Determination of Equilibrium Constants for Binding of Acrnidine Orange and Its 10-Alkyl Derivatives to Dissolved Humic Substances by a Fluorescence Quenching Method

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Fluorescence intensities of Acrnidine Orange and its 10-alkyl derivatives were reduced by the presence of commercial humic acid or dissolved humic substances obtained from river water, seawater and pond water samples. Synchronous changes in the absorption spectra and the virtually unchanged fluorescence lifetime of the 10-dodecyl derivative suggested that the fluorescence quenching could be explained on the basis of a static quenching mechanism, in which the fluorescence dyes were bound to humic substances to form non-fluorescent dye aggregates. Curve fitting procedures for the upwardly-curved Stern–Volmer plots yielded the equilibrium binding constants normalized to organic carbon content. The binding constants at pH 7.0 for fractions eluting at pH 2.4–7.5 in a pH-gradient chromatographic separation of the aquatic humic substances were larger than those for fractions eluting at pH 8.2–11.4. Some chemical properties of the binding have also been discussed in terms of the electrostatic interaction and/or the hydrophobic interaction.

Keywords Equilibrium binding constant, aquatic humic substance, humic acid, Acrnidine Orange dye, static fluorescence quenching

One of the important chemical properties of humic substances, which are a major fraction of dissolved organic carbon (DOC) in natural water, is an ability to enhance water solubility values of hydrophobic organic contaminants such as polycyclic aromatic hydrocarbons and polychlorinated pesticides, through an action of hydrophobic moieties within the polymer matrix. The binding of the hydrophobic compounds to aquatic humic substances has been investigated in detail using equilibrium dialysis, reverse-phase separation and fluorescence quenching techniques; it was primarily evaluated by determining partition coefficients.

On the other hand, humic substances can associate with cationic organic compounds as well, since they are negatively-charged polymers. For example, bipyridinium herbicides are bound to soil humic and fulvic acids and are adsorbed on various cation-saturated humic acid particles. Absorption spectra of cationic aromatic dyes are changed by the presence of soil humic substances, though mechanistic details remain vague. Cationic surfactants have been listed as organic compounds which are bound to humic substances in the environment. It is also interesting that trioctylamine in chloroform has been used to extract aquatic humic substances, probably through formation of hydrophobic ion pairs. In this paper, we report a spectrophotometric study on the binding of Acrnidine Orange (n=0) and its 10-alkyl derivatives.

Fig. 1 Acrnidine Orange (n=0) and its 10-alkyl derivatives.
gical impacts of effluents from dyeworks on receiving waters.

Experimental

Reagents

AO base (Aldrich Chemical Company) was purified by a method described in the literature\textsuperscript{13}, after being pretreated by chromatography on alumina using chloroform as an eluent. The 10-dodecyl derivative of AO (AO\textsubscript{C12}) was purchased from Dojindo Laboratories and used without further purification. The butyl (AO\textsubscript{C4}), hexyl (AO\textsubscript{C6}) and octyl (AO\textsubscript{C8}) derivatives were synthesized and purified according to the method described by Yamagishi et al.\textsuperscript{16}

Humic acid (Aldrich Chemical Company) dissolved in a 0.1 M NaOH solution was reprecipitated by adjusting the pH to 1.0 with HCl. After this reprecipitation procedure was repeated twice, the humic acid was suspended in deionized water and then dialyzed against deionized water using a Visking VT351 cellulose tube with a nominal molecular weight cutoff level of 3500 in order to remove Cl\textsuperscript{-}. The purified humic acid (HA) was recovered by centrifugation and dried under vacuum; the elemental analysis gave C of 53.32\%, H of 4.13\%, N of 2.00\% and the ash content of 6.1\%. A stock HA solution was prepared by dissolving desired amounts of HA in 0.1 M NaOH and then diluting with water by a factor of 10.

Water was purified with both a Millipore Milli-RO15 water purification system and an activated charcoal column. XAD-8 resin was purified according to Thurman and Malcolm\textsuperscript{17} and was stored in methanol. Tannic acid was purchased from Wako Pure Chemical Industries Ltd. and was used in its original condition. All other reagents were of reagent grade or better and used without further purification.

Aquatic humic substances

A river water sample (3 dm\textsuperscript{3}) was taken from the Oita River (Oita, Japan) about 7 km upstream from the river mouth. Water samples were also taken from a pond (3 dm\textsuperscript{3}) near the university and from the Oita harbor mouth. Water samples were filtered through a 0.5-µm glass fiber filter (Togo Roshi GC-50; pretreated at 400°C for 3 h) and were then adjusted to pH 2.0 with phosphoric acid. The respective acidified sample was pumped onto a XAD-8 column (12 mm i.d.×180 mm) at a rate of 1.0 cm\textsuperscript{3} min\textsuperscript{-1}. The humic substances adsorbed were eluted with 0.1 M NaOH (100 cm\textsuperscript{3}) after the column was washed with 0.01 M phosphoric acid (50 cm\textsuperscript{3}). The column effluent was again adjusted to pH 2.0 with phosphoric acid and pumped onto a smaller XAD-8 column (10 mm i.d.×90 mm) at a rate of 0.5 cm\textsuperscript{3} min\textsuperscript{-1}. The readsorbed humic substances were separated into two fractions by a pH-gradient elution using the same apparatus as described previously\textsuperscript{18} the first fraction (34 cm\textsuperscript{3}) was collected at pH 2.4 – 7.5 (fraction I) and the second fraction (34 cm\textsuperscript{3}) was collected at pH 8.2 – 11.4 (fraction II). The pH-gradient solution was prepared by titrating 0.01 M phosphoric acid with 0.01 M trisodium phosphate and was passed through the column at a flow rate of 0.55 cm\textsuperscript{3} min\textsuperscript{-1}.

Apparatus

Fluorescence and UV-VIS absorption spectra were recorded on a Shimadzu RF-510 spectrophotometer and a JASCO UVIDEC-610A spectrophotometer, respectively. pH values were measured with a TOA HM-5B pH meter. The DOC values of the humic fractions from the natural water were determined with a JASCO 524 total carbon analyzer. Fluorescence lifetime measurements were made with a PRA nanosecond time-resolved fluorescence spectrophotometer based on a single-photon counting technique. The light source was a hydrogen-filled (0.5 atm) flash lamp thyratron-triggered at ca. 33 kHz. Data of exciting flash lamp profiles and fluorescence decay curves accumulated on a Norland MCA IT-5300 multichannel analyzer were transferred to a NEC PC-9801 Vm computer through an EIA/RS-232 C connector.

Changes in the dye absorption spectra

An aliquot of the stock HA solution (5 – 10 mm\textsuperscript{3}) was added to a 1×1×4 cm cell containing a dye solution (3 cm\textsuperscript{3}) in 0.01 M phosphate buffer (pH 7.0) and was then rapidly mixed. The cell had been previously thermostated at 25±1°C. The buffer contained 10 v/v% methanol unless otherwise noted. An initial decrease in the absorbance of AO\textsubscript{C12} due to adsorption to the walls of the cell could not be depressed even by the addition of the 10% methanol. Thus, the dye solution was allowed to stand in the cell for at least 12 h prior to the addition of the HA solution. The concentration of AO\textsubscript{C12} in the preequilibrated solution was estimated from Beer’s law: the molar absorptivity (6.25×10\textsuperscript{4} M\textsuperscript{-1} cm\textsuperscript{-1}) at λ\textsubscript{max} of 496 nm was determined by using the limiting absorbance, which was obtained by extrapolation of a linear -\ln(A\textsubscript{f}−A\textsubscript{e}) vs. time plot to zero time, where A\textsubscript{f} and A\textsubscript{e} are the absorbances at time t and at equilibrium, respectively.

Association of the dyes with the added HA attained equilibrium within less than 3 min. Absorption spectra were recorded for the equilibrated solutions. This addition/spectral measurement step was repeated until a total of five to ten aliquots of HA had been added. The same procedure was carried out also in the cases of addition of the aquatic humic fractions. In order to avoid any dimerization of the dyes\textsuperscript{13}, the dye concentrations were kept lower than 3 µM throughout this work.

Fluorescence quenching experiments

The fluorescence spectra of the dye solutions were taken in a 1×1×4 cm quartz fluorescence cell according to the same procedure as was used in the measurements of the absorption spectral changes. The excitation
wavelength was 460 nm. Quenching of the dye fluorescences was quantified as the ratio of \( I_0/I \), where \( I_0 \) is the fluorescence intensity at \( \lambda_{\text{max}} \) of 517 nm when no humic substances are present and \( I \) is the intensity when humic substances are present. The fluorescence intensities were corrected for the inner filter effect according to Gauthier et al.; the correction factors were not more than 1.15.

**pH effects on quenching of the AOC12 fluorescence**

The two humic fractions from the river water sample were used as quenchers. Each fraction was diluted with an NaOH or phosphoric acid aqueous solution in order to obtain the desired pHSs, and was then added to an AOC12 methanolic solution. The sample solutions were allowed to stand for 12 h prior to taking the fluorescence spectra.

**Fluorescence lifetime measurements**

Fluorescence intensities were monitored at \( \lambda_{\text{max}} \) of 517 nm. The optimized lifetimes were determined by superimposing the curves due to convolution integrals, which were computed with the flash lamp profiles, on the observed fluorescence decay curves, checking the weighed residuals. All the measurements were carried out at room temperature using sample solutions prepared by the same method as in the measurements of the fluorescence spectra. The lifetime of AO (1.75±0.05 ns) obtained in the absence of humic substances was in good agreement with the value (1.7 ns in 5 mM phosphate buffer at 23°C) determined by Kubota and Steiner.19

**Results and Discussion**

**Spectral changes**

Figure 2 shows typical absorption spectra obtained upon addition of HA to an AOC12 solution; similar spectral changes were observed with AO, AOC4, AOC6 and AOC8-HA systems as well. A gradual substitution of the \( \alpha \) free dye band with a broader band around 470 nm bears a striking resemblance to the spectral change of AO induced by its binding to DNA or synthetic anionic polyelectrolytes, which is known as the metachromatic effect.13 Further, the addition of HA induced marked decreases in the fluorescence intensities of the dyes, closely linking with the changes in the absorption spectra, as shown in Figs. 3 and 4,

![Fig. 2](image)

**Fig. 2** Absorption spectra of AOC12 (1.7 \( \mu \)M) in the presence of HA in 10% methanol/0.01 M phosphate buffer (pH 7.0). Concentrations of HA: a, 0; b, 0.0533; c, 0.107; d, 0.133; e, 0.160 mgC dm\(^{-3}\).

![Fig. 3](image)

**Fig. 3** Plots of \( I_0/I (\bigcirc) \) and \( \tau_0/\tau (\triangle) v. C_{DOC} \) in the AOC12-HA system. \( \tau_0 \) and \( \tau \) are the fluorescence lifetimes in the absence and presence of HA, respectively. The concentration of AOC12 was 1.38 \( \mu \)M; \( \tau_0 \) was 1.37±0.05 ns.

![Fig. 4](image)

**Fig. 4** Stern–Volmer plots for quenching of the fluorescence (\( \lambda_{\text{max}}=517 \) nm) of AO (\( \bigcirc \)), AOC4 (\( \bigtriangleup \)), AOC6 (\( \bigcirc \)) and AOC8 (\( \triangle \)) by HA in 10% methanol/0.01 M phosphate buffer (pH 7.0). Shaded triangles represent the data obtained with AOC8 in the buffer containing 0.05% methanol. The concentrations of the dyes were all 2.5 \( \mu \)M.
where the HA concentration on the abscissa is expressed as CDOC, the DOC calculated from the carbon content. The HA fluorescence was negligible as compared to the dye fluorescence.

The AOC12 fluorescence was not much altered by addition of low-molecular-weight organic compounds (hydroquinone, vanillin, salicylic acid and propionic acid of 1×10⁻⁵ M) whose structures are included in constituents of humic substances.²⁰ But it was quenched by tannic acid, which is a mixture of high-molecular-weight compounds having analogous molecular structures to those of humic substances, with a lower quenching efficiency: I₀/I values were 1.11 and 2.67 with 0.02 and 0.2 mg dm⁻³ of tannic acid, respectively, when the AOC₁₂ concentration was 0.86 µM. Accordingly, the spectral changes in the present HA systems would be explained on the basis of an analogous mechanism to that in the above-mentioned metachromatic AO-polyelectrolyte systems; that is, a binding of the dye molecules to the humic polymers would raise the local concentrations of the dyes, resulting in a ready formation of stacked dye aggregates. In addition, the result that the shapes and positions of the dye fluorescence spectra were little affected by the presence of HA appears to indicate that the ground-state dye aggregates are almost non-fluorescent.

**Determination of binding constants**

The Stern-Volmer plots of I₀/I vs. CDOC in Figs. 3 and 4 were curved upwards. In general, fluorescence quenching can be classified into two types: one is static quenching and the other is dynamic.⁶,²¹-²⁴ The present AO dyes-HA systems undoubtedly include static quenching due to the dye aggregate formation. Also, since the fluorescence lifetimes of the dyes were quite short (1.75±0.05 ns for AO; 1.37±0.05 ns for AOC₁₂) and moreover the concentrations of the HA added were exceedingly low, it would be unreasonable to explain the positive deviations from the linear Stern-Volmer plot in terms of the coexistence of dynamic quenching, assuming that the quenching process is diffusion-controlled. In fact, as shown in Fig. 3, the fluorescence lifetime of AOC₁₂ was little affected by the presence of HA within the HA concentration range examined. This suggested that, at least in the AOC₁₂ system, the fluorescence quenching substantially consisted of the static quenching, which is shown in Scheme 1. Here, AOC₁₂⁺ is the excited singlet state of AOC₁₂ which may be converted to the ground state through both radiative and non-radiative processes; Iₘₙ is the rate of absorption of photons by AOC₁₂ in the absence of HA. The non-fluorescent dye aggregates are formed inside the ground-state AOC₁₂-HA complex. The binding of AOC₁₂ by HA may be described by a binding constant, Kₛ=[AOC₁₂-HA]/[AOC₁₂][HA], where [AOC₁₂-HA] and [HA] represent the concentrations of the bound AOC₁₂ and vacant binding sites, respectively. The decrease in the concentration of free AOC₁₂ ([AOC₁₂]) which is induced by the binding is accompanied by the reduction of the light absorption to Iₘₙδ.

An analogous argument to that of Gauthier et al.⁶ or Moon et al.²² gives the following equation, which has the form of the Stern-Volmer equation, on the assumption that the fluorescence intensity is proportional to the concentration of free AOC₁₂ in solution:

\[
\text{I₀/I} = 1/\delta = 1 + Kₘ[\text{HA}].
\]

If a significant excess of the quencher HA is present, [HA] can be approximated to correspond to the total concentration of the quencher added. This type of situation happens in quenching of polycyclic aromatic hydrocarbon fluorescences by humic substances.⁶ As seen in Fig. 3, however, small amounts of HA efficiently quenched the AOC₁₂ fluorescence and thus the approximation may not be applied to the present system. In such a situation,

\[
[\text{HA}] = \frac{-(Kₛ Cₐ - K_{\text{DOC}} C_{\text{DOC}} + 1)^2 + 4K_{\text{DOC}} C_{\text{DOC}}}{2Kₛ}. \quad (2)
\]

Here, Cₐ is the total stoichiometric concentration of AOC₁₂; K_{DOC} is the binding constant normalized to the organic carbon content of the dissolved HA. K_{DOC} is equal to KₛS, where S is the number of moles of the available binding sites per unit organic carbon content. Combining Eqs. (1) and (2) gives Eq. (3):

\[
\text{I₀/I} = 1 + (Kₛ Cₐ - K_{\text{DOC}} C_{\text{DOC}} + 1)^2 + 4K_{\text{DOC}} C_{\text{DOC}})^{1/2}. \quad (3)
\]

The observed plots of I₀/I vs. CDOC in Fig. 3 were analyzed with the aid of the Marquardt nonlinear least-squares method. The parameters obtained, Kₛ and K_{DOC}, are listed in Table 1. The same analysis of the data shown in Fig. 4 gave K_{DOC} values of 2.9, 2.3, 4.6 and 8.3 dm³/mgC for AO, AOC₄, AOC₆ and AOCR, respectively, on the assumption of the nonparticipation of dynamic quenching. Solid lines in Figs. 3 and 4 represent the I₀/I values calculated from Eq. (3).

When pyrene was used as a neutral fluorophore, the system gave a K_{DOC} of 0.085 dm³/mgC according to the data treatment reported by Gauthier et al.⁹ This value was 34-times smaller than that for AO, suggesting that electrostatic interaction played a significant role in...
the present cationic dye systems. On the other hand, it is important to note that $K_{DOC}$ for the AO dyes increased with an increase in the length of the alkyl chain except for AOC$_4$ (vide supra). The extents of change in the absorption spectra were also in the same order, when the spectra were taken at a fixed HA concentration. It is known that the affinity for binding of hydrophobic organic solutes to humic substances increases with an increase in the hydrophobicity of the solute$^{3-5}$, and moreover, it has been reported that AO derivatives with longer 10-alkyl chains have dimerization-equilibrium constants more enlarged by hydrophobic interaction between the alkyl chains$^{16,25}$, if the alkyl groups are longer than the hexyl one. Thus, it is probable that the effect of the alkyl group on the $K_{DOC}$ resulted from the enhanced hydrophobic interactions in both the binding to HA and the self-association of the bound dyes in the polymer domains. This significance of hydrophobic interaction was supported by the fact that reduction of the methanol content in the buffer solution from 10% to 0.05% enhanced the quenching efficiency in the AOC$_8$ (Fig. 4). The smaller $K_{DOC}$ for AOC$_4$ would be attributable to a more predominant steric hindrance due to the butyl group than due to the hydrophobic effect, in analogy with the case of the dimerization equilibrium.$^{25}$

A $S$ value of 11 for the AOC$_{12}$-HA system signified that HA contained about 8 carbon atoms per unit binding site. In addition, calculations of [HA] by using Eq. (2) showed that, in the case of AOC$_{12}$, the available binding sites of 83% or above were occupied by the dye molecules under the conditions in Fig. 3; even in the case of AO having the smallest $K_{DOC}$ (Fig. 4), more than 49% of the sites was occupied. Such a dense occupancy might account for the results that the so-called $\gamma$ absorption bands$^{13}$ due to the dye aggregates were observed, whereas absorption bands due to bound monomeric and/or dimeric species were not.

**Fluorescence quenching by aquatic humic substances**

Figure 5 shows Stern–Volmer plots for quenching of the AOC$_{12}$ fluorescence by aquatic humic substances which were fractionated using a pH-gradient elution technique. Fluorescences from the humic fractions were negligible and the humic fractions were confirmed not to affect the AOC$_{12}$ fluorescence lifetime. Changes in the absorption spectrum by the addition of the humic fractions were also analogous to those obtained by the addition of HA. Further, the fluorescence spectrum was little altered by addition of several inorganic ions (NaCl, CaCl$_2$, MgCl$_2$, and NaNO$_3$ of $5.0 \times 10^{-4}$ M; Na$_2$SO$_4$ of $1.0 \times 10^{-3}$ M; CuCl$_2$ and FeCl$_3$ of $5.0 \times 10^{-4}$ M) which were assumed to have got into the quencher solutions from the original water samples.

The parameters obtained by the same analysis as performed on the HA systems are summarized in Table 1. All of the humic fractions examined had far smaller $K_{DOC}$ values than that for HA. Analogous differences between aquatic humic substances and commercial humic acids have been observed in the water solubility enhancement of chlorinated aromatic hydrocarbons by humic substances, and have been attributed to the higher content of nonpolar molecular moieties in commercial humic acids.$^2$ Thus, the results in this study would also be explained in terms of reduced hydrophobic interaction with the aquatic humic substances. In other words, electrostatic interaction may be assumed to contribute to the binding to the aquatic humic substances more highly than to the binding to HA. In addition, these results substantiated the warning given by Malcolm and MacCarthy$^{26}$ that commercial humic acids are not representative of

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**Table 1 Parameters for the binding of AOC$_{12}$ to humic substances in 10% methanol/0.01 M phosphate buffer (pH 7.0)**

<table>
<thead>
<tr>
<th>Humic Substance</th>
<th>$K_{DOC}$ (M$^{-1}$)</th>
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<tbody>
<tr>
<td>AOC$_{12}$</td>
<td>10$^{-4}$</td>
</tr>
<tr>
<td>AOC$_{4}$</td>
<td>10$^{-5}$</td>
</tr>
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**Fig. 5** Stern–Volmer plots for quenching of the fluorescence of AOC$_{12}$ (0.82 µM) by aquatic humic substances in 10% methanol/0.01 M phosphate buffer (pH 7.0). The aquatic humic substances used are fractions I and II of the river water (○), pond water (▲) and seawater (◆, ■). Shaded marks represent the plots for fractions II.
aquatic humic substances.

As shown in Fig. 6, the quenching efficiencies by fractions I and II from the river water increased with an increase in pH; the efficiency of fraction I remained nearly unchanged above pH 7 and that of fraction II remained unchanged above pH 10. Fraction I is a humic fraction eluting at pH 2.4 - 7.5 from a hydrophobic XAD-8 resin column and fraction II is that eluting at pH 8.2 - 11.4. Accordingly, as expected from the significant contribution of electrostatic interaction, the pH dependences appear to reflect titrations of the humic fractions with a base. Moreover, differences in S between fractions I and II of all the water samples (Table 1) would also be accounted for at least in part by the extents of ionization may be higher for fractions I at pH 7.0 where the fluorescence quenching experiments were conducted. It should be noted here that the larger S values for fractions I govern larger K_{DOC} values for fractions I in the cases of river and pond waters.

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References


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