5.1	362	0.591	−13.5	
{18-crown-6-K$^+$-C$_2$H$_5$CO$_2$−}	19.8	−10.9	7.6	325	0.813	−15.3	
{18-crown-6-Na$^+$-C$_2$H$_5$CO$_2$−}	20.6	−10.7	5.0	284	0.598	−15.0	

ΔG_{lipo} values in n-octane, all other properties in water. Changes in ΔG and $T\Delta S$ are relative to the free ionophore as the reference point (ref pt). Ionophore in bound configuration is the {ionophore-M$^+$} less the M$^+$ configuration. Ionophore in the free configuration is the unconstrained optimised configuration. Charges on M$^+$ are calculated by the CHELPG method. $T\Delta S$ is calculated at 298.15K. *Complex with water molecules bonded to Na$^+$ and intramolecularly hydrogen bonded from water molecule to free O atom of the crown ether. # refers to binding free energies between the {18-crown-6-M$^+$} complexes and the n- C$_9$H$_{19}$CO$_2^-$ or n-C$_2$H$_5$CO$_2^-$ anions in kcal/mol in water.

The dipole moment for the {valinomycin-K$^+$} complex μ 3.9D in water does not vary in comparison with the free valinomycin μ 3.4D, and the molecular volume of the complex is ca. 15% smaller than the free valinomycin. However for {valinomycin-Na$^+$} μ 9.9D is significantly more polar than the {valinomycin-K$^+$} and has water solvated molecular

volume that is 20% larger. The calculated CHELPG atomic charge on K in {valinomycin-K^+} 0.85AU compared with a value of 0.97AU on Na^+ in {valinomycin-Na^+} which is indicative of the greater bonding interaction between the K^+ and valinomycin and consistent with the dipole moments.

The transport model in equation (3) indicates that the passive permeability within the hydrophobic environment of the membrane of the {valinomycin-K^+} is greater than the {valinomycin-Na^+} because of a dominant large difference in dipole moments (potassium complex has a smaller μ by 6.0D), and smaller differences in molecular volume and lipophilicity, but little difference in desolvation energies. The much lower dipole moment within the membrane environment is the dominant reason that the {valinomycin-K^+} complex has a greater diffusion rate through the cell membrane than the {valinomycin-Na^+} complex. The permeabilities of the {valinomycin-K^+} complex through lipid membranes has been shown to be about 282–285 times as fast as that of the {valinomycin-Na^+} complex (Eisenman et al., 1972; Papahadjopoulos, 1973).

The enthalpy of activation for valinomycin induced transport of $^{42}K^+$ across phosphatidic acid-phosphatidyl choline liposomes is 15.4 kcal/mol, with an entropy of activation of 35 cal/mol/K, giving a free energy of activation of 5.0 kcal/mol at 298 K (Johnson and Bangham, 1969).

The free valinomycin is expected to diffuse through the hydrophobic membrane environment at about a similar rate to the {valinomycin-K^+}, as it has a lower hydrophobicity, but closely comparable dipole and molecular volume, but as a lower water desolvation energy to offset the lower hydrophobicity (Table 1). This is in accord with experimental findings (Stark et al., 1971).

It has been postulated that formation and transport of the {valinomycin-K^+} complex first involves adsorption to the membrane surface by the free valinomycin (Stark et al., 1971) (which requires some desolvation, and possibly ionic interaction by the zwitterionic phosphatidylcholine (PC) head group to one of the amide carbonyl oxygens of valinomycin). This is followed by insertion of the desolvated K^+ into the valinomycin which undergoes configurational rearrangement at the same time, with accompanying desolvation and desorption of the complex, and subsequent permeation into the lipid bilayer. The configurational rearrangement of the valinomycin during insertion of the K^+ may assist the desorption from the membrane surface, facilitating permeation into the membrane environment. This stepwise mechanism helps overcome some of the large desolvation requirements involved in formation and transport of the complex. *However, the competing mechanism where the {valinomycin-M^+} complex can also bind to the PC head group via an amide carbonyl oxygen may also be energetically favourable.* There is some evidence from molecular dynamic studies that initial insertion of the K^+ into the valinomycin while it is loosely bound to the membrane surface is via the amide carbonyl oxygens, which are more polar than the ester carbonyl oxygens, before finally bonding to the ester carbonyl oxygens (Forester et al., 1997). Calculations in this study (not reported) which show that the six amide carbonyl oxygens can easily complex with K^+, also support this hypothesis. There is also evidence that the interactions between the zwitterionic head group of phosphatidylcholine moiety of cell membranes and polar molecular species can assist transport of these species across cell membranes by altering the membrane dipole potential (Carpenter et al., 2014; Fong, 2015b) The formation of a {valinomycin-PC} complex at the interface surface of the membrane (most probably via an amide carbonyl oxygen) which then allows insertion of K^+ would affect the alter

the dipole potential V_D (possibly by ca. 45 mV (Warshaviak et al., 2011)) facilitating permeation of the {valinomycin-K$^+$} after it desorbs from the membrane surface or is displaced from the surface by another free valinomycin molecule. A large change in V_M (via V_D) would significantly increase $(z)(F)(V_m)$ in equation (1). The formation of a transient {valinomycin-M$^+$-PC} complex would exhibit K$^+$ and Na$^+$ selectivity similar to the free {valinomycin-M$^+$} species in the extracellular environment. In some Gram-positive bacteria, an additional proton motive force PMF mechanism is known to occur when valinomycin acts an anti-bacterial agent.

It is noted that equation (1) applies to the species concentrations of the *desolvated* complexes prior to diffusing into and after leaving the membrane environment, and the electrochemical component, $(z)(F)(V_m)$, applies to the charged complexes which both have a notional charge of +1, but would have differing spatially charged characteristics when interacting with the electrochemical gradient. For bacterial cell membranes, an additional driving force for transport can come from the PMF, as per equation (2), since many bacteria cells have membrane potentials and comparably high PMF as well.

The {18-crown-6 ether –K$^+$} and {18-crown-6 ether –Na$^+$} complexes can be compared with their valinomycin counterparts directly as they show similar ionophore behaviour with K$^+$ and Na$^+$, but are less complicated in terms of their reduced conformational isomers.

The dominant conformation of 18-crown-6 ether in the free state in water is taken to be the computationally optimised geometry and the conformation in the bound state is taken to be the optimised geometry of the {18-crown-6 ether-K$^+$} complex without the K$^+$. The geometry of {18-crown-6-ether-K$^+$} is essentially the same as the crystal X-ray structure. The changes in crown ether conformation from the free state to the bound state can be quantified in terms of configurational energy (TΔS) (Chang et al., 2007; Kar et al., 2013; Fong, 2014d, 2015a) and desolvation energy ΔG_{desolv} changes. The configurational and desolvation energy changes in water for free and bound crown ether configurations are 13.7 and 19.4 kcal/mol and the changes from the bound state of crown ether to the {18-crown-6-ether-K$^+$} complex are 4.0 and 14.0 kcal/mol respectively. Overall, it can be seen that the changes in configurational energy and desolvation energy are large (changes of 17.4 and 33.4 kcal/mol) as the crown ether in the free state is transformed into the {18-crown-6-ether-K$^+$} complex.

The {18-crown-6-Na$^+$} complex, as per the X-ray structure, has a lower ΔG_{desolv} than the {18-crown-6-K$^+$} by 9.0 kcal/mol. The configurational and desolvation energy changes in water for free and bound crown ether configurations are 6.2 and 15.9 kcal/mol and the changes from the bound state of crown ether to the {18-crown-6-Na$^+$} complex are 5.2 and 7.7 kcal/mol respectively. Overall there are large changes in configurational energy and desolvation energies (changes of 11.4 and 23.6 kcal/mol) as the crown ether in the free state is transformed into the {18-crown-6-Na$^+$} complex. Compared with the values for the K$^+$ complex, these values appear to suggest that the crown ether is more selective towards Na$^+$ than K$^+$, which is at variance with the observed greater selectivity for the K$^+$ (Eisenman et al., 1972, 1973). However there is a likelihood that the {18-crown-6-Na$^+$} exists as a di-hydrated species in solution since the crystal structure of {18-crown-6-Na$^+$.(H$_2$O)$_2$} has 2 methanol molecules bonded to the {18-crown-6-Na$^+$} above and below the quasi plane of the {18-crown-6-Na$^+$} at a Na$^+$ – O distance of 2.33-2.4Å (Steed et al., 2003). The overall change in configurational energy and desolvation energy from the free state of the crown ether to the {18-crown-6-Na$^+$.(H$_2$O)$_2$} complex is 15.3 and 32.1 kcal/mol, compared with the values of 17.7 kcal/mol and 23.4 kcal/mol for

{18-crown-6-K$^+$}. These data for pre-organisation of the complex prior to entering the membrane environment are very different with the lower desolvation energy for the {18-crown-6-K$^+$} indicating a greater selectivity for K$^+$ over that for Na$^+$, as found experimentally (Eisenman et al., 1972, 1973).

The ΔG_{lipo}, dipole moment and molecular volume values for the {18-crown-6-K$^+$}, {18-crown-6-Na$^+$.(H$_2$O)$_2$} and {18-crown-6-Na$^+$} complexes are {–26.2 kcal/mol, 1.0 D, 249 cm^3/mol}, {–27.0 kcal/mol, 0.6 D, 243} and {–27.0 kcal/mol, 1.5 D, 227 cm^3/mol} respectively. These data suggest that these species have comparable permeability within the lipophilic membrane environment as found experimentally (Kimura and Shono, 1990) and as per equation (3).

Overall, the greater desolvation and configurational energies, lipophilicities, dipole moments and molecular volumes suggest that the crown ether has a greater selectivity towards K$^+$ than Na$^+$, and similar membrane transport properties for the complexes, which are in accord with experimental findings (stability constants K$^+$ 120, Na$^+$ 26 M^{-1}). (Eisenman et al., 1972, 1973).

The ΔG_{desolv}, ΔG_{lipo}, dipole moment and molecular volume values for the free crown ether were 25.4 kcal/mol, –9.6 kcal/mol, 0 D, 224 cm^3/mol, indicating a closely comparable membrane transport rate to the complexes since it has a lower preoganisational desolvation energy prior to entering the membrane environment, but a lower lipophilicity (with dipole and molecular size being very similar to those of the complexes) which decreases the permeation in the lipid environment of the membrane.

The {18-crown-6-Na$^+$.(H$_2$O)$_2$} complex in the crystal state is expected to show strong intramolecular hydrogen bonding between one of the water molecule attached to Na$^+$ and the free ether O atom of the crown ether, based on the equivalent crystal structure of the {18-crown-6-Na$^+$.(MeOH)$_2$} complex (Steed et al., 2003). However in solution, the intramolecular hydrogen bonding may be eliminated since water molecules in the inner solvation sphere would compete with the intramolecular hydrogen bonding. This possibility was examined by comparing the two structures where strong intramolecular hydrogen bonding is available by a distortion of the ring structure and the tilting of the water molecules linked to Na$^+$ so a strong intramolecular hydrogen bond can occur with the free O of the crown ether, and the equivalent structure where there was no distortion of the {18-crown-6-Na$^+$.(H$_2$O)$_2$} complex. It can be seen in Table 1 that there are significant changes in desolvation energies, the configurational entropies and charges on Na$^+$. Based on the known stability constants for the sodium-crown ether complex in water, it is unlikely that the intramolecular hydrogen bonded species is present in any significant concentrations, as its properties would predict this species to be the result of greater Na$^+$ selectivity by the crown ether than that towards the K$^+$ species.

There is evidence that the solution chemistry of the valinomycin-Na$^+$ complex may include dimeric species, since the dimeric species [Na$_2$(18-crown-6)$_2$(H$_2$O)$_3$][BPh$_4$]$_2$ has been characterised (Steed and Junk, 1999). The presence of small quantities of such species in solution would probably lower membrane permeability rates since lower quantities of the monomeric species are present to permeate the membrane, and have a smaller impact on stability constants due to the dynamic equilibrium between monomer and dimer species being shifted towards the monomer when complexation with Na$^+$ occurs. There appears to be no unambiguous membrane permeability data for the {18-crown-6-M$^+$} complexes, with some studies showing similar permeability rates (Kimura and Shono, 1990) but studies of the more soluble {bis-t-butylclyclohexyl-crown-6-M$^+$} shows that the K$^+$ complex permeates through lipid membranes faster than the Na$^+$

complex by a factor of 21.4. However, it was found that the permeability ratios were only independent of concentration above 10^{-6} M, and *below this concentration the permeability ratio depends on crown ether concentration* (Eisenman et al., 1973). This observation indicates that the crown ether selectivity constants and permeability ratios are closely intertwined factors governing transport of K^+ and Na^+ in these systems.

It is worth noting that the proposed mechanism for formation of the {valinomycin-M^+) complexes being absorbed at the membrane surface interface with the extracellular medium is highly unlikely to apply to the crown ether, because the crown ether has no externally accessible polar groups like valinomycin to interact with the zwitterionic PC head group, but only inward facing ether groups, which are not particularly polar. Clearly valinomycin has greater selectivity towards metal ions and induces faster permeability of lipid membranes by orders of magnitude than the crown ether.

It is clear that the larger configurational entropy changes are required for crown ether complexation to K^+ and Na^+ compared to the valinomycin complexes. This appears to be a result of changes to the molecule to allow the Na^+ and K^+ ions to fit within the molecular cavity, which are greater than the equivalent process for valinomycin. Since valinomycin has 12 carbonyl groups, this result is a little surprising, but the optimised structures for valinomycin have the majority of carbonyls facing inwards towards the central cavity, with the isopropyl and methyl groups facing outwards. The crown ether rings is buckled to allow the co-ordination to the metal ions, and the data shows this buckling process is greater than the buckling required of the valinomycin ring to co-ordinate to the metal ions.

A cursory investigation of the reported increased selectivity of some crown ethers in the presence of alkanoic acids (Inokuma et al., 1984) in transporting K^+ but not Na^+ has been undertaken. It was reported that the addition of longer chain alkanoic acids *above* $C_7H_{15}CO_2H$ under alkaline conditions increased the transport of the 18-crown-6/K^+ system from a zero base through a $C_{11}H_{23}CO_2H/C_{17}H_{35}CO_2H/CHCl_3$ membrane barrier, but no effect was seen for Na^+, nor for alkanoic acids with shorter hydrocarbon chains than $C_3H_7CO_2H$ and below. The technique appears to be a simple means of increasing the selectivity and efficacy of K^+ transport over that of the Na^+. It can be seen from Table 1 that the {18-crown-6-M^+-$C_9H_{19}CO_2^-$} and {18-crown-6-M^+-$C_2H_5CO_2^-$} complexes show very different properties from the corresponding {18-crown-6-M^+} complexes, with far lower ΔG_{desolv} (by about 30–37 kcal/mol) and higher ΔG_{lipo} values (by about 10–15 kcal/mol). It is noted that the {18-crown-6-M^+-$C_9H_{19}CO_2^-$} and {18-crown-6-M^+-$C_2H_5CO_2^-$} complexes are nominally *neutral species*, compared nominally positively charged {18-crown-6-M^+} complexes. The actual charges on the metal atom in the various complexes are shown in Table 1 as CHELPG atomic charges. The optimised K^+-O-C(O)- bond length was 2.7 Å, compared with the 2.4 Å distance found in the X-ray structure of potassium hydrogen phthalate or the hydrated K^+ in solution (Li et al., 2003; Mahler and Persson, 2012) but K-O bond lengths can vary from 2.9–3.2 Å in Li-Fe-K clusters, or 2.7–2.8 Å in potassium sulphate or potassium chromate (Newton et al., 2009, McGinnetty, 1972). The optimised Na^+-O-C(O)- bond length was 2.4 Å, compared with the average 2.2–2.4 Å distance found in similar compounds (Royal Society Chemistry, 2012; Tonnessen et al., 1996). The very large desolvation penalties for the {18-crown-6-M^+} complexes compared to the {18-crown-6-M^+-$C_9H_{19}CO_2^-$} and {18-crown-6-M^+-$C_2H_5CO_2^-$} complexes would lead to large decreases in permeation rates through lipid membranes for the {18-crown-6-M^+} complexes. In addition, the ΔG_{lipo} values of the {18-crown-6-K^+-$C_2H_5CO_2^-$} and {18-crown-6-K^+-$C_9H_{19}CO_2^-$}complexes are lower than

those of the corresponding Na$^+$ complexes by about 4 kcal/mol, indicating an enhanced permeability for the K$^+$ complexes. The binding constants for addition of the alkanoic acid anions to the {18-crown-6-M$^+$} species are very similar, ruling out a binding process as being a significant driver of the enhanced effect. In summary, the data is consistent with the original findings that the addition of alkanoic acid anions to the crown ether can selectively transport K$^+$ faster than the Na$^+$ ion, and this approach could be the basis of a simple design method for enhancing the transport of alkali ions by crown ethers.

3   Conclusions

The processes involved in transport of K$^+$ and Na$^+$ by the carrier ionophores valinomycin and 18-crown-6 ether across cell membranes have been elucidated using quantum mechanical modelling. The critical features of ionophore transport process are:

1   Formation of the {ionophore-M$^+$} complex, which involves desolvation (ΔG_{desolv}) of the central cavity of the ionophore, accompanied by configurational or conformational reorganisation (changes in configurational energy $T\Delta S$ are minor compared to desolvation effects) of the ionophore to accommodate the insertion of the metal ion. Desolvation of the M(H$_2$O)$_{6-7}^+$ is also required to allow the bare M$^+$ to insert into the cavity of the ionophore. Relatively large changes in free energy are required in these steps.

2   Desolvation of the {ionophore-M$^+$} complex prior to entering the membrane environment, which requires relatively large changes in free energy.

3   Permeation through the lipophilic environment of the membrane, which is dependent on the lipophilicity (ΔG_{lipo}), dipole moment μ, and molecular volume of the {ionophore-M$^+$} complex.

4   Release of the M$^+$ on the intracellular side, and diffusion of the free ionophore back towards the extracellular side to restart the process.

The structure of the {valinomycin-Na$^+$} complex in solution is different from that of the crystal structure. The selectivity and membrane diffusion properties of the {valinomycin-K$^+$} complex are dominated by higher desolvation energy requirements during formation of the complex and before the carrier ionophore complex enters the membrane environment, and by lower dipole interaction effects within the membrane compared to the {valinomycin-Na$^+$} complex. A mechanism is proposed that suggests that valinomycin acts by adsorption to the membrane surface and forming a transient complex with the zwitterionic phosphatidylcholine head group followed by insertion of K$^+$ into the complex, and altering the membrane dipole potential, thereby increasing the transport rate of the {valinomycin-K$^+$} complex across the cell membrane. Steps 1 and 2 above may occur at the membrane interface.

The 18-crown-6 ether complexes with Na$^+$ to predominantly form the complex {18-crown-6--Na$^+$.(H$_2$O)$_2$} in solution. The desolvation and configurational energies, lipophilicities, dipole moments and molecular volumes show that the crown ether has higher selectivity towards K$^+$ than Na$^+$, and similar membrane transport properties for the complexes.

The required changes to configuration or conformation of the free ionophores to allow insertion of the metal ions into the central cavities have been calculated in terms of configurational energy changes. To form the {valinomycin-K$^+$} complex, the valinomycin requires 2.2 kcal/mol less configurational energy than is required for valinomycin to change its configuration to be able to form the {valinomycin-Na$^+$} complex. It has been found that the configurational energy changes for the 18-crown-6 ether to complex with the metal ions are greater than those for valinomycin, with the {18-crown-6--K$^+$}, and {18-crown-6--Na$^+$.(H$_2$O)$_2$} complexes having configurational energy values of 17.7 and 17.9 kcal/mol compared to the values for {valinomycin-K$^+$} and {valinomycin-Na$^+$} of 2.7 kcal/mol and 4.9 kcal/mol.

The elucidation of the energy requirements for ionophore-metal ion formation and cell membrane transport should provide ready input into the prediction of the efficacy of ionophore-metal ion complexes as potential drugs against for example, the (SARS-CoV) virus or as an anti-tumour agent under hypoglycemic conditions. Results from this study show that it is possible to design molecular structures to enhance the ability of crown ethers to selectively transport alkali metal ions across lipid membranes.

4 Experimental methods

All calculations were carried out as previously described (Fong, 2014a, 2014b) using the Gaussian 09 package at the B3LYP/6-31G**(6d, 7f) level of theory with optimised geometries, as this level has been shown to give accurate electrostatic atomic charges, and was used to optimise the IEFPCM/SMD solvent model. With the 6-31G* basis set, the SMD model achieves mean unsigned errors of 0.6–1.0 kcal/mol in the solvation free energies of tested neutrals and mean unsigned errors of 4 kcal/mol on average for bare ions (Marenich et al., 2009). The 6-31G* basis set has been used to calculate absolute free energies of solvation and compare these data with experimental results for more than 500 neutral and charged compounds. The calculated values were in good agreement with experimental results across a wide range of compounds (Rayne and Forest, 2010; Rizzo et al., 2006). Adding diffuse functions to the 6-31G* basis set (i.e., 6-31$^+$*) had no significant effect on the solvation energies with a difference of ca 1% observed in solvents, which is within the literature error range for the IEFPCM/SMD solvent model. Desolvation energies are essentially the reverse of solvation energies.

Electrostatic potential at nuclei were calculated using the CHELPG method in Gaussian 09. The atomic charges produced by CHELPG are not strongly dependant on basis set selection. Using the B3LYP level of theory, calculated atomic charges were almost invariant amongst the basis sets 6-31G(d), 6.311(d,p), 6-311+(2d,2p), 6-311G++(3df,3dp). Errors between calculated and experimental dipole moments were 3%. (Martin and Zipse, 2005; Kubelka) All calculations were at the B3LYP/6-31 G**(6d, 7f) level of theory for all atoms except for Na and K where the relativistic ECP SDD Stuttgart-Dresden basis set was used. The atomic radii used for neutral Na$^+$ and K$^+$ (6 coordinated octahedral configuration) in the CHELPG calculations were 1.16Å and 1.52Å respectively. (http://www.webelements.com/potassium (or sodium)/atom_sizes .html). CHELPG calculations using the ECP SDD basis set were in good agreement with those using the B3LYP/6-31 G**(6d, 7f) for the sodium compounds tested.

It is noted that high computational accuracy for each species in different environments is not the focus of this study, but comparative differences between various

species is the aim of the study. The Basis Set Supposition Error was within 1% for the thermochemical energy calculations.

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