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Correlation between the Antibacterial Activity and Alkali Metal Ion Transport Efficiency of Crown Ether

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The antibacterial activity of five crown ethers (15-crown-5, 18-crown-6, benzo-18-crown-6, 4'-methyl-benzo-18-crown-6 and dicyclohexano-19-crown-6) along with their corresponding ion transport efficiency and ion selectivity among five alkali metal ions (lithium, sodium, potassium, rubidium and cesium) are studied. It is found that the toxicity of the crown ethers bears an identical trend as that exists in their capability of transporting potassium ion.

Introduction

In spite of the fact that crown ether (macrocyclic polyether) was first reported in 1937 [1], systematic investigations of its chemical synthesis, physical properties, biochemical toxicology and potential application in industry have only been started after Pedersen's work in 1967 [2]. Crown ether, as a family of compounds, is characterized by having different number of $-(OCH_2CH_2)-$ constituting units either substituted or nonsubstituted, joined covalently in a macrocyclic ring. The number of repeating units normally varies from three to ten; of which, only those containing five (15-crown-5) or six (18-crown-6) oxygen atoms in the ring have attracted the widest attention.

Chemically, crown ether has a central hydrophilic cavity with a hydrophobic external ring which enables it to form stable complex either with alkali or alkaline earth [2, 3], or transition and lanthanide metal ion [4–6]. The central oxygens help to stabilize the salt in organic solvent by the formation of ion pair which can be utilized to promote chemical reactions involving cations [7, 8]. Because of this unique nature, crown ether has been used extensively as a reagent to promote phase transfer reactions in binary solvent systems. The complexability of crown ether depends on its ring size and is highly ion-specific: 15-crown-5 is specific for sodium and 18-crown-6 is specific for potassium. In industry, this ion selectivity of crown ether is indispensable for its application in ion selective electrode, and its use as a selective agent and an ion chelating resin [9, 10].

In biochemistry, a great variety of naturally occurring macrocyclic ionophoretic antibiotics has been shown to exhibit various degrees of ion selectivity effect in ion transport phenomena, in photosynthesis and in oxidative phosphorylation [11]. Essentially, this ion selective power is a key factor in the known biosynthetic antibiotics of the valinomycin and monactin type that affects ion transport across cell membranes [12–14]. Crown ether is the first synthetic alkali metal ion according to the relative size of the cation and the cavity of the crown ether; the number, the arrangement, and the basicity of the oxygen atoms in the crown ether ring; the steric hindrance and the tendency of the cation to associate with the solvent. These factors taken as a whole, affect the stability of the complex and its specific interaction in the membranous system [2, 4, 5]. In general, the parent 18-crown-6 is most widely used to substitute for natural ionophores in the study of active ion transport across biomembranes.

In prokaryotes as well as in mammals, crown ether is found to be toxic [2, 11, 15]. Its presence in the growth medium at high concentrations, induced a lag in the bacterial growth curve. We have recently shown that this toxicity is influenced by the presence of alkaline metal ions [16] and that the toxicity profile varies very sensitively with the structure of crown ether even within substituted members of the 18-crown-6 series that are slightly modified [17]. In biological systems, the distribution of sodium ion and potassium ion is of great physiological significance [18]. In order to examine the physiological effect of crown ether on biological systems, an understanding of the interactions of these two ions with crown ether is required. In this paper, the lethal toxicity of several common crown ethers was examined along with the ion transport efficiency and the ion selectivity of the compounds.

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TABLE I. The Absorbance of Crown Ether-Picrate Salt Complexes Extracted in the Organic Phase.[a]

Crown Ether	Absorbance of Crown Ether–Picrate Salt Complex				
	Li^+	Na^+	K^+	Rb^+	Cs^+
15-Crown-5	0.082(352)[b]	0.805(352)	0.228(355)	0.163(354)	0.130(358)
18-Crown-6	0.130(356)	0.285(356)	3.545(361)	3.424(360)	1.960(365)
Benzo-18-crown-6	0.072(352)	0.275(354)	2.765(361)	1.976(360)	0.808(365)
4'-Methyl-benzo-18-crown-6	0.072(352)	0.287(360)	3.185(363)	2.272(362)	1.020(365)
Dicyclohexano-18-crown-6	0.104(358)	0.670(360)	3.645(363)	3.152(363)	1.928(367)
Control (without crown)[c]	0.056(352)	0.070(340)	0.015(356)	0.082(344)	0.048(356)

[a]The amount of complex in the organic phase was determined by the solvent extraction method as described in Experimental. In the binary aqueous–chloroform system, the initial concentration of various chemical components were: Crown ether: 2.5×10^{-4} *M*, Picric acid: 3.93×10^{-4} *M*, and the alkali metal chloride: 1.0×10^{-1} *M*. [b]Absorption maximum (λ max) of the corresponding complex, in unit of nm. [c]Note a small background in the absence of crown ether with a distinct absorption maximum.

Experimental

Chemicals

High purity (98%) 15-crown-5 and 18-crown-6 were purchased from Sigma and used without further purification. Dicyclohexano-18-crown-6 was purchased from Aldrich Chemical Company and was purified by column chromatography on activated neutral alumina with n-heptane as eluent. 4'-methyl-benzo-18-crown-6 and benzo-18-crown-6, obtained from K. H. Wong as gift, were synthesized according to the procedures described by Pedersen [2] and Smid [19]. The crown ether stock solution was prepared at its highest concentration, depending on its solubility, and sterilized by filtering through sterile membrane filter (Millipore, pore size 0.45 μm).

RbCl, LiCl, KCl and NaCl were obtained from Merck and CsCl was from BDH. All these salts were of analytical grade.

Determination of Extinction Coefficient (ϵ) of Crown Ether-Potassium Picrate Complexes

An accurately weighed potassium picrate was dissolved in a chloroform solution of crown ether, in which crown ether was slightly in excess. On complete solvation, which usually took 2–3 days at room temperature, the absorption spectrum of the crown ether-potassium picrate was determined spectrophotometrically with a Beckman spectrophotometer (Model 25), and the extinction coefficient (ϵ) of the complex was calculated according to the Beer's law.

Determination of Complexability between Various Crown Ethers and Alkali Metal Ions by the Solvent Extraction Method

Essentially, 4 ml of water saturated chloroform solution of crown ether and 4 ml of chloroform saturated aqueous solution of picrate acid and an alkali metal chloride were mixed in a test tube, as modified from the method of Wong *et al.* [20]. The binary system was thoroughly mixed by stirring vigorously to ensure an equilibrium had reached. This equilibrated system was left undisturbed at room temperature overnight to allow complete separation of the two phases. The chloroform layer which contained the crown ether-picrate salt complex was then collected and the absorption spectrum (300 nm to 500 nm) was determined with a Jasco spectrophotometer (Model ORD/UV-5).

Determination of the Susceptibility of Escherichia coli to Crown Ether Toxicity by the Test Tube Serial Dilution Method

The tryptone broth contained 1% (w/v) Difco tryptone in glass distilled water and sterilized by autoclaving at 120 °C for 25 minutes. A serial two-fold dilution of crown ether in tryptone broth was prepared according to the susceptibility test described in the method of Bailey and Scott [21]. The crown ether serially supplemented growth media with their control were then inoculated with aliquotes of logarithmic phase freshly cultured *E. coli* AW405, a derivation of K12, and incubated overnight in a gyrotory shaking water bath (New Brunswick Scientific, Model G76).

In all determinations, the chemical composition of the growth medium, the incubation period as well as the incubation temperature were maintained constant so as to see only the toxic effect of the tested chemical, crown ether [22]. The turbidity of the culture media was measured at wavelength 590 nm, with a photometer (Bausch and Lomb, Spectronic-70), using a cuvette of 5 mm light path. The lowest chemical concentration in the series that showed no turbidity increment was taken as the minimal inhibitory concentration (MIC) as described by Bailey and

TABLE II. Extraction Equilibrium Constants (K_e) of Crown Ether with Alkali Metal Picrate Salts in an Aqueous–Chloroform System at 20 °C.

Crown Ether	Extraction Equilibrium Constant[a] K_e ($\times 10^4$ M^{-2})				
	LiPi	NaPi	KPi	RbPi	CsPi
15-Crown-5	0.05	0.75	0.16	0.11	0.09
18-Crown-6	0.09	0.21	51.99	36.91	3.66
Benzo-18-crown-6	0.05	0.21	13.44	4.26	0.81
4'-Methyl-benzo-18-crown-6	0.05	0.23	36.91	6.78	1.19
Dicyclohexano-18-crown-6	0.07	0.58	73.65	20.42	3.52

[a]The molar absorptivities (ϵ) employed were determined in a calibration experiment. When metal ions other than potassium were used, the value were still assumed to be equal to their potassium counterparts. Similarly, value of 15-crown-5 complex was based on that determined for the 18-crown-6 complex.

Scott [21]. Similarly, the inhibition dosage (ID_{50}) value was determined at a chemical concentration that allowed only fifty percent growth.

Results

Complexability of Various Crown Ethers towards Alkali Metal Ions

Picrate anion is mainly soluble in aqueous solution and its presence in the organic solvent depends on its formation with the alkali metal ion as an ion pair which in turn is complexed with crown ether. On account of the quantitative nature of the ion pair formation, the amount of picrate present in the organic phase has been used as a measure of the complexability of the crown ether with the alkali metal ion [12, 20, 23, 24]. Picrate salt is chosen because of its large structure and its high polarizability to give an extraordinary stable picrate-crown ether complex [25]. In addition to this, an extra merit of the yellowish picrate complex is its high absorptivity in the visible range.

The absorption maxima of various crown ether-alkali metal picrate complexes in chloroform solution were determined and their values found to be between the range of 352 nm–367 nm (Table I). It has been suggested that the absorption maximum depends on the mode of the complexation, and that at least two types of ion pairs (the crown-complexed tight ion pair and the crown separated ion pair) can be formed in low polarity medium, such as chloroform [25]. The tight ion pair has its cation located at the centre of the crown cavity forming a 1:1 complex which gives an absorption maximum around 350–370 nm. With the loose ion pair type, however, the cation is located in between two crown molecules forming a sandwiched complex, with a crown ether and cation ratio of 2:1. This latter complex has its absorption maximum at a longer wavelength, around 370–380 nm. According to this criterion, all the complexes involved in this study are of the tight 1:1 ion pair complex type.

In the solvent extraction method, the overall reaction of crown ether with picrate salt can be described according to

$$M^+_{aq} + Cr_{org} + Pi^-_{aq} \overset{K_e}{\rightleftharpoons} M^+, Cr, Pi^-_{org}$$

as in Eisenman *et al.* [12]. In the equation, M^+_{aq} and Pi^-_{aq} denote the alkali cation and picrate anion in aqueous phase; M^+, Cr, Pi^-_{org} and Cr_{org} denote the crown-picrate salt complex and non-complexed crown ether in the organic phase. The overall extraction process involves the transfer of alkali cation and picrate anion from the aqueous phase into the organic phase by the formation of the ion pair M^+Pi^-,

$$M^+_{aq} + Pi^-_{aq} \overset{K}{\rightleftharpoons} M^+Pi^-_{org}$$

and the complexability between the ion pair and the crown ether in the organic phase

$$M^+Pi^-_{org} + Cr_{org} \overset{K_c}{\rightleftharpoons} M^+, Cr, Pi^-_{org}$$

The overall extraction equilibrium constant (K_e) is

$$K_e = KK_c$$

If the alkali metal ion forms a 1:1 complex with the crown ether and the concentration of the picrate salt in the aqueous phase is much greater than the initial crown ether concentration in the organic layer, the extraction equilibrium constant, K_e, can be calculated by the following expression,

$$K_e = \frac{[MCrPi_{org}]}{\gamma\pm^2 [M^+_{aq}]\,[Pi^-_{aq}]\,\{[Cr_{org}]_o - [MCrPi_{org}]\}}$$

TABLE III. Selectivity of Crown Ethers towards Alkali Metal Ions.[a]

Crown Ether	Selectivity Trend	Ratio
15-Crown-5	Na > K > Rb > Cs > Li	15.0: 3.2: 2.2:1.8:1.0
18-Crown-6	K > Rb > Cs > Na > Li	577.7:410.1:40.7:2.3:1.0
Benzo-18-crown-6	K > Rb > Cs > Na > Li	268.8: 85.2:16.2:4.2:1.0
4'-Methyl-benzo-18-crown-6	K > Rb > Cs > Na> Li	738.2:135.6:23.8:4.6:1.0
Dicyclohexano-18-crown-6	K > Rb > Cs > Na > Li	1052.1:291.7:50.3:8.3:1.0

[a]Selectivity was calculated from data given in Table II and a trend was arranged accordingly.

TABLE IV. A Comparison of the Antibacterial Properties and the Physical Properties of Various Crown Ethers.

Class	Crown Ether	Ion Transport Efficiency[a]		Toxic Effect[b]		Ion Selectivity[a]
		K	Na	ID_{50}	MIC	K/Na
I	15-Crown-5	1.0	3.6	4.20	50.0	0.2
II_A	18-Crown-6	324.9	1.0	0.21	5.0	247.6
II_B	Benzo-18-crown-6	84.0	1.0	0.40	7.5	64.0
II_C	4'-Methyl-benzo-18-crown-6	230.7	1.1	0.40	5.0	160.5
II_D	Dicyclohexano-18-crown-6	460.3	2.8	0.13	5.0	127.0

[a]Data taken and recalculated from absorbance study (Table II). [b]Data taken from Figure 1.

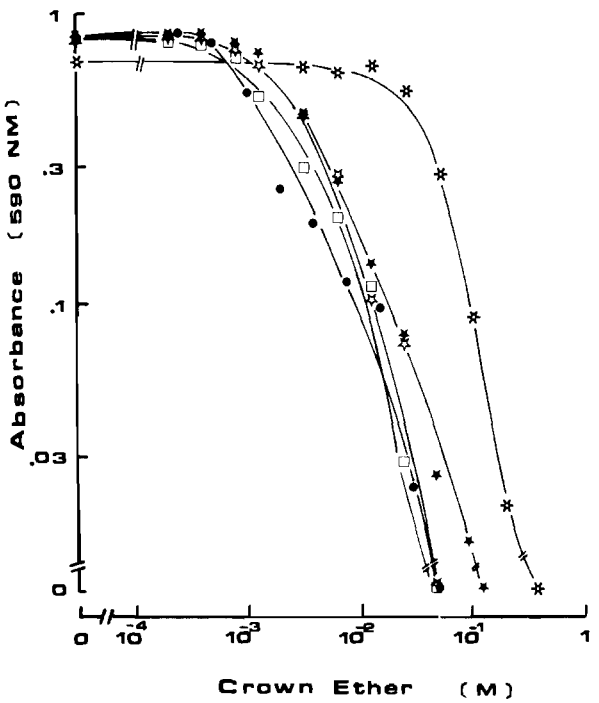

Fig. 1. MIC Test of Crown Ether Toxicity on *E. coli*. Turbidity measurement was done on overnight culture (approximately 16 hours). Symbols for crown ethers: (✱) 15-crown-5, (□) 18-crown-6, (★) benzo-18-crown-6, (☆) 4'-methyl-benzo-18-crown-6 and (●) dicyclohexano-18-crown-6.

where $\gamma\pm$ is the mean activity coefficient of the picrate salt in water and $[Cr_{org}]_o$, $[MCrPi_{org}]$ are the initial crown ether concentration and crown ether-picrate salt complex concentration in organic phase, respectively. At low component concentrations, γ can be considered to be approaching unity. Experimentally, the concentration of the crown ether-picrate salt complex in organic phase is derived from its corresponding absorbance (Table I).

The extraction equilibrium constants of various crown ethers with five alkali metal salts in Table II. This K_e value represents the complexability of the crown ether towards the alkali metal ion. In addition, a comparison of the K_e values of the same crown ether with each alkali metal ion reflects its ion selectivity. These properties are given in Table III. It is apparent that except 15-crown-5, all 18-crown-6s exhibit more specific selectivity towards potassium ion. The selectivity trends within the 18-crown-6s are essentially identical.

Toxicity of Crown Ether on E. coli

The MIC susceptibility test, which is a simple test for the chemical toxicity on bacterial cells, showed that compounds of the 18-crown-6 family exhibit similar but not identical toxicity profile while 15-crown-5 is much less toxic (Fig. 1). The ID_{50}, which is calculated at 50 percent growth, gives essentially the same pattern (Table IV). For all 18-crown-

6s, the toxicity thresholds lie above 10^{-3} *M* crown ether concentration. This finding is in consistent with the previous observation that ionophores are often mildly toxic to microbial organisms but extremely toxic to higher organisms [11].

Correlation of Toxicity and Physical Properties

Crown ether, being lipid soluble, is able to be incorporated in the membrane fraction of the biological system interfering with the transport of metal ions. Of all metal ions affected by crown ether, potassium and sodium, both being important physiologically, have received much attention recently.

It has been suggested by Ward that the crown ether facilitated ion transport rate is proportional to the extraction equilibrium constant of the corresponding crown-cation complex [26]. According to this consideration, the efficiency of crown ether facilitated ion transport can be estimated by its corresponding K_e value. This value as well as the ion selectivity value of the same crown ether can then be compared to the toxicity to shed light on the nature of the antibacterial mode of action. Table IV shows a comparison of the crown ether toxicity and two physical properties of crown ether-potassium or sodium complex in a synthetic membrane system. A combination of two toxicity parameters (ID_{50} and MIC) gives a trend of

$$II_D > II_A > II_C > II_B \gg I$$

with dicyclohexano-18-crown-6 as the most potent ionophore. Apparently a better correlation can be found between toxicity and potassium ion transport efficiency.

It is worthwhile to note that although 15-crown-5 has a higher selectivity for sodium, its sodium ion transport efficiency, however, is not much departed from the 18-crown-6s. The parent 18-crown-6 has the highest selectivity for potassium and the aromatic substituted 18-crown-6 is the least selective one among the 18-crown-6s. And as far as transport efficiency is concerned, dicyclohexano-18-crown-6 is the most efficient potassium ion transport carrier. At the same time the rubidium ion, is the only ion that resembles potassium in a great deal.

Discussion

For 1:1 crown ether metal ion complexes, as all those appeared in this study, the metal ion is located at the centre of the crown ether cavity which makes the relative sizes of the cation and the cavity the key factor in determining the stability of the complex. Metal ions of sizes either too small or too big will not form stable complexes. The extraction equilibrium constants presented in the results show the trend of this stability. The members of 18-crown-6 series with a cavity diameter around 2.6–3.2 Å, show the highest complexability towards potassium ion (diameter 2.66 Å). 15-Crown-5, with cavity diameter of 1.7–2.2 Å, forms the most stable complex with sodium ion (diameter 1.9 Å).

The members of the 18-crown-6 series do show some minor variations in their extraction equilibrium constants. This variation may have stemmed from the substitution on the skeleton, thus leading to a change in the electron density available at the complexing oxygen. Crown ether with substituted groups containing only aliphatic carbon atoms has higher basicity than that having substituted group with aromatic carbon atoms. This is illustrated by the high extraction equilibrium constant of dicyclohexano-18-crown-6 towards metal ions, while much smaller value for benzo-18-crown-6. The presence of an electron donating methyl group on the benzene ring of the crown ether, however, increases the basicity of the oxygen atoms which has been weaken by the aromatic ring. This is also reflected on the complexability in K_e values determined for 4′-methyl-benzo-18-crown-6 and benzo-18-crown-6.

Complexation may involve a spatial change of the crown ether ring to achieve maximal interaction between the cation and the binding oxygen atoms. Any steric hindrance introduced in the crown ether ring which is unfavorable for a conformational change will decrease the stability of the complex [27]. A lower value in benzo-18-crown-6 than the parent 18-crown-6 may be partly resulted from a steric hindrance introduced by the presence of the bulky benzene ring.

For the monovalent alkali metal ions, the smaller the ionic size, the higher is its solvation. In general, the solvation energy is an inverse function of the ionic diameter in a given group of elements. For highly solvated cations, more energy is needed for the crown ether to compete with the solvent in complexation. The much lower crown ether complexability to lithium ion clearly illustrates this factor. A rule of thumb for these complexes is that stability of the complex usually reflects the complexability.

For ion selectivity, in general, the factors that affect the stabilities of crown ether–cation complexes are also factors that influence the selectivity of the corresponding crown ether towards the ion. A rigid crown ether ring which suits only one specific cation and discriminates against other cations will be highly selective. The fact that similar ion selectivity trends exist in all 18-crown-6s indicates that cavity *versus* ion size effect is the dominant factor in determining complexability. Minor differences among 18-crown-6 family may thus be resulted from the steric factor or the reduction of oxygen

basicity in the ring, as described in earlier paragraphs.

A parallelism found between toxicity and potassium ion transport efficiency indicates the importance of potassium ion (its distribution) in *E. coli.* Contrary to this, sodium ion is not greatly influenced by the presence of crown ether in the synthetic membrane system. In an early publication, we have shown that the *E. coli* growth lag is affected by the presence of 18-crown-6 and that the intracellular potassium ion might be a governing factor in determining cell growth [16]. The importance of potassium was recognized more than two decades ago. Although there are numerous cellular interactions involving potassium which makes its study never too easy, an intensive study on the role of potassium in life process should get started now.

It must be pointed out here that for crown ether facilitated ion transport through organic layer, besides its effect on the stability of the complex, the solubility of the crown ether in the binary phase also affects the extraction efficiency from which the transport values were determined [5]. Although early studies showed that 18-crown-6 compounds do positively facilitate the potassium permeability in both the artificial as well as the natural membranes [12–14], the extraction method with water–chloroform binary system should be considered only as a primitive artificial membrane model for crown ether facilitated ion transport. As the chemical composition of bacterial membrane has proved to be in great similarity to that of other biological membranous systems [28], it is hoped that the crown ether effect on the advance organisms might behave the same. Further study on crown ether interactions in biological systems as well as their potassium effect is in progress in this laboratory.

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