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Inhibition of Stomatal Opening by Cyclic 'Crown' Polyethers in *Commelina Communis*, and a Correlation with Lipophilicity and Bonding

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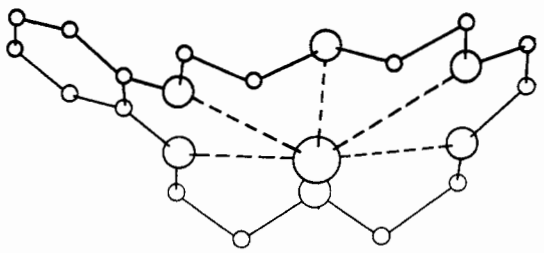
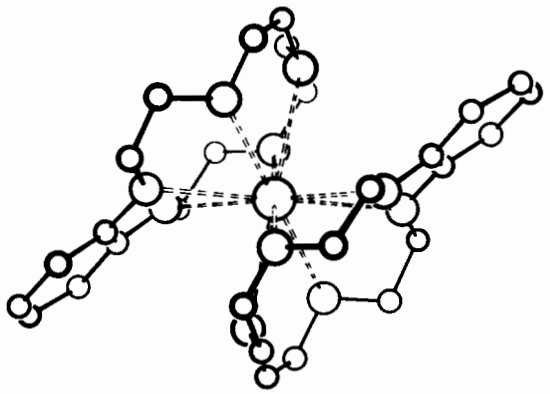
Epidermal peels of Commelina communis *have been treated with three series of cyclic crown polyethers based respectively on substituted 15-crown-5, 18-crown-6 and 30-crown-10 ligands. The concentrations of the crown compounds required just to inhibit stomatal opening under the test conditions correlate well with their lipophilicities measured by partition, and also with bonding stoichiometries for potassium as observed in organic solvents and in isolated crystalline complexes.*

Introduction

In an initial study [1], we reported the effect of a series of macrocyclic 'crown' polyethers on fluxes of potassium (rubidium and caesium) into respiring rat liver mitochondria, a system used for the original work on antibiotic ionophores [2]. Results showed qualitatively that a 1:1 wrap round complexing agent was much more effective in inducing ion uptake than one which would sandwich the cation between two molecules, while compounds which formed complexes holding the cation only in one plane, had relatively little effect (Fig. 1). Within the second two classes there was no quantitative correlation with measured physical properties such as formation constant or ion extraction into chlorobenzene.



(a)



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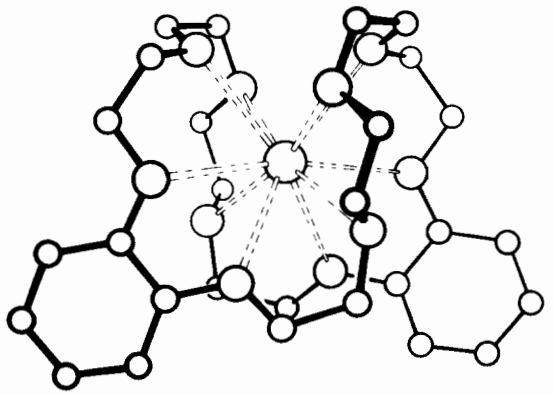

(c)

Fig. 1. Diagram showing three modes of bonding. In each the largest circle represents the cation, the smallest ones show carbon atoms, and the intermediate ones oxygen atoms. The hydrogen atoms are omitted. (1a) A cation surrounded by six oxygen atoms in a plane; (1b) a cation sandwiched between two molecules each containing five oxygen atoms, and (1c) a cation completely enclosed by one molecule containing ten oxygen atoms.

butanol (80:20). We therefore sought another biological membrane system for assay purposes and particularly one from the plant, not the animal, kingdom.

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Potassium ions are known to move into the guard cells of stomata as they open [3–8]. The water potential of the guard cell is lowered and water flows in, increasing the turgor pressure. Because of the radial orientation of microfibrils in the guard cell [9, 10], expansion takes place longitudinally, and as the two ends are fixed, the stomata open by the guard cells bulging outwards. In a preliminary paper, we investigated the effect of benzo-18-crown-6 on the potassium ion flux in guard cells of epidermal peels of *C. communis*, and under test conditions observed a reduction in opening of crown treated peels when compared with controls [11]. In this paper, we have extended the investigation to cover three different ring sizes 15-crown-5, 18-crown-6 and 30-crown-10, which are known to form complexes with potassium by ion–dipole interaction. However, the mode of complexation varies as illustrated in Fig. 1. The partition coefficients of the cyclic polyethers between n-octanol and 50 m*M* aqueous potassium chloride solution have also been determined and compared with the inhibition to stomatal opening caused by the crowns.

Experimental

Materials and Methods

Seeds of *C. communis* were germinated by sandwiching them between damp tissue paper in a shallow beaker kept in the dark. When the seeds had germinated (3–5 days) they were potted in 5″ pots in EFF compost and transferred to a growth cabinet. The seedlings were subjected to a 14 hr photoperiod supplying 125 Wm^{-2} at a light time temperature of 24° falling to 20° during darkness, and kept at a relative humidity of 65%. Demineralised water was used for watering. 4–6 week old plants were used for experimental material and kept in darkness 1 hr prior to peeling. The youngest fully developed leaves were excised, and peels of the lower epidermis were made and cut into 5 × 10 mm strips. These were floated cuticle side up in petri dishes containing 10 ml of 50 m*M* KCl and 10 m*M* MES (4-morpholine-ethanesulphonic acid) buffered at pH 6.15 with potassium hydroxide, and containing a series of concentrations of the cyclic polyether being tested. At this stage, all stomata were closed. The dishes were then illuminated on a light box (21,000 lux), and carbon dioxide-free air bubbled through the solutions at a rate of 0.16 L min^{-1} per dish. After a three hour incubation period, three strips were selected at random from each dish. Stomatal apertures were obtained by taking photographs through a Vickers M17 microscope fitted with an automatic camera attachment, and then making measurements on enlargements from the films. On average, 90 stomatal measurements were made for each dish. In this way, the concentration of crown just to inhibit stomatal opening was found. Some crowns were not sufficiently soluble in water, and therefore were dissolved in the minimum amount of absolute alcohol and then diluted in the buffer solution. The same quantity of alcohol was tested on control epidermal peels and shown to have no effect on stomatal aperture.

Recovery experiments were also carried out using the concentration of crown just to inhibit stomatal opening. These were to determine whether the crown was causing damage to the stomata, and were performed as follows:

Peels (5 × 10 mm) were placed in four dishes, two containing control solutions of 10 ml of 50 m*M* KCl and 10 m*M* MES, and two containing 0.2 m*M* 4-t.-butylbenzo-15-crown-5 in 10 ml of 50 m*M* KCl and 10 m*M* MES. These were then illuminated for 3½ hr in carbon dioxide-free air, after which time stomata from three peels out of each dish were measured. The stomata of the controls were open, but those of the crown treatment were all closed. Three peels from each of the treatment dishes were then quickly washed in control solution (50 m*M* KCl and 10 m*M* MES) and then transferred to two dishes containing fresh control solution. Illumination was continued for 15 hr when the peels originally treated with crown and then transferred to control solution had as wide open stomata as controls illuminated for the same period. Peels left in the crown solution and illuminated during this period had closed stomata (Table I). A similar recovery experiment has been reported earlier for benzo-18-crown-6 [10]. At the concentra-

TABLE I. Stomatal Apertures on Treatment with 0.2 m*M* t.-butylbenzo-15-crown-5.

	3½ hr		18½ hr		
	Control	Treatment	Control	Treatment	Recovery[a]
Number of stomata	60	60	40	40	40
Aperture (μm)	15 9	all closed	18.8	all closed	19.7
Standard error[b]	0.18	–	0.25	–	0.24

[a]Treated for 3½ hr with 0.2 m*M* t.-butylbenzo-15-crown-5, then transferred to fresh control solution. [b]Standard error = standard deviation/$\sqrt{N}$.

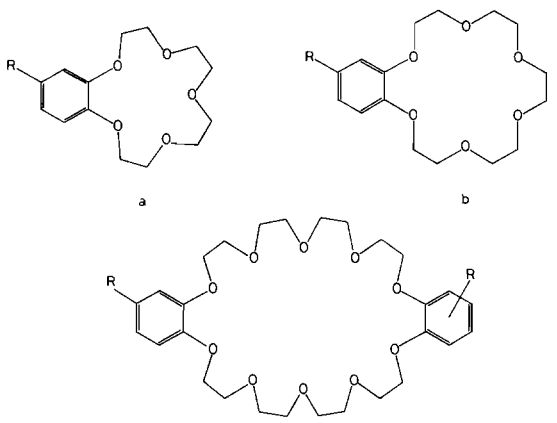

Fig. 2. (a) R = H, t.-butyl; (b) R = H, ethyl, acetyl, carboxy, t.-butyl; (c) R = H, t.-butyl.

tion just to inhibit stomatal opening, the effect is completely reversible, but at higher concentrations treated peels fail to reach the apertures of controls during recovery experiments.

The cyclic polyethers used (Fig. 2) were mostly prepared by literature methods [12]. Preparations of acetylbenzo-18-crown-6, ethylbenzo-18-crown-6 and carboxybenzo-18-crown-6 will be described elsewhere [13]. The polyethers were either sublimed or crystallised to literature melting points before use.

The partition coefficient of crowns between n-octanol and 50 m*M* aqueous potassium chloride solution were determined as follows:

The extinction coefficient of each crown was determined at 273 nm by making 5 m*M* solutions in methanol, diluting and then measuring the solution absorbance using 1 cm matched silica cells on a Cecil CE 5095 UV/visible spectrophotometer. 4 ml of 5 m*M* crown solution in n-octanol was shaken vigorously with 4 ml of 50 m*M* KCl in water in a stoppered test tube for 30 minutes, when a very fine emulsion resulted. The solution was centrifuged for 15 minutes, and then the octanol layer was removed and discarded. The absorbance of the water layer at 273 nm was measured, and hence the concentration in the water layer calculated. By difference, the concentration of crown in the octanol layer was calculated, and hence the partition coefficient $[\text{Crown}]_{\text{octanol}}/[\text{Crown}]_{\text{aqKCl}}$ was determined.

Stability constants were determined as previously described by titrations in Analar methanol at 25 °C by a potentiometric method using a potassium electrode [1]. The titration curves were then converted to stability constants using the Miniquad program [14, 15].

Results and Discussion

50 m*M* potassium chloride and 10 m*M* MES buffer have been shown to give optimal stomatal opening in *C. communis* on illumination in a carbon dioxide-free atmosphere [16]. These conditions were therefore chosen for this study, and the first crown investigated was the water soluble benzo-18-crown-6 which previously had been shown to reduce stomatal opening at a concentration of 10 m*M* [5]. Under the test conditions used here, 12 m*M* benzo-18-crown-6 was found as the concentration required just to inhibit stomatal opening. Unsubstituted 18-crown-6, even at 20 m*M* concentration, had no inhibitory effect on stomatal opening, and carboxybenzo-18-crown-6, at 10 m*M*, showed no inhibition to opening. Ethylbenzo-18-crown-6, however, was much more effective and prevented opening at 1.5 m*M*, and finally t.-butylbenzo-18-crown-6 only required 0.25 m*M* concentration to inhibit opening. The results cannot be explained on the basis of stability constants of the crowns with potassium ions, since 18-crown-6 binds potassium more strongly than does benzo-18-crown-6 and t.-butylbenzo-18-crown-6. However, as the crown becomes more lipophilic, the required concentration for inhibition to stomatal opening decreases. Crystal structure determinations of potassium complexes with 18-crown-6 ligands show the potassium ion in or near the plane of the oxygens of the ring, and it is open to further coordination above and below the ring by solvent or counter-ion [17, 18]. For transport through a membrane, therefore, it may be advantageous to form a sandwich, where two ligands completely shield the cation, and give the complexed cation a totally lipophilic exterior. Experiments with crowns on the uptake of potassium by rat liver mitochondria showed that, although the 18-crown-6 crowns had little effect on the uptake of potassium, they had a greater influence on the uptake of the larger cations rubidium and caesium where a 2:1 sandwich complex ion could be formed [1].

15-Crown-5 rings have invariably been found to give sandwich compounds with potassium [19, 20], and the concentrations required just to prevent stomatal opening reported here are all lower than those found for the corresponding 18-crown-6 ligands. This parallels the results found with the rat liver mitochondria. In addition, the more lipophilic the crown, the lower is the concentration required to be effective.

There is an even more dramatic reduction in the effective concentration for both stomata and rat liver mitochondria when the 30-crown-10 system is studied. Thus, di-t.-butyldibenzo-30-crown-10 prevents stomatal opening at 0.005 m*M* concentration, which is near to the active concentration of abscisic acid, a natural plant inhibitor to stomatal activity. The crystal structure of a dibenzo-30-crown-10 molecule shows it to be capable of enclosing completely a potassium ion, coordinating it with all ten oxygen atoms, and giving a uniform lipohilic

exterior to the complexed cation [21]. As before, the introduction of lipophilic groups greatly enhances the activity of the crown.

The recovery experiments using the concentration of crown required just to prevent stomatal opening show that the influence of the crown is reversible at this concentration. Also guard cells of stomata treated at these concentrations stain cherry red with Neutral Red [22]. These facts suggest that compounds capable of complexing potassium ions and rendering them more lipophilic could be used as anti-transpirants. It is therefore of interest to propose the mode of action of the crowns. It is possible that they act as surfactants, or that they partition into the membrane and act as passive ionophores enabling potassium ions to diffuse down their chemical gradient. Traube's rule [23, 24] states that in a homologous series the addition of each CH_2 group reduces the surface tension of the solution in which the molecule is dissolved by a factor of three. We find only a qualitative fit on this basis, and likewise with calculated partition coefficients. We therefore decided to determine the experimental coefficients of the crowns between n-octanol and aqueous 50 m*M* potassium chloride. n-Octanol has been used in the past as a membrane model [25], and the aqueous

TABLE II. Hansch Treatment.

Compound	$P^{\neq}$	C* in mol	log 1/C	log P
Carboxybenzo-18-crown-6	0.53			−0.28
	0.63			−0.20
	0.43			−0.37
Acetylbenzo-18-crown-6	0.82	1.6×10^{-2}	1.8	−0.09
	0.95			−0.03
	0.96			−0.02
Benzo-18-crown-6	1.37	1.2×10^{-2}	1 92	0.14
	1.84			0.26
	1.10			0.04
Benzo-15-crown-5	4.43	5×10^{-3}	2.3	0.65
	5.73	(2.5×10^{-3})	(2.6)	0.76
	4.96			0.70
Ethylbenzo-18-crown-6	9.49	1.5×10^{-3}	2.82	0.98
	9.57			0.98
	9.47			0.98
t.-butylbenzo-18-crown-6	55.8	2.5×10^{-4}	3.6	1.75
	52 0			1.72
	45.5			1.66
Dibenzo-30-crown-10	80.7	1×10^{-4}	4.0	1.91
	61.3			1.78
	68.8			1.84
	79.8			1.90
	57 0			1.76
Dibenzo-15-crown-5	195.3	3×10^{-4}	3.52	2.29
	187.6	(1.5×10^{-4})	(3.82)	2.27
	118.8			2.07
t.-Butylbenzo-15-crown-5	193.6	2×10^{-4}	3.7	2.29
	177.6	(1×10^{-4})	(4.0)	2.25
	153.4			2.19
	199.6			2.30
di-t -Butyldibenzo-30-crown-10	1592	5×10^{-6}	5.3	3.2
	2026			3.31
	2786			3.44

$^{\neq}$Partition coefficient of crown between n-octanol and 50 m*M* KCl in water. *Concentration of crown in mol at which stomata just fail to open. ( ) Concentration adjusted for two ligands coordinating each potassium ion.

TABLE III. Least Squares Regression.

	1	2
a	0.99	1.02
b	1.79	1.85
R sq	0.93	0.97
Res error	0.08	0.03
Max. abs. res	0.54	0.36

1) Using concentration of crown in the bathing medium. 2) Correcting the concentration of 15-crown-5 ligands to take account of sandwich formation. $C_{eff} = C/2$. For the equation $Y = aX + b$ ($Y = \log 1/C$, $X = \log P$)

$$\text{R square is } 1 - \frac{\Sigma(Y_i - g(X_i))^2}{\Sigma(Y_i - Y)^2} \text{ where } Y = \Sigma Yi/N$$

$$\text{Residual error is } \frac{\Sigma(Y_i - g)X_i))^2}{N - 2}$$

$$\text{Maximum absolute error is } \max(|Y_i - g(X_i)|)$$

50 m*M* KCl solution parallels that used in the stomatal bathing medium. The Hansch approach was then applied by testing an empirical fit of the data to eqn. 1 [25],

$$\log 1/C = a \log P + b \quad (1)$$

where C is the concentration in mol required to bring about a particular response (in this case just to prevent stomatal opening), P is the partition coefficient, and *a* and *b* are constants. Table II shows the experimental values for C and P, and the calculated log 1/C and log P. A plot of log 1/C against log P gives a reasonable straight line, and Table III shows the constants obtained when the data are subjected to a least squares regression analysis. When the concentrations of the 15-crown-5 ligands are adjusted for the fact that two molecules of crown are required to coordinate each potassium ion (values in brackets, Table II) then the data give a very good straight line fit (Table III). If the measured partition coefficients and the limiting crown concentrations are now used to calculate the apparent concentration of crown in the membrane, then the values in Table IV are obtained. Within experimental error, these are remarkably similar, especially when the 15-crown-5 series is adjusted for sandwich type bonding. Thus, the mode of action seems to involve partition of the crown into the membrane, and as the concentrations correlate well with the known binding stoichiometries for potassium, the crowns appear to be acting in the same way as the neutral ionophores valinomycin and nonactin [26–31]. The concentration of carboxybenzo-18-crown-6 needed just to inhibit stomatal opening may be calculated from the derived line at a value of 31 m*M*. This explains why there is no detectable response at 10 m*M*. The partition coefficient of 18-crown-6 cannot be determined by the UV/visible spectrophotometric method, but it is very soluble in water, again indicating that a high concentration would be required to inhibit stomatal opening.

There is apparently little effect due to the known differences in stability constants of the crowns for potassium (Table V) on the effective concentration for stomatal activity. Previous studies on ion transport in organic phases suggest that there is an optimum value for the stability constant for maximum rate of transport [32, 33], so the values for the ligands used in our study may not be sufficiently

TABLE IV. Effective Membrane Concentrations.

Compound	Calculated effective membrane concentration* (m*M*)
Acetylbenzo-18-crown-6	14.6
Benzo-18-crown-6	17.2
Benzo-15-crown-5	25.2 (12.6)[a]
Ethylbenzo-18-crown-6	14.3
t.-Butylbenzo-18-crown-6	12.8
Dibenzo-30-crown-10	7.0
Dibenzo-15-crown-5	50(25)[a]
t.-Butylbenzo-15-crown-5	36.2 (18.1)[a]
di-t.-Butyldibenzo-30-crown-10	10.7

*Calculated from effective bathing solution crown concentration and the octanol/aqueous potassium chloride partition coefficient. [a]( ) concentration adjusted for two ligands coordinating each potassium ion.

TABLE V. Log_{10} Stability Constants Measured in Methanol at 25 °C with Potassium Bromide.[a]

Ligand	Stability Constant	
	1:1	2:1 (ligand/metal)
Carboxybenzo-18-crown-6	4.9	
Acetylbenzo-18-crown-6	4.93	
Benzo-18-crown-6	5.2[b]	
Benzo-15-crown-5		5.9[b]
Ethylbenzo-18-crown-6	5.2	
t.-Butylbenzo-18-crown-6	5.12	
Dibenzo-30-crown-10	4.94[b]	
t.-Butylbenzo-15-crown-5		6.07[b]
Dibenzo-15-crown-5		5.7
di-t.-Butyldibenzo-30-crown-10	4.83[b]	

[a]Values quoted are the $\log_{10}K_1 = [(M.\ crown)^+]/[M^+][crown]$ and $\log_{10}\beta_2 = [(M{\cdot}2crown)^+]/[M^+][crown]^2$. [b]Values as given in reference 1.

different to overcome the obvious lipophilic effect. We are therefore extending the study to both more powerful and weaker potassium ion complexing agents. Work in the past has also been carried out on the variation of potassium ion concentration in guard cell and subsidiary cell with changing stomatal aperture [8, 34], and we have attempted to measure guard cell potassium ion concentrations by electron probe analysis in order to determine any potassium ion fluxes on treatment with cyclic polyethers. The method involved plasma ashing the samples prior to measurement, but this produced very variable results from one stoma to the next. We are in the process of modifying the method, and will report the results in due course.

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