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Proton-driven Ion Transport and Metal-assisted Amino Acid Transport with an Anion-capped Azacrown Ether[1)]

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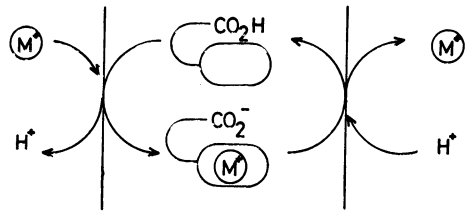
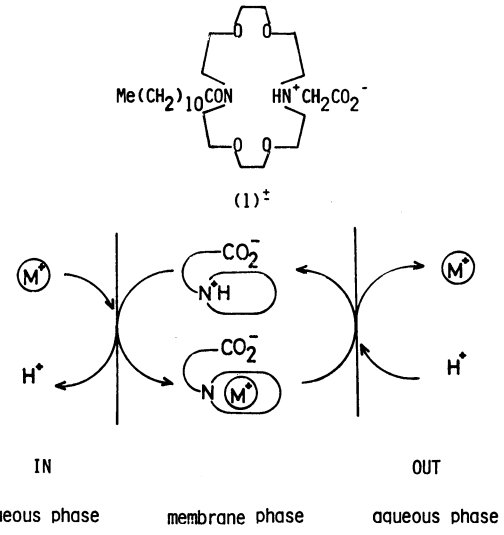
We have found that 1,10-diaza-4,7,13,16-tetraoxa-18-crown-6 with a carboxylate cap and a hydrophobic dodecanoyl group (**1**) acts as a new class of ion carrier for membrane transport. The rate of K^+ transport across a chloroform liquid membrane was significantly affected by pH of the OUT aqueous phase: The rate decreased with increasing or decreasing pH, providing a maximum at around pH 5. The main species of **1** in the neutral pH region should be zwitterionic $\mathbf{1}^{\pm}$, so that one may rationalize the pH-rate profile in terms of (i) "enforced ion release" due to protonation of the ring nitrogen and (ii) high lipophilic nature of zwitterionic $\mathbf{1}^{\pm}$. The efficiency of **1** was further demonstrated by active K^+ transport from the basic IN aqueous phase to the neutral OUT aqueous phase. Also, **1** was capable of carrying amino acids in the presence of alkaline earth metal cations. These results suggest that, being different from conventional anion-capped crown ethers, the aza crown ethers carrying the *N*-substituted carboxylate cap exhibit a new class of ionophore functions.

Cations are known to be transported through biological membranes with the aid of antibiotics.[2,3)] In particular, polyether antibiotics such as nigericin and monension feature "enforced ion-release" and coupled counter-transport of cations and protons due to interconversion between cyclic and acyclic forms.[2-5)] Although coupled counter-transport can be mimicked in an artificial system using anion-capped crown ethers,[6-8)] acyclic polyethers,[9-13)] and metal complexes,[14-16)] few of them permit as ready an enforced ion release as do the polyether antibiotics.[17,18)] As show in Scheme 1, for example, the conventional anion-capped crown ethers mediate the ion-transport, the basic functions of these carriers being related only to the ion-binding ability due to charge neutralization by an intramolecular anionic cap.



Scheme 1.

Recently, several new ionophores have been synthesized from 1,10-diaza-4,7,13,16-tetraoxa-18-crown-6 which is commercially available.[19-24)] In order to attain the "facile enforced ion-release" like natural ionophores, we sythesized an ionophore **1** for which the following novel behaviors are expected (Scheme 2): (i) Ion-release is enforced by protonation of the ring nitrogen, (ii) the main species in the neutral pH region, $\mathbf{1}^{\pm}$, should be highly lipophilic owing to its zwitterionic structure, and (iii) the counter-current of protons should be mediated by the ring nitrogen but not by the carboxylato group and should occur from the neutral OUT aqueous phase. With these expectations in view, we carried out the proton-driven ion-transport and the metal-assisted amino acid transport through a liquid membrane.



Scheme 2.

Experimental

Materials. 1-Dodecanoyl-10-carboxymethyl-1,10-diaza-4,7,13,16-tetraoxa-18-crown-6 (**1**) was prepared by mono-*N*-alkylation of 1,10-diaza-4,7,13,16-tetraoxa-18-crown-6 with chloroacetic acid, followed by acylation of the remaining amine with dodecanoyl chloride. The aqueous solution (20 ml) containing 1.19 g (12.5 mmol) of chloroacetic acid was adjusted to pH 9.0 with sodium carbonate. After the addition of 3.00 g (11.4 mmol) of 1,10-diaza-4,7,13,16-tetraoxa-18-crown-6, the reaction mixture was heated at 90—100 °C with stirring. The progress of the reaction was followed by the potentiometric titration of the aliquot withdrawn from the solution with silver nitrate. The titration showed that the stoichiometric amount of Cl^- was produced after 8 h. The reaction mixture was diluted to 100 ml with water and adjusted to pH 11 with sodium carbonate. The aqueous solution was extracted with chloroform to remove the unreacted diaza crown ether. The aqueous layer was concentrated *in vacuo*, the residue being taken with absolute ethanol. Thus, 3.47 g of the oily product (monocarboxymethylated diaza crown ether) was obtained. The HPLC analysis showed that the product contained a

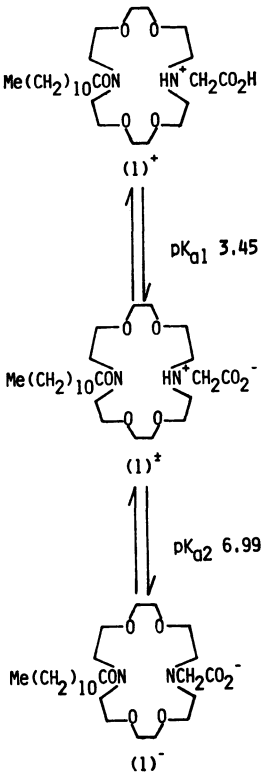
small amount of bis(carboxymethylated) diaza crown ether, but we used the oil without further purification. 3.47 g of the oil was dissolved 34 ml of *N,N*-dimethylformamide (DMF) containing 4 ml of triethylamine. While stirring the DMF solution at room temperature, 2.50 g (11.4 mmol) of dodecanoyl chloride in 10 ml of DMF was added dropwise. After the addition, the reaction mixture was heated at 60 °C for 1 h. The solution was diluted with 50 ml of water, extracted with chloroform, and the chloroform solvent was evaporated to dryness. The oily residue was dissolved in water adjusted to pH 6.5 and extracted with chloroform. The chloroform solution was concentrated *in vacuo*. Finally, the product (**1**) was isolated by the TLC separation (silica gel, chloroform: ethanol=4 : 1 in volume); oil, yield 16.7%. NMR ($CDCl_3$): CH_3, 0.88 ppm (3H); $(CH_2)_9$, 1.28 ppm (18H); $NCO-CH_2$, 2.30 ppm (2H); $C-N-CH_2$ (ring proton), 2.76 ppm (4H); $N-CH_2-CO$, 3.12 ppm (2H); OCH_2, 3.64 ppm (16H); $CO-N-CH_2$, 3.80 ppm (4H). IR (KBr disk) of the sodium salt; $\nu_{C=O}$ 1630 and 1600 cm^{-1}, ν_{C-O-C} 1100 cm^{-1}. Found: C, 59.42; H, 10.04; N, 4.95%. Calcd for $C_{26}H_{49}N_2O_7Na$: C, 59.21; H, 9.77; N, 5.31%.

Method of Ion-transport. The ion-transport was carried out using a U-tube apparatus immersed in a thermostated water bath (30 °C). The transport system consists of 50 ml of a liquid membrane phase (chloroform) and 25 ml of IN and OUT aqueous phases in a U-tube with 2.5 cm diameter. The membrane phase was stirred slowly at a constant speed (180 rpm). The concentrations of alkali and alkaline earth metal cations were determined by an atomic absorption spectrophotometer (Shimadzu AA-640), while those of amino acids were determined by the treatment with fluorescamine.[25] Usually, the sampling was continued for 12 h at intervals of 1 h. After an induction period (about 1 h) a linear increase in metal (or amino acid) was observed, from which we determined the transport rates across a chloroform membrane barrier.

Results and Discussion

Transport of Alkali and Alkaline Earth Metal Cations. Potentiometric titration of **1** (30 °C) gave two pK_a values: $pK_{a1}=3.45$ and $pK_{a2}=6.99$. The fraction of zwitterionic $\mathbf{1}^{\pm}$ thus becomes maximum at pH 5.22 ($=(pK_{a1}+pK_{a2})/2$). As a preliminary step to ion-transport, we estimated the partition of **1** between chloroform and aqueous solution by its absorbance at 270—300 nm which is on the lower slope of the absorption band of **1**. The partitions of **1** to the chloroform layer after agitation with equal volumes of following aqueous solutions were: 90% with 0.01 M HCl,† 96% with pH 5.23 (0.05 M Tris–AcOH), 73% with 0.01 M Et_4NOH, and 99% with 0.01 M KOH. The result indicates that the solubility of **1** in chloroform is in the order, $\mathbf{1}^-$–K^+ complex$>\mathbf{1}^{\pm}>\mathbf{1}^+>\mathbf{1}^-$.

The transport of alkali and alkaline earth metal cations across a chloroform membrane was studied using a U-tube apparatus immersed in a thermostated water-bath (30 °C). The typical experimental results are summarized in Table 1. In passive transport of K^+ (Runs No. 1—5), the rate of K^+ transport gave a maximum when the OUT aqueous phase was maintained in the neutral pH region. When the OUT aqueous phase was strongly basic or acidic, the rates of K^+ transport were significantly suppressed. The plot of K^+ transport rate *vs.* pH of the OUT aqueous phase is well correlated with the pH-dependence of the fraction of zwitterionic $\mathbf{1}^{\pm}$ (Fig. 1). These results establish that the best K^+ transport system consists of the coupled counter-transport of K^+ and protons by interconversion between the $\mathbf{1}^-$–K^+ complex (IN→OUT) and $\mathbf{1}^{\pm}$ (OUT→IN) (see Scheme 2).



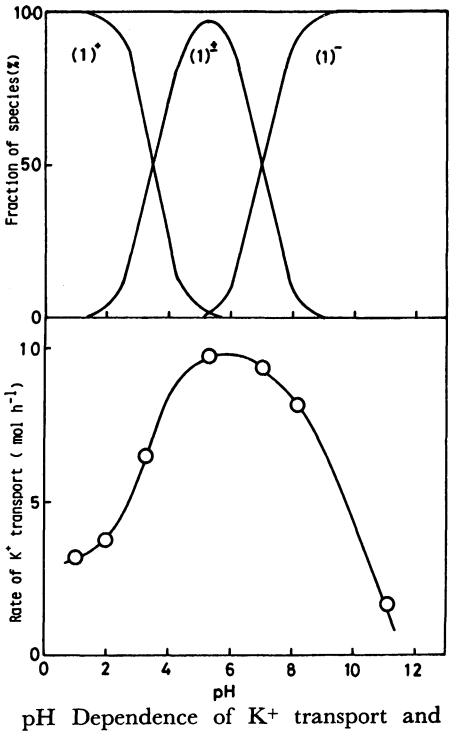


Fig. 1. pH Dependence of K^+ transport and carrier species.

† 1 M=1 mol dm^{-3}.

Table 1. Ion transport of alkali and alkaline earth metal cations with carrier **1**

Run No.	IN Aqueous phase (c/mM)	[**1**]	pH or additive in OUT aqueous phase(c/mM)	Rate of ion-transport (μmol h^{-1})	Relative rate
1	KOH(10)	1.0	HCl(100)	3.25	1.9
2	KOH(10)	1.0	HCl(10.0)	3.80	2.2
3	KOH(10)	1.0	pH 5.24[a]	9.80	5.8
4	KOH(10)	1.0	pH 6.99[b]	9.40	5.5
5	KOH(10)	1.0	Et_4NOH(10.0)	1.70	1.0
6	NaOH(10)	1.0	pH 6.99[b]	2.51	1.5
7	KOH(10)	0.50	KCl(10)+HCl(50.0)	$\approx$0	0
8	KOH(10)	0.50	KCl(10), pH 5.24[c]	0.97	0.57
9	$CaCl_2$(8.9)[d] $Ca(OH)_2$(2.0)	1.0	HCl(10.0)	2.33	1.4
10	$CaCl_2$(8.9)[d] $Ca(OH)_2$(2.0)	1.0	pH 5.25[c]	0.95	0.56
11	$CaCl_2$(8.9)[d] $Ca(OH)_2$(2.0)	1.0	Et_4NOH(10.0)	$\approx$0	0
12	$Ba(OH)_2$(12.5)	1.0	pH 5.25[c]	22.6	13.3

a) Buffer: tartarate (50 mM) and Tris. b) Buffer: Tris (50 mM) and HCl. c) Buffer: AcOH (50 mM) and Tris. d) Picric acid (3.8 mM) was added. e) Picric acid (4.3 mM) was added.

Being different from the conventional ion transport with anioncapped carriers (Scheme 1), the transport with (**1**) features that (i) the ion release is enforced by protonation of the ring nitrogen and (ii) proton flux in the counter-current is carried by the ring nitrogen but not by the carboxylate cap. The fact that the rate of the ion-transport is dependent upon the pH of the OUT aqueous phase supports the involvement of the ion-release step in the rate-limiting step. Conceivably, the ion-release is a slow, rate-limiting step when the OUT aqueous phase is basic, whereas the ion-release is facilitated by the "enforced" ion-release due to protonation of the ring nitrogen when the OUT aqueous phase is neutral. A similar enforced ion-release may be expected for the acidic OUT aqueous phase. However, $\mathbf{1}^+$, the main species in acidic pH region, is less soluble in the membrane phase, so that overall transport efficiency is inferior to that of $\mathbf{1}^\pm$.

The proton-driven active transport of K^+ demonstrates the high transport efficiency of $\mathbf{1}^\pm$ more clearly. As recorded in Table 1 (Runs No. 7, 8), the net active transport from the basic IN aqueous phase to the neutral OUT aqueous phase took place (0.97 μmol h^{-1}), whereas that to the acidic OUT aqueous phase was not observed at all. The result implies that only $\mathbf{1}^\pm$ is capable of serving as a recycle ion carrier and carring K^+ against its concentration gradient. As described in introduction, the $\mathbf{1}^-$–K^+ complex is converted to $\mathbf{1}^\pm$ when it contacts with the neutral OUT aqueous phase. Since $\mathbf{1}^\pm$ is highly lipophilic, the species is easily carried back to the side in contact with the IN aqueous plase (see Scheme 2). In case the OUT aqueous phase is strongly acidic, $\mathbf{1}^+$ is the main species after the ion-release. In order to stay in the membrane phase, $\mathbf{1}^+$ must extract some anion, X^-(probably Cl^-) from the OUT aqueous phase. Although we did not estimate the extraction of anion from the acidic aqueous phase, the result in Run No. 7 suggests that the counter-current of $\mathbf{1}^+X^-$ is not sufficient enough to mediate the active transport.

In passive transport, Na^+ was carried less effectively than K^+. This is attributed to the higher affinity of the diaza crown ring toward K^+ in preference to Na^+. Similarly, Ca^{2+} was carried less effectively than Ba^{2+}. Interestingly, Ca^{2+} was transported to the acidic OUT aqueous phase more efficiently than to the neutral OUT aqueous phase. This implies that the enforced Ca^{2+} release occurs only from $\mathbf{1}^+$ and the protonation of the ring nitrogen is not enough to eject the bound Ca^{2+} ion. The trend is quite different from that of K^+ transport. The difference probably stems from the high affinity of Ca^{2+} with the carboxylato group.[2,6]

Amino Acid Transport with ***1****–Metal Complexes.* The transport of amino acids across biological membranes is assumed nowadays to be coupled with Na^+ transport.[26] The transport of amino acids has been investigated by several groups,[27–31] but they mostly employed protected amino acids instead of biologically relevant "free" amino acids. An efficient transport system of free amino acids was reported by Behr and Lehn,[27] who demonstrated that a cationic surfactant is useful for the transport from the basic IN aqueous phase to the acidic OUT aqueous phase, while an anionic surfactant is useful for the transport from the acidic IN aqueous phase to the basic OUT aqueous phase.

It occurred to us that zwitterionic $\mathbf{1}^\pm$ may carry zwitterionic amino acids from the neutral IN aqueous phase to the basic OUT aqueous phase, while alkali metal cations may be carried back from the OUT to the IN aqueous phase. If it occurs, the system has characteristic, like the biological system, of the amino acid transport coupled with the counter-current of alkali metal cations. We thus measured the rate of the transport of phenylalanine from the neutral IN aqueous phase to the basic OUT aqueous phase containing KOH.[32] However, phenylalanine was not transported in an appreciable velocity. This implicates the difficulty

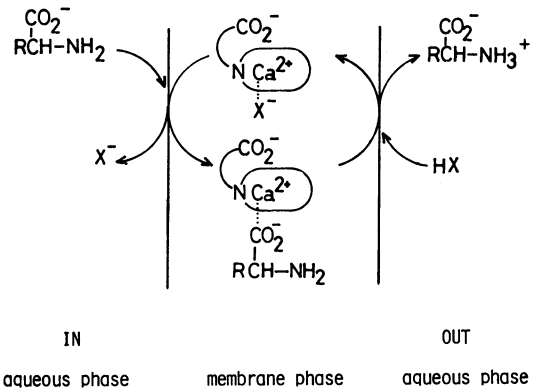
TABLE 2. TRANSPORT OF AMINO ACIDS WITH CARRIER **1**[a)]

Run No.	IN Aqueous phase (c/mM)		OUT Aqueous phase (c/mM)	IN → OUT (mol h^{-1})		OUT → IN (μmol h^{-1})
	Amino acid	Metal		Amino acid	Ca^{2+}	Li^+
13	Phe(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	pH 2.6[b)]	0.12	1.67	0
14	Phe(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	pH 8.0[b)]	0.84	0.81	0.038
15	Phe(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	pH 8.0[c),d)]	0.90	1.13	0.054
16	Phe(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	LiOH(6.25)	1.33	0	2.08[e)]
17	Phe(20)	$BaCl_2$(150), $Ba(OH)_2$(50)	pH 8.0[c)]	0.86	1.08[g)]	0.050
18	Phe(20)	KOH(200)	pH 8.0[c)]	0.031	2.14[f)]	0.052
19	Phe(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	KOH(6.25)	0.060	0	0.625[f)]
20	Leu(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	pH 8.0[c)]	0.079	1.70	0.047
21	Ala(20)	$CaCl_2$(190), $Ca(OH)_2$(10)	pH 8.0[c)]	0	1.30	0.035

a) Chloroform membrane phase: [**1**]$=1.0\times10^{-3}$ M. b) Buffer: H_3PO_4 (50 mM) and LiOH (6.25 mM). c) Buffer: H_3BO_3 (50 mM) and LiOH (6.25 mM). d) [$LiClO_4$]$=1.0$ mM in the OUT aqueous phase. e) The rate of Li^+ transport decreased with time and became undected after 5 h. 2.08 μmol h^{-1} is the initial rate of Li^+ transport. f) Rate of K^+ transport. g) Rate of Ba^{2+} transport.

to extract zwitterionic amino acids to the membrane phase.

Here, a new idea arises that the **1**$^-$–Ca^{2+} complex may be capable of extracting free amino acids from the basic IN aqueous phase and carrying them to the slightly basic OUT aqueous phase, because Ca^{2+} is scarcely ejected to the basic OUT aqueous phase (Table 1) and the extremely low solubility of zwitterionic amino acids in the membrane phase is rather favorable in the ion-release process. We thus constructed an amino acid transport system with the OUT aqueous phase adjusted mainly to pH 8.0, expecting the **1**$^-$–Ca^{2+} complex to extract an anionic amino acid from the basic IN aqueous phase as a counteranion and to release it to the slightly basic OUT aqueous phase as a zwitterionic amino acid (Scheme 3). The pH of the OUT aqueous phase was adjusted with boric acid and LiOH. LiOH was selected because of its low affinity for **1**. The results are summerized in Table 2.



Scheme 3.

Experimental Runs No. 13—16 show the effect of pH in the OUT aqueous phase on the rate of phenylalanine transport. The maximum rate was observed for the strongly basic OUT aqueous phase where Ca^{2+} is not released at all (Run No. 16). Usually, the Li^+ concentration in the IN aqueous phase increased linearly with time. In Run No. 16, however, the counter-current of Li^+ was very fast in the initial stage but became undetected after 5 h. This implies that all the carrier molecules are occupied by Ca^{2+} after 5 h. When the OUT aqueous phase was adjusted to pH 8.0, the rate of phenylalanine transport was somewaht slower than that observed for the basic OUT aqueous phase and Ca^{2+} was released in a speed comparable with phenylalanine. These results show that the release of phenylalanine is not necessarily facilitated in its zwitterionic structure. We added $LiClO_4$ to the OUT aqueous in order to estimate the influence of the X^- concentration. Run No. 15 shows, however, that the effect is almost negligible. In case K^+ was used instead of Li^+ (compare Run No. 19 with Run No. 16), the rate of phenylalanine transport was reduced by 14 fold. Since K^+ is efficiently bound to **1**$^-$ in the counter-current path, the rate suppression is attributed to the exchange between K^+ and Ca^{2+} at the interface between the membrane and the IN aqueous phase.

Runs No. 17 and 18 demonstrate that the phenylalanine transport is mediated by Ba^{2+} but not by K^+. In other words, only the **1**$^-$–alkaline earth metal complexes are capable of extracting phenylalanine. The comparison of Runs No. 10 and 20 with Run No. 14 reveals that the transport rate is associated with the hydrophobicity of amino acids: The rate of leucine transport is smaller by one order of magnitude than that of phenylalanine transport, and the rate of alanine

transport is undetected in the present experimental precision.

Concluding Remark. The present paper has demonstrated a new class of ion transport involving the rate acceleration due to the enforced ion release and an amino acid transport system with the $\mathbf{1}^-$–metal complexes. The former system well mimicks the basic functions of the natural antibiotics, while the latter system seems still incomplete to call a biomimetic amino acid transport system. We believe, however, that the crown–metal complexes would have greater possibilities as amino acid carriers than simple phase transfer catalysts. Further study is now continued in this laboratory.

References

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31) Recently, Sunamoto *et al.* have found that spiropyrans carry free amino acid under photoirradiation.
32) Membrane phase: $[\mathbf{1}]=1.0\times10^{-3}$ M. OUT aqueous phase: $[KOH]=1.0\times10^{-2}$ M. IN aqueous phase: [phenylalanine]$=1.0\times10^{-1}$ M, pH 5.26 with tartrate (5.0×10^{-2} M)–triethanolamine. The significant increase in the concentration of phenylalanine was not detected in the OUT aqueous phase.