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Conductivity, Emf, and Spectrophotometric Study on the Participation of Na^+ and Cl^- into Acridine Orange-Poly(α,L-Glutamic Acid) Complex

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Conductivity, emf and spectroscopic investigations were made for the dimerization process of Acridine Orange (AO) and for the complex formation of AO with poly(α,L-glutamic acid) (PLG) in water. The increase of the dimer species of AO by adding the neutral salt was quantitatively explained, taking the ionic strength into account. From the emf measurements, the inclusions of Cl^- into PLG–AO complex were concluded to be negligible, whereas Na^+ was included into this complex to a fairly appreciable extent. The association of Na^+ with carboxylate groups in the PLG–AO complex was described on the basis of the ordinary law of mass actions. The association constant K_1 was unaffected by the presence of AO even in the helical region of PLG. These results strongly support Schwarz's theory on cooperative binding.

Many authors have studied the interactions between Acridine Orange (AO) and a number of polyelectrolytes from various standpoints.[1–7] These investigations have shown that the addition of electrolytes affects the properties of the poly(α,L-glutamic acid) (PLG)–AO complex such as absorption spectra, circular dichroism, helix-coil transition, and so on.

To interpret the effects of Cl^- on the PLG–AO complex, two different assumptions were adopted. Sato *et al.* assumed that (i) Cl^- associates with AO dimer in the following manner:[5,8]

$$2AO^+ + Cl^- \rightleftharpoons (AO)_2Cl^+ \qquad (1)$$

and then (ii) this $(AO)_2Cl^+$ is bound to the ionized carboxyl groups ($-COO^-$) of PLG. On the other hand, Schwarz *et al.* have assumed that AO dimer does not involve Cl^-.[3,4,9,10]

$$AO^+ + AO^+ \rightleftharpoons (AO)_2^{++} \qquad (2)$$

Lamm and Neville, however, have found that it is impossible to distinguish the two mechanisms above by spectral means alone.[11]

To interpret the kinetic results for the reaction between AO and PLG, Schwarz offered the cooperative binding theory in which the binding of Na^+ to $-COO^-$ plays an important part in determining the rates.[2,3] He assumed that (i) AO^+ binds to $-COO^-$ cooperatively, while Na^+ binds to $-COO^-$ non-cooperatively and (ii) and Na^+ bind to $-COO^-$ competitively one another.

Now the following questions arise:

(a) What kind of a participation of Cl^- occurs when AO self-associates to dimer species?

(b) How many Cl^- and/or Na^+ ions are included into PLG–AO complex?

(c) Are Schwarz's assumptions above described correct?

No complete study, however, has been made on the equilibrium among many ionic species in the present system. In order to answer the questions above, the present paper reports the results obtained by conductivity, emf and spectrophotometric methods.

Experimental

Materials. Sodium salt of PLG (Na·PLG) purchased from Protein Research Foundation was used without further purification. The molecular weight of the sample (66000 in the acid form) was deduced from the intrinsic viscosity (1.42 dl/g) in 0.2 M NaCl at pH 7.3, using the calibration determined by Wada.[12] The concentration of the glutamyl residue was calculated using a value of 169 for the mean residue weight of monosodium glutamate monohydrate.

Acridine orange hydrochloride (AO·HCl), purchased from Wako Fine Chemical was recrystallized five times from water-methanol mixture. The apparent molar absorption coefficient at 492 nm was $5.0\times10^4\ M^{-1}\ cm^{-1}$ under the following condition; $[AO]=1.0\times10^{-5}$ M, pH=6.0 and 293 K. The elemental analysis showed that the AO·HCl purified in this way contained 3% NaCl as a contaminant. For the measurement of the activity of Cl^-, the total amount of Cl^- added was corrected by this excess Cl^-. The conductivity of AO-HCl solution was corrected in the following way:

$$k(\text{corrected}) = k(\text{observed}) - k(\text{excess NaCl})$$

where k(corrected) and k(observed) denote a corrected specific conductance and an observed specific conductance, respectively. k(excess NaCl) was the specific conductance of the solution containing the same amount of NaCl only as in the sample solution. For the spectroscopic measurements, this excess Cl^- was neglected, considering that an added NaCl was in large excess at least by a factor of 100.

Acridine orange zinc chloride $AO\cdot HZnCl_3$ (Merck) was three times recrystallized from ethanol. Other materials were reagent grade. Water was deionized and distilled once.

Instruments. The absorption spectra were obtained by a Hitachi EPS-3T recording spectrophotometer. The emf was measured with a Radiometer "PHM-4d." The electrochemical cells used for the measurements of Cl^- and Na^+ activities are

$$\begin{array}{l|c||l||c} HgCl_2(s)/Hg & \text{satd KCl} & \begin{array}{l} AO\cdot HCl \\ \text{or} \\ AO\cdot HCl+Na\cdot PLG \end{array} & Ag/AgCl \end{array} \qquad (3)$$

and

$$\begin{array}{l|c||l|l} HgCl_2(s)/Hg & \text{satd KCl} & \begin{array}{l} Na\cdot PLG+ \\ \quad AO\cdot HCl \\ \text{or} \\ Na\cdot PLG \end{array} & \begin{array}{l} Na^+ \text{ selective} \\ \text{glass electrode} \end{array} \end{array} \qquad (4)$$

respectively. A Na^+ selective glass electrode No. 1512-05T was offered by the Horiba Co., Ltd. A silver-silvechloride electrode was prepared by slowly depositing crystalline silver electrolytically onto a platinum wire sealed into a pyrex glass tube. The silver chloride was formed by electro-deposition in a 0.1 M aqueous solution of hydrochloride with the silvered platinum as the anode.[14] Horiba No. 2010A was used as a saturated calomel electrode. The reproducibility of the emf measurements was within ±1 mV.

Conductivity was measured with a universal bridge BV-Z-3B (Yokogawa Electric Work Ltd.) at a frequency of 1 kHz, using an ordinary conductivity cell whose cell constant was 0.45. The pH values of the solutions were measured with a Horiba M-7 pH meter.

Preparations and Measurements. All the measurements were performed at 298±0.5 K. The pH values in the Na·PLG–AO·HCl solutions were adjusted at pH=4.53 (±0.03) and pH=7.51 (±0.03). 5 mM sodiu macetate-acetic acid buffer and 5 mM phosphate buffer were used for the measurements of Cl^- activity. For the measurements of Na^+ activity, 5 mM tetraethylammonium $(Et)_4N^+$ acetate-acetic acid buffer and 5 mM $(Et)_4N^+$-phosphate-phosphoric acid buffer were used. Considering that tetrapropylammonium ion is known to bind only slightly to polyanion,[15] the effect of $(Et)_4N^+$ on the association of Na^+ with $-COO^-$ was neglected. The activity of Na^+ was determined by comparing the measured emf values at each pH with those for NaCl solutions adjusted to pH=4.53 or pH=7.51 by the corresponding buffer.[13]

The specific conductivity of water used was $2\times10^{-6}\ \Omega^{-1}\ cm^{-1}$. The equivalent conductivity was obtained by the usual procedures[16] and was reproducible to about ±(5—10) $\Omega^{-1}\ mol^{-1}\ cm^2$ on repeated measurements.

The dimerization constant K_d of AO was obtained by the same procedure as Schwarz and Balthasar.[3]

Results and Discussion

I) *The Role of Cl^- on the Dimerization Process of AO.*

A) Conductance Measurements of AO–HCl: It is well known that the increase of the concentration of the aqueous AO causes the spectral change due to the increase of the aggregates containing more than two AO molecules.[5,11,17] In Fig. 1 the equivalent conductivity Λ is plotted against the square root of the concentration of the aqueous AO·HCl. Λ increases as [AOHCl] increases at first and then reaches the maximum value. If AO·HCl behaves as a univalent electrolyte, Λ would show a linear depence on [AO–HCl] as shown in Fig. 1 with a dashed line (A) in arbitrary unit. If the association between AO aggregates and Cl^- occurs, Λ of AO–HCl would fall down more rapidly than the case of (A). The increase of Λ at the concentration region from 2×10^{-4} M to 8×10^{-4} M, therefore, may be explained by the formation of the AO aggregates which scarcely contain Cl^- ions. An extrapolation of this curve to [AO·HCl]=0 gives a rough value to 85; Λ_0(AO·HCl)=85. Substracting the limiting equivalent conductance of Cl^-, $\Lambda_0(Cl^-)=76$,[16] from this value, we have the coarse limiting equivalent value of AO^+, $\Lambda_0(AO^+)=7$. Adopting this value, $\Lambda_0(AO^+)=7$, to the Stokes equation,[18] an apparent diameter of AO^+ is calculated to be $r=10$ Å.

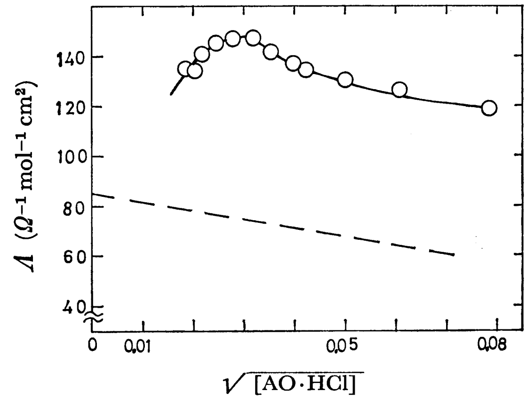

Fig. 1. The equivalent conductivity Λ of the aqueous AO·HCl plotted against square root of the concentration; ---- represents the equivalent conductivity in arbitrary unit, assuming that AO·HCl behaves as "strong electrolyte."

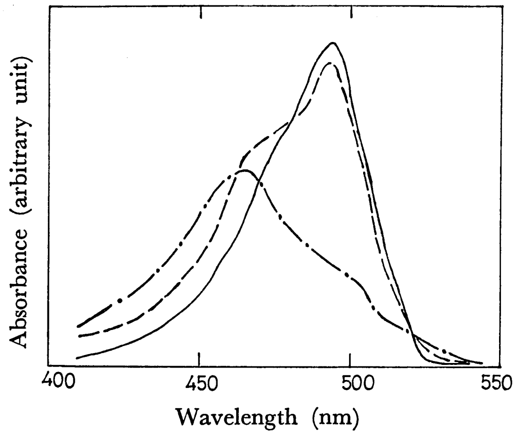

Fig. 2. Solvent dependence of AO-HCl spectrum: at 298 K.
—— in water/methanol 30/70 wt% mixture at [AO·HCl]$=3\times10^{-4}$ M. ---- in water at [AO·HCl]$=1\times10^{-5}$ M. —·— in water at [AO·HCl]$=5\times10^{-4}$ M. Coordinate is represented in arbitrary unit.

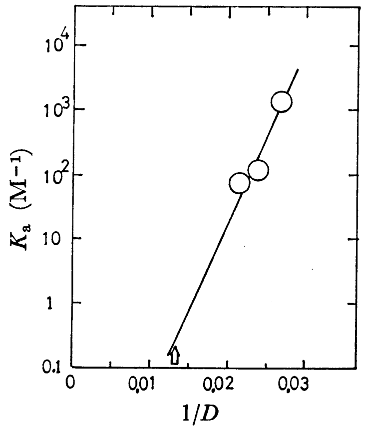

Fig. 3. The logarithm of the association constant K_a between AO^+ and Cl^- as a function of the reciprocal of the dielectric constant D. The notation ↑ indicates the point at $D=78.5$, which corresponds to that of water.

The conductivity measurements of AO–HCl were performed in water–methanol mixture at H_2O/CH_3OH $=30/70$, 20/80 and 10/90 (in wt.%). As shown in Fig. 2, the spectra of AO in these solvents clearly indicate that AO is present as monomer species.[17] Therefore the association constants, K_a, in the following equilibrium were obtained by the Shedlovsky's method.[19,20]

$$AO^+ + Cl \rightleftharpoons AOCl;\ K_a \tag{5}$$

As shown in Fig. 3, the logarithm of K_a linearly decreases with the decrease of the reciprocal of the dielectric constant ($1/D$). From this linearity, it is reasonable to presume that even in water K_a should be dominated by a function of D in a way predicted by Bjerrum's theory.[16] Extrapolating the linear plot of log K_a *vs.* $1/D$ to the point $D=78.5$ which corresponds to the dielectric constant in water at 298 K, we obtained a rough value of K_a in water; $K_a=0.2\ M^{-1}$. It is concluded, therefore, that the association between AO^+ and Cl^- can be thoroughly neglected in the investigated AO–HCl concentration range.

B) Emf Measurements of Cl^- in the AO–$HZnCl_3$ System: In previous paper there have been reported the results of emf measurements of Cl^- activity in the aqueous AO–HCl solutions.[21] Figure 4 shows emf values plotted against the logarithm of the total amount of Cl^- added as AO·$HZnCl_3$, log[Cl]. The emf values for the AO–$HZnCl_3$ solution are in excellent agreement with those for the AO–HCl solution, that is, the emf values linearly decrease with log[Cl] at all concentrations from 1×10^{-5} to 5×10^{-3} M.

These results exclude the possibility that Cl^- might participate into the dimerization equilibrium of AO. This conclusion coincides with that in the results in section (*A*).

C) Effects of Ionic Strength on the AO Dimerization Equilibrium: The results in (*A*) and (*B*) exclude the possibility of the mechanism (1). Then the mechanism (2) should describe the dimerization process of AO correctly. The effect of the electrolyte on the dimerization equilibrium, therefore, would be interpreted in terms of the ionic strength.

Over the low concentration region, $(1-5)\times10^{-5}$ M, of AO at 298 K in water, there is an isosbestic point corresponding effectively to a monomer–dimer equilibrium.[11,22] The dimerization constant K_d is given by Eq. 6.

$$K_d = \frac{[(AO)_2{}^{2+}]}{[AO^+]^2} \tag{6}$$

where $[AO^+]$ and $[(AO)_2{}^{2+}]$ denote the equilibrium concentrations of AO mono-cation and AO dimer, respectively. After Scatchard,[23] it is assumed that the activity coefficient, f_z, of a z-valent ion at an ionic strength I is given by the expression

$$-\log f_z = AZ^2\frac{\sqrt{I}}{1+1.5\sqrt{I}} \tag{7}$$

where A is a constant. For aqueous solutions at 298 K, $A=0.5$. Assuming Eqs. 6 and 7, the Brønsted-Bjerrum equation becomes as follows:[24,25]

$$-\log K_d = \log K_t + 1.02\frac{\sqrt{I}}{1+1.5\sqrt{I}} \tag{8}$$

where K_t is a tentative thermodynamic dimerization constant at $I=0$. In Fig. 5 the dimerization constant K_d was plotted against $\sqrt{I}/(1+1.5\sqrt{I})$. Ionic strength was varied from 7×10^{-3} to 0.2 M by NaCl, $NaNO_3$ and KCl. From the intercept of the straight line K_t was obtained to be $1\times10^4\ M^{-1}$. This value well coincides with Schwarz's value.[22] Thus the promotion of the dimerization of AO by an addition of electrolyte is explained quantitatively, considering the increase of the ionic strength.

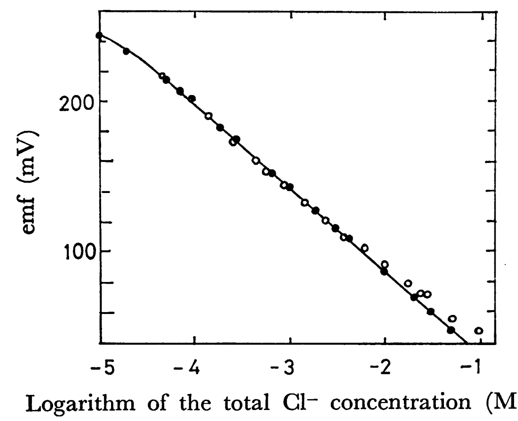

Fig. 4. Reversible emf values plotted against the logarithm of the total amount of Cl^- added as: ● NaCl (in water), ○ $AOHZnCl_3$ (in water) at 298 K.

II) Inclusion of Cl^- into PLG–AO Complex. From the results obtained in (*I*) and also the large negative charges of PLG, the inclusions of Cl^- into PLG–AO complex would be concluded to be improbable. This is confirmed by emf measurements as described below.

The emf measurements of Cl^- activity were performed in the following systems:

(a) Na·PLG + NaCl at pH = 4.53,

(b) Na·PLG + AO·HCl at pH = 4.53,

where the concentration of glutamyl residue was kept constant, varying the concentration of AO·HCl, and finally,

(c) Na·PLG + AO·HCl at pH = 4.53,

where the glutamyl residue to dye ratio (R/D) was kept constant at R/D=10. The results were exhibited in Fig. 6. The emf values for both the Na·PLG+AO·HCl systems (b) and (c) coincide with those for the Na·PLG+NaCl system. The results exactly show that there is no essential difference in the Cl^- activity among the systems (a), (b) and (c). An effect of PLG on the emf of Ag/AgCl electrode was discussed in ref. 21. It is reasonable to conclude that no signifi-

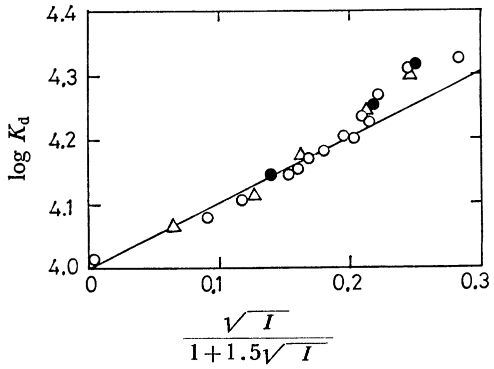

Fig. 5. The logarithm of the dimerization constant K_d of AO plotted as a function of the ionic strength I. ○ NaCl △ KCl ● $NaNO_3$.

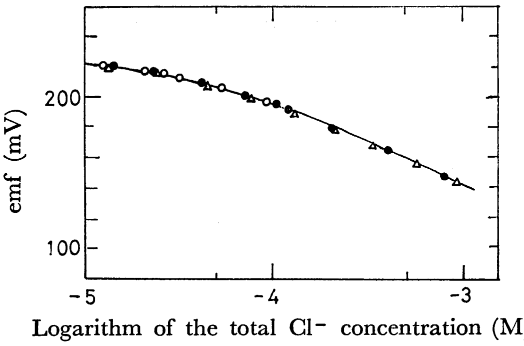

Fig. 6. Reversible emf of the cell (3) in the text as a function of the logarithm of the total chloride concentration added as AO-HCl or NaCl.
(a) ● Na·PLG+NaCl at pH=4.53, and at 298 K.
(b) ○ Na·PLG+AO·HCl at pH=4.53; [Na·PLG] is constant at 4×10^{-3} M, and at 298 K. (c) △ Na·PLG+AO·HCl at pH=4.53; R/D=10 at 298 K in water.

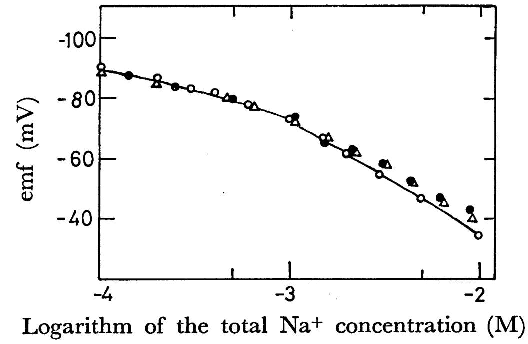

Fig. 7. Reversible emf of the cell (4) in the text as a fuuction of the logarithm of the total Na^+ concentration at pH=4.53 and 298 K in water.
(a) ○ NaCl+buffer, (b) ● Na·PLG+buffer,
(c) △ Na·PLG+AO·HCl+buffer.

cant inclusion of Cl^- takes place into PLG–AO complex.

III) Effects of Na^+ on the PLG–AO Complex. As stated previously, Schwarz and Balthasar explained the interaction between AO and PLG in the random coil conformation in terms of the cooperative binding theory.[3] The assumptions made in this theory have been verified to be valid.[26] In order to interpret the interaction between AO and PLG in helical conformation, it would be worthwhile to examine the validity of the assumptions even in the helical region.

Figure 7 exhibits the emf values of the following systems at pH=4.53:

(a) NaCl in water,

(b) Na·PLG in water,

(c) Na·PLG + AO·HCl in water at R/D = 10.

At pH=4.53 PLG is present in the helix conformation with carboxyl groups partially ionized. The concentration of the ionized carboxyl groups, [–COO⁻], was calculated from the degree of PLG ionization, α, defined by

TABLE 1. ASSOCIATION CONSTANT K_1 BETWEEN Na^+ AND -COO⁻ [28]

	In the random coil region (pH=7.51)	In the helix region (pH=4.53)
In the presence of AO	$90\pm20\ M^{-1}$ [a]	$48\pm20\ M^{-1}$
In the absence of AO	$87\pm20\ M^{-1}$ [a]	$50\pm20\ M^{-1}$

a) Cited from Ref. 26.

$$\alpha=\frac{[\text{-COO}^-]}{[\text{-COOH}]+[\text{-COO}^-]} \tag{9}$$

where [–COOH] denotes the concentration of the protonated carboxyl groups in PLG. The value of α was assumed to be α=0.35 at pH=4.53 according to Jacobson's data.[27] The association constant K_1 between Na^+ and $-COO^-$ in the helical Na–PLG was obtained according to Eq. 10,

$$K_1=\frac{[\text{-COONa}]}{[\text{-COO}^-][\text{Na}]} \tag{10}$$

where [Na] indicates the free Na^+ concentration obtained from the emf measurement. [–COO⁻] and [–COONa] are free carboxylate ions and ion-paired carboxylate ions, respectively. They were calculated by Eq. 11,

$$\begin{aligned}[\text{-COO}^-]&=[\text{-COO}^-]_t-[\text{-COONa}]\\ [\text{-COONa}]&=[\text{Na}]_t-[\text{Na}]\end{aligned} \tag{11}$$

where $[\ ]_t$ indicates the total concentration of each ion. K_1 was obtained to be $50\pm25\ M^{-1}$ in the range of $[Na]_t$ from 1×10^{-4} M to 1×10^{-3} M.[28] This value well agreed with Nitta and Sugai's data;[29] 20—30% of $[Na]_t$ are present as the ion-pair at [Na·PLG]=0.011 M. In the presence of AO, K_1 was calculated to be $48\pm20\ M^{-1}$, assuming that the three carboxyl groups are bound to the three nitrogen atoms in one AO molecule.[6,7]

The agreement between the two K_1 values reveals that the association between Na^+ and $-COO^-$ can be described with the single association constant K_1 irrespective of the presence of AO. This conclusion has been found to hold also in the random coil region (at pH=7.51) as represented in Table 1.

These results show that the assumption in Schwarz's cooperative binding theory as stated in the introduction section is valid; namely Na^+ binds with $-COO^-$ to a certain extent non-cooperatively even in the helical region. It has been also known that the reaction between AO and PLG in the helix conformation displays a non-exponential relaxation curve.[4]

Considering these findings, it is expected that this reaction may be described by the theory which is similar to Schwarz's cooperative binding theory and in which the association between Na^+ and $-COO^-$ is taken into account.

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28) The association constant K_1 between Na^+ and $-COO^-$ varied considerably with the variation of α; for example, $K_1=78\pm35$ for $\alpha=0.30$, $K_1=50\pm20$ for $\alpha=0.35$, $K_1=36\pm15$ for $\alpha=0.40$ and $K_1=28\pm15$ for $\alpha=0.45$. At the present stage, therefore, we do not discuss whether K_1 in the helical region agrees with that in the random coil region or not.

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