

Studies Directed Toward the Fabrication of a Synthetic Cation-conducting Channel Based on Lariat Ethers: the Feeble Forces Concept for Self-assembly

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Abstract. The work described here derives from our observation that while Nature certainly utilizes covalent interactions and other forces of great strength in the construction of biomolecules, many 'feeble forces' are involved as well. We have used feeble forces in model studies directed eventually to the synthesis of a self-assembling, cation-conducting channel. Specifically, we have prepared three models for portions of such a cation-conducting channel. The first is based on the steroidal lariat ethers that clearly self-assemble. This process involves *inter alia* entropy as a feeble force of cumulative importance. Second, we have prepared three *tris*(macrocyclic) systems that are simplified versions of the proposed channel-former. Third, we have demonstrated the strength of carefully-conceived hydrogen bonding interactions by constructing a molecular box based on the interaction between adenine and thymine.

Key words. Lariat ether, self-assembly, channel, cation-conduction.

1. Introduction

During the past decade, numerous studies have been undertaken to develop synthetic ionophores that might permit cations or molecules to pass through a lipid bilayer [1]. Naturally-occurring gramicidin A is known to form transmembrane channels [2] and efforts to prepare a cation-conducting channel have been reported as well [3]. In our work, we have studied the selectivity of numerous crown ethers, lariat ethers [4, 5], and multi-armed versions of the latter [6]. Much has been learned about flexible ionophores and we have now attempted to utilize the concepts of flexibility and self-assembly to permit construction of a cation- or molecule-conducting channel.

Although Nature utilizes covalent bonds and rigid structural arrangements to establish steric relationships, weak or 'feeble' forces such as conformational change, hydrogen bonding, the formation of salt bridges, entropic forces, and even chirality play a major role in determining the three-dimensional structures of natural systems. In our approach to the self-assembly problem, we have utilized two of these feeble forces. By feeble forces, we mean interactions that individually contribute a small amount of energy, but in concert can lead to well-defined structural arrangements. Specifically, we have utilized entropic driving forces, to prepare novel cholesteryl lariat ether-based bilayers, and hydrogen bonding, to induce the formation of a molecular box. These are preliminary steps required for development of the cation channel molecule.

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2. Experimental Methods

Melting points were determined on a Thomas-Hoover capillary device and are corrected. Infrared spectra were recorded as neat films on NaCl plates and are calibrated against the 1601 cm^{-1} band of polystyrene. Proton NMR spectra were recorded in CDCl_3 or $\text{DMSO}-d_6$ using internal Me_4Si as standard. Combustion analyses were performed by Atlantic Microlab, Atlanta, GA and molecular weights were determined using a Wescor 5100C vapor pressure osmometer. Vapor pressure osmometer readings were evaluated using calibration curves described in the text.

2.1. SYNTHESSES OF *tris*(MACROCYCLIC) COMPOUNDS

To a stirred solution of *N,N'*-bis(6-chlorohexyl)-4,13-diaza-18-crown-6 (1 mmole) in MeCN (15 mL) was added 2 mmole of aza-3*n*-crown-*n* in which $n = 4, 5, \text{ or } 6$ in 5 mL of MeCN. The solution was heated at reflux for 3 days. Workup involved extraction and chromatography over alumina (2-propanol : hexane mixtures as eluents).

2.2. *N,N'*-bis[6-(*N*-AZA-12-CROWN-4)HEXYL]-DIAZA-18-CROWN-6

The compound was obtained as described above (0.45 g, 58%). *Anal.* calcd. for $\text{C}_{40}\text{H}_{80}\text{N}_4\text{O}_{10}$: C, 61.81; H, 10.40%. Found: C, 60.58; H, 10.42%.

2.3. *N,N'*-bis[6-(*N*-AZA-15-CROWN-5)HEXYL]-DIAZA-18-CROWN-6

The compound was obtained as described above (0.53 g, 61%). IR: 3390, 2870, 1450, 1350, 1240, 1105 cm^{-1} . *Anal.* calcd. for $\text{C}_{44}\text{H}_{88}\text{N}_4\text{O}_{12} \cdot \text{H}_2\text{O}$: C, 59.82; H, 10.29%. Found: C, 59.88; H, 10.28%.

2.4. *N,N'*-bis[6-(*N*-AZA-18-CROWN-6)HEXYL]-DIAZA-18-CROWN-6

The compound was obtained as described above (0.64 g, 67%). *Anal.* calcd for $\text{C}_{48}\text{H}_{96}\text{N}_4\text{O}_{14}$: C, 60.46; H, 10.17%. Found: C, 60.21; H, 10.23%. Osmometric molecular weight (3 trials, 1, 2-dichloroethane), theory: 953. Found 988 (3.7% error).

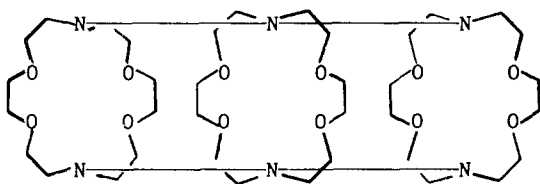
3. Results and Discussion

3.1. SCHEMATIC FOR THE SYNTHESIS OF A CATION CHANNEL

The vast majority of cation transport model studies have been conducted using crown ethers as ionophores and bulk liquid membranes as bilayer models. Much information has been obtained about carrier-mediated transport [1, 7] but the mechanism for transport of molecules through biological membranes is predominantly of the channel type [8]. The synthesis of a cation-conducting channel is a daunting task. We felt that a suitable compound would require at least three basic features. First, it must be capable of insertion into a lipid bilayer. Second, it must span the membrane. Third it must have some residue integral to it that would

exhibit a cation or molecule affinity. An additional consideration is that it must have polar groups positioned at distances appropriate for the relay of a cation through it, should the surface polar groups be spaced too far apart for a direct jump.

Based on our experience with macrocyclic polyether compounds, we reasoned that three parallel crown ether rings might form the basis of such a channel. As envisioned, two of the rings would be on the external and internal surfaces of the bilayer and a third would lie halfway between the other two. Since membrane thickness varies considerably, we based our first design on the well-characterized rat liver plasma membrane. It contains more than 50% phosphatidyl choline and nearly 20% cholesterol [9]. Phosphatidyl choline (PC) is derived from palmitic and oleic acids, each of which contains 16 carbons. The extended length of PC is thus about 26 atoms including the trimethylammonium and phosphate residues. A straight span of 26 carbon atoms would be about 40 Å long so the dimer of such a system falls well within the normal 60–100 Å membrane thickness. It should not be overlooked, however, that the crystal structure of coiled gramicidin A shows a surface-to-surface distance of only 32.5 Å [2]. The basic, schematic outline of a cation-conducting channel might thus be as shown in the figure below.

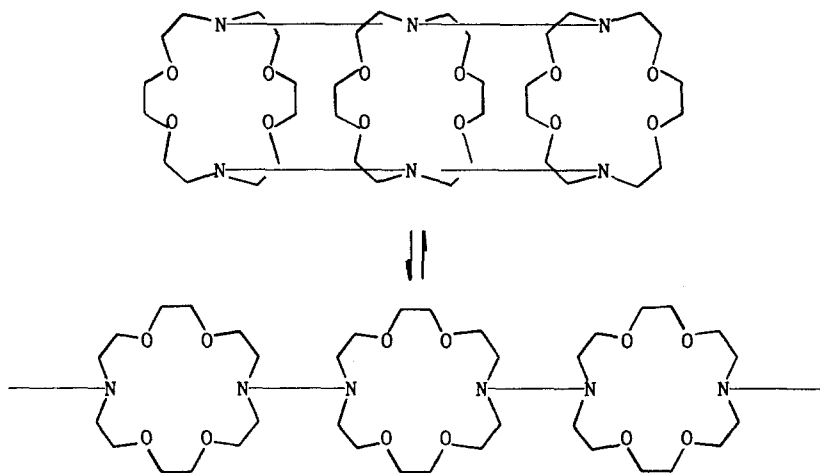


3.2. STEROIDAL LARIAT ETHERS

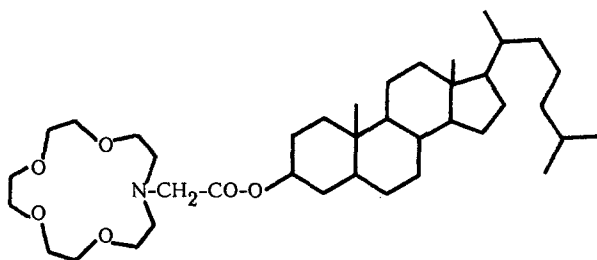
As noted above, cholesterol comprises nearly 20% of rat liver plasma membrane [9]. Its presence controls membrane fluidity and cholesterol is obviously quite lipophilic. It seemed reasonable to consider incorporating cholesterol directly into the spacer units, shown above by a simple, straight line. Since cholesterol exists in many membranes in free form, it must be a highly organizing molecule, capable of significantly stabilizing a self-assembling structure.

Entropy is the major force for membrane formation. If enough elements of a membrane itself were incorporated in a model cation-channel-former, the normally feeble entropic force might assist the assembly and/or insertion process. If so, then the compound illustrated schematically could be reduced by two covalent linkages to that shown below. It, in turn, could exist in a fully extended conformation as shown.

In principle, the equilibrium between the *tris*(macrocyclic) compound shown overleaf and the folded form shown above it, would be driven *inter alia* by entropy. The credibility of such a proposition would be enhanced if a cholesteryl lariat ether, a compound having the macroring and spacer portions of the system shown, could be prepared. Such a structure has been synthesized [10] and its self-assembly into



niosomes, in the absence of cations, and vesicles, in their presence, has now been demonstrated [11]. The self-assembling, steroidal lariat ethers, are based upon both carbon-pivot [5] and nitrogen-pivot [6] systems. The most versatile structure has thus far proved to be the steroidal ester of an *N*-glycyl-azacrown. The structure of a typical steroidal lariat ether compound is shown below.

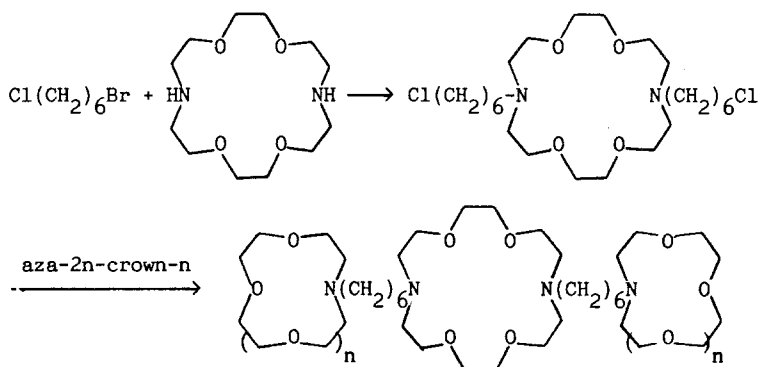


The self-assembly of steroidal lariat ethers bodes well for the possible self-assembly of a cation-conducting channel. Clearly, the entropic driving force, although feeble compared to a covalent bond, manifests itself with considerable vigor in this system. It is also interesting to note that ESR studies of these membrane systems utilizing a steroidal nitroxide spin label indicates that the resulting bilayers are very rigid (non-fluid) structures [12].

3.3. *Tris*(MACROCYCLIC) COMPOUNDS

The *tris*(macrocylic) compounds required for the present studies pose some significant difficulties. As models, somewhat simpler systems were prepared in two steps from diaza-18-crown-6. 1-Bromo-6-chlorohexane, 4,13-diaza-18-crown-6, and sodium carbonate were stirred at reflux in acetonitrile solution for 48 hours. *N,N'*-bis(6-chlorohexyl)-4,13-diaza-18-crown-6 was obtained as a colorless oil in

70% yield after chromatography. This dichloride could then be converted into compounds that we can illustrate schematically as O–O–O or o–O–o to indicate that the same or differently-sized macrocycles may be attached to each other. The former structure could represent three 18-membered rings while the latter could represent an array of three rings as follows: 12–18–12. A schematic representation of the channel-forming precursor containing three 18-membered rings might be –O–O–O–. The general synthetic sequence is straightforward and an example is shown below.



Of course, the molecules shown above lack two crucial features required for the envisioned channel formation in membranes. First, the interring spacers are far too short but represent, in most respects, the required spacers in a synthetically accessible form. Second, there is no second chain on the terminal (first and third) macrocycles. Nevertheless, the essential features of the desired system have been brought to hand and this shows, at least in general, synthetic feasibility.

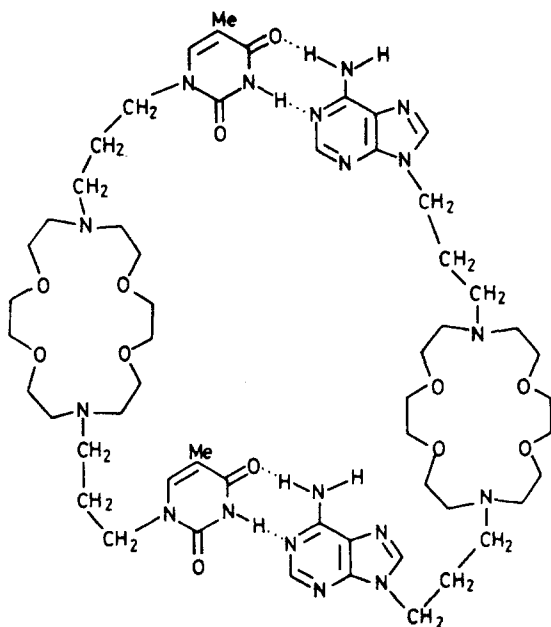
3.4. HYDROGEN BONDING IN MODEL SELF-ASSEMBLY SYSTEMS

Hydrogen bonding is a well-known and much studied phenomenon [13]. In general, hydrogen bonds exhibit strengths from 1–6 kcal/mole although higher and lower values are known. In addition, they generally exhibit a predictable directionality. Although not specifically incorporated in the self-assembling, cation-conducting channel model illustrated schematically above, hydrogen bonding is a feeble force whose cumulative effect on the assembly process could be considerable. Our model to assess cumulative hydrogen bonding strength and to incorporate potential molecular recognition was based on the well-established base-pairing of purines and pyrimidines. In the matrix of DNA, two hydrogen bonds hold together adenine and thymine while cytosine and guanine base-pair use three such interactions. While the latter is more promising from the energetic point of view, synthetic access to model systems involving these two residues is less propitious.

We envisioned the formation of a molecular box that comprises essentially half (although not to scale) of the channel molecule shown above. We anticipated that a diaza-18-crown-6 macrocyclic ring would be substituted by two sidearms, each terminated in adenine or thymine. Leonard and coworkers [14] had reported the

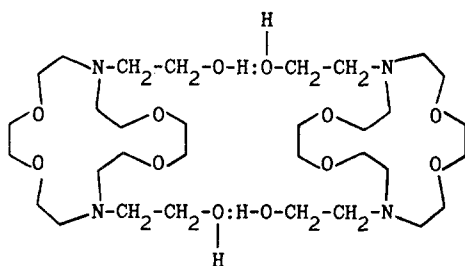
syntheses of adenine- and thymine-terminated propyl chains so these were chosen as bibrachial lariat ether (BiBLE) [6] sidearms. Although the preparation of a single macrocycle having one adenine- and one thymine-terminated chain was the most intellectually pleasing prospect, its synthesis appeared beyond our grasp. Instead, two BiBLE subunits were prepared, each having two identical propyl sidearms terminated either in adenine or thymine. These may be represented schematically as A-O-A and T-O-T in which A and T stand for adenine and thymine respectively and O is 4,13-diaza-18-crown-6.

Once these compounds were brought to hand [15] their affinity for each other in aqueous solution was assessed using vapor pressure osmometry (VPO, see experimental section). A calibration curve was obtained using (65, 114, 155, 203, and 265 mmolar, 5 trials each) 18-crown-6 in water. The calibration curve was a straight line: $Y = 0.785X + 59.43$. Based on this, the apparent molecular weight of the particles in solution was assessed. When A-O-A and T-O-T (each approximately 100 mmolar) were dissolved in pure water, an apparent molecular weight of 771.2 was observed. The molecular weights of the individual species are: A-O-A, 612.84; T-O-T, 594. In the absence of any association, a simple average of 603 should be observed. The apparent molecular weight of 771.2 suggests that there is about 25% association. Thus the feeble hydrogen bonding forces compete quite effectively with water, a solvent of considerable organization and hydrogen bonding strength itself. The envisioned complex is illustrated below.



For comparison, we examined the behavior of *N,N'*-bis-(2-hydroxyethyl)-diazia-18-crown-6. This differs from the 'DNA-box' precursors in the chain length (two, rather than three, carbons) and in the terminal hydroxyl group. Even so, the systems are closely related and the hydroxyl function is known to be both a

hydrogen-bond receptor and donor. Thus, its similarity to A-O-A or T-O-T is clear. In principle, a dimer of the type shown below could form.



Studies similar to those described for the DNA-box (above) were undertaken using *N,N'*-bis-(2-hydroxyethyl)-diazia-18-crown-6 [16]. Thus, 12.5 mg of *N,N'*-bis-(2-hydroxyethyl)-diazia-18-crown-6 was dissolved in 0.6249 g of H₂O. Again using the 18-crown-6 calibration curve, an apparent molecular weight was determined. *N,N'*-bis-(2-hydroxyethyl)-diazia-18-crown-6 has a molecular weight of 350.52. The observed molecular weight of 346.38 differs from the expected weight for a completely monomeric system by less than 2%. Thus, the self-association of *N,N'*-bis-(2-hydroxyethyl)-diazia-18-crown-6 is $\leq 2\%$ and well within experimental error for a monomeric species. Compared to this, the association of A-O-A with T-O-T is substantial.

It occurred to us that the DNA-box is, in a sense, an induced-fit receptor. The affinity of primary ammonium salts for 18-membered crown ethers is well known [17] and the enthalpy of this weak interaction has been assessed at 2–3 kcal/mole in water [18]. Studies of CPK molecular models suggested that the macroring-to-macroring span across the DNA box could effectively accommodate 1,12-dodecanediammonium salt. The ideal interaction would increase the number of hydrogen bonds from 4 to 10. Addition of 1,12-dodecanediammonium dichloride to a solution containing equimolar A-O-A and T-O-T, affords an apparent molecular weight of 1123 while the ternary complex should have a molecular weight of 1498. The meaning of these numbers is unclear. One might assume that the simple ratio $1123/1498 = 75\%$ suggests extensive association and, indeed, it may. Unfortunately, the problem is far too complicated to quote such a percentage based on current data.

The greatest difficulty is that any salt in the structure $C1H_3N(CH_2)_nNH_3Cl$ dissociates into three particles in water. Thus the apparent molecular weight of these species should be one-third of the expected value. Measurements of $C1H_3N(CH_2)_3NH_3Cl$, $C1H_3N(CH_2)_6NH_3Cl$, $C1H_3N(CH_2)_{10}NH_3Cl$, and $C1H_3N(CH_2)_{12}NH_3Cl$ all gave correct molecular weights when a calibration curve based on $C1H_3N(CH_2)_3NH_3Cl$ rather than 18-crown-6 was used. Thus correct (by a factor of 2.0–2.5 rather than the expected factor of 3) molecular weights were obtained for diammonium salts using the crown calibration curve and *vice versa*. In our original report of DNA box formation, we also noted that the system was, so to say, complex [15]. There are thus two essential difficulties in evaluating complex formation between ammonium salts and neutral species. First, complexes having

either arrangements or stoichiometries other than the expected one may form. Second, the analytical method must be fully compatible with and respond similarly to all chemical species present. These problems do not affect the results for association between A—O—A and T—O—T in the absence of $\text{C1H}_3\text{N}(\text{CH}_2)_n\text{NH}_3\text{Cl}$. In the presence of the latter, conclusions must currently remain tentative.

In order to obtain some additional calibration on the association problem, we examined apparent molecular weights for mixtures of 4,10,16-triaza-18-crown-6 and $\text{CH}_3\text{NH}_3\text{Cl}^-$. Our assumption was that the ammonium salt should bind strongly to the triaza-macrocycle even in water because of the excellent threefold N—H—N complementarity. Equimolar amounts of triaza-18-crown-6 and $\text{CH}_3\text{NH}_3^+\text{Cl}^-$ in water should give an apparent molecular weight (by VPO) of $328.8 D$ if fully associated. Using the 18-crown-6 calibration curve to evaluate the VPO readings, the apparent molecular weight was $119 \pm 5 D$. When the independently determined calibration curves for triaza-18-crown-6 or methylammonium chloride were used, the apparent molecular weights were, respectively, $216 \pm 10 D$ and $216 \pm 10 D$. The difference of the above results from those obtained using the 18-crown-6 calibration curve is less troubling than the similarity of the latter two values. We have thus obtained clear evidence of association driven, at least in part, by hydrogen bonding. A quantitative evaluation of the association remains elusive.

3.5. SUMMARY

The basis of the effort described here is our observation that while Nature certainly uses covalent interactions and other forces of great strength, many feeble forces are involved as well. We have attempted to utilize these feeble forces in model studies directed eventually to the synthesis of a self-assembling, cation-conducting channel. In particular, we have prepared three types of models for such a cation-conducting channel. The first is the steroidal lariat ethers that clearly self-assemble. This self-assembly involves entropy as a feeble force of cumulative importance. Second, we have prepared three *tris*(macrocyclic) systems that are simplified versions of the desired channel-former. Third, we have demonstrated the strength of carefully-conceived hydrogen bonding interactions by constructing a molecular box based on hydrogen bonds formed between adenine and thymine.

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