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INFLUENCE OF ORGANIZED MEDIA ON THE ABSORPTION AND FLUORESCENCE SPECTRA OF AURAMINE-O DYE

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Summary—The spectral characteristics of the aqueous solutions of Auramine O dye in the presence of organized media have been examined. The absorbance and fluorescence of the dye are significantly enhanced in cyclodextrins and surfactants. The dye forms a predominant 1:1 complex with β-CD, whereas a 1:2 complex is formed with α-CD. The dissociation constants of the complexes have been determined by use of a non-linear regression method. Premicellar solutions of the sodium dodecyl sulphate exert maximum influence on the fluorescence and absorbance spectra of the dye. The microviscosity in cyclodextrins and micelles of SDS and Brij-78 have been estimated from fluorescence measurements.

INTRODUCTION

Auramine O dye is a fluorescent, cationic diphenylmethane derivative. Its fluorescence is sensitive to the microviscosity of the environment.[1] In aqueous solution, this fluorescence is very weak, but is significantly enhanced in a viscous solution. The fluorescence enhancement upon binding to DNA and proteins have been reported.[2–4] The fluorescence quantum yield of the probe decreases by dissipation of electronic energy through intramolecular rotations. Therefore, the fluorescence intensity of the dye reflects the effective microviscosity of its microenvironment and it has been used to estimate the microviscosity in micelles and membranes.[5–7]

Cyclodextrins (CDs) and surfactants are organized media that have been reported to influence the absorbance and fluorescence of many dyes.[8–12] Cyclodextrins are cyclic oligosaccharides formed by an α-(1,4) linkage of glucopyranose units. Most common are α, β, and γ-CDs corresponding to six, seven and eight units. These compounds are known to form non-covalent inclusion complexes with various molecules of appropriate size and polarity.[13] The binding forces contributing to the inclusion complex have been attributed to weak interactions such as hydrogen bonding, van de Waals and hydrophobic interactions.[14] The inclusion complex equilibria are governed by a negative enthalpy change which is due to the displacement of the high energy water from the CD cavity and release of the CD strain energy upon complexation.[14] The intrinsic spectroscopic properties of the dye may be altered as a result of the inclusion of the dye into the hydrophobic cavity of CD. However, spectroscopic changes may also occur as a result of deaggregation of the multimeric dyes by the selective inclusion of the monomeric form.[10] The interaction between CDs and fluorescent dyes has been studied by Cramer *et al.*[15] They reported that azo dyes of the type 4′-dimethylaminophenylazo-1-naphthalene-1-sulfonate form a 1:1 complex with CD, and suggested a mechanism in which the dyes are enclosed in the cyclodextrin cavity. Harada *et al.* studied the interaction of fluorescent dyes with CDs and CD containing polymers.[16] They reported the formation of a 2:1 complex between the dye and the polymer β-CD, while β-CD formed a 1:1 complex at low concentration and 2:1 at higher concentration. A much higher enhancement was obtained when a polymer-CD was used. Conversion of the thionin dimer into the monomeric form upon addition of α and β-CD was accompanied by changes in the absorption and fluorescence spectra of the dye.[10] The association constants for the binding of the dye to α and β-CD were

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estimated from absorption measurements. Deaggregation in concentrated solutions and variable complex formation in dilute solutions have been proposed to account for the effect of β-CD on the fluorescence, absorbance, and lasing of the xanthene dyes.[12]

Numerous dyes display a pronounced tendency to interact with surfactants.[9] Surfactants are molecules which possess both a hydrophobic (hydrocarbon) chain and a hydrophilic head group. The latter may be cationic, anionic or nonionic. The concentration of monomer at which surfactants aggregate into micelles is called the critical micelle concentration (cmc). Three types of interactions are commonly found in the dye-surfactant systems.[17] At surfactant concentrations above the cmc, the dyes are generally bound individually to micelles. In the premicellar region, dye-surfactant aggregates are usually formed, and at concentrations near the cmc, premicelles with large dye content are formed with structures similar to micelles. The effects of various surfactants on the absorption and fluorescence spectra of the dyes have been attributed to these interactions and the subsequent aggregation and deaggregation processes.

Both the cyclodextrins and the surfactants used in this work provide the hydrophobic and charged regions for interaction with amphiphilic molecules such as AuO. In this paper, we report the influence of α, β, and γ-CD and surfactants on the absorption and fluorescence spectra of the AuO dye.

EXPERIMENTAL

Instrumentation

Steady state fluorescence measurements were acquired with a Fluorog-2 spectrofluorometer model F2T21I (SPEX Industries, Edison, NJ) equipped with a thermostated cell housing and a water cooled Hamamatsu R928 photomultiplier tube. Fluorescence emission spectra were acquired with an excitation wavelength of 365 nm. Excitation and emission bandwidths were set at 5 and 3 nm, respectively. All data were processed through a SPEX DM 3000F computer interfaced to the spectrofluorometer. Absorption measurements were performed with a Perkin-Elmer Lambda 3 UV–Vis spectrophotometer using a 1-cm path length cell. Data were processed using the Perkin-Elmer computerized spectroscopy software. All measurements were performed at room temperature.

Materials

Auramine O, Brij-78, and SDS, were purchased from Aldrich Chemical Co., Milwaukee, WI and used as received. The α, β, and γ-CD were obtained from American Maize Products (Hammond, IN).

Method

A $5 \times 10^{-5}M$ aqueous AuO solution was prepared by pipetting a 500-μl aliquot of $10^{-3}M$ aqueous AuO stock solution into a 10-ml flask. The corresponding measured amount of aqueous CD or surfactant solution was added to achieve the desired concentration. A 2-ml volume of the phosphate buffer (pH = 7) was added and the flasks were filled to the mark with deionized water. All solutions were allowed to equilibrate overnight prior to analysis. In addition to these samples, blanks were prepared with appropriate concentrations of CD and deionized water for absorbance measurements.

RESULTS AND DISCUSSIONS

Absorption measurements

The absorption spectrum of the Auramine O dye consists of two major bands, one at 368 and another at 430 nm. These bands correspond to the transitions from the ground state to the second and first excited electronic state, respectively.[1] The absorption spectrum of AuO in aqueous solution follows Beer's law between 5×10^{-6} and $1 \times 10^{-4}M$ for a 368-nm band and between 1×10^{-6} and $6 \times 10^{-5}M$ for a 430-nm band. It is already known that the absorption spectra of most organic dyes in aqueous solutions deviate from Beer's law.[18] It has been hypothesized that such deviations are due to reversible polymerization of the dye molecules. The second absorption band of AuO at $2 \times 10^{-4}M$ is 8-nm red shifted which suggest the presence of molecular aggregates of the dye.

The addition of α-CD to AuO resulted in a small and inconsistent increase of the absorbance of the dye. However, a significant spectral change was observed when β-CD was added. Figure 1 shows the absorption spectra of AuO in aqueous solutions at various concentrations of β-CD. The absorption maxima of AuO are slightly red-shifted upon addition of the CD. This effect has been attributed to the interaction of the dye with a non-absorbing electron donating component.[19] This result indicates the interaction between AuO and the

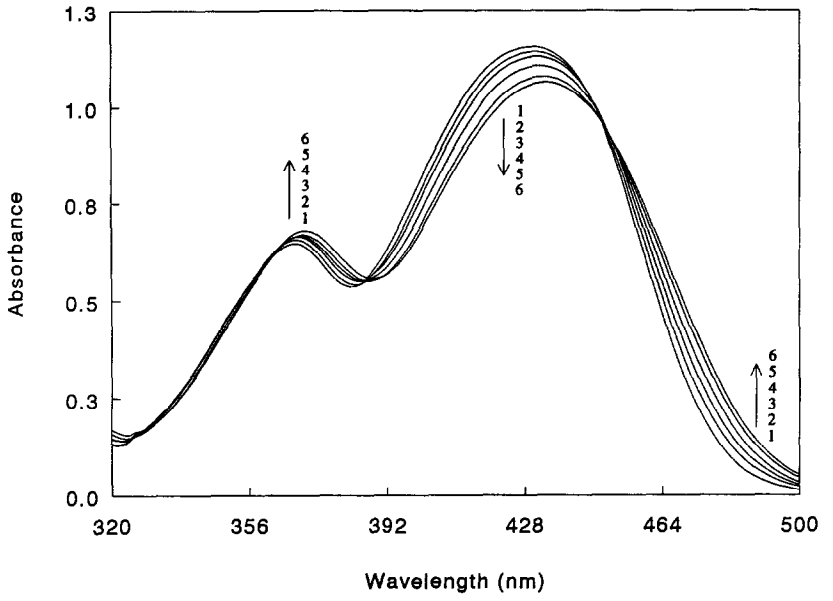

Fig. 1. Absorption spectra of AuO in various concentration of β-CD.

electron donating hydroxyl and glycosidic oxygen of the cyclodextrin. The absorption band at 368 nm increases while the band at 430 nm decreases upon addition of β-CD. The isobestic points appearing at 338 and 446 nm suggest a 1:1 equilibrium between the dye and the CD. These spectral changes can be attributed to an equilibrium represented by the following equation:

$$\mathrm{AuO} + \mathrm{CD} \rightleftharpoons \mathrm{AuO}\,.\,\mathrm{CD} \qquad (1)$$

The equilibrium constant for this reaction can be obtained by plotting the absorption values according to the Hilderbrand-Benesi relationship.[20]

$$\frac{[\mathrm{AuO}][\mathrm{CD}]}{\Delta E} = \frac{K}{\Delta\epsilon} + \frac{[\mathrm{CD}]}{\Delta\epsilon} \qquad (2)$$

where [AuO] is the concentration of the dye, [CD] is the concentration of β-CD, K is the equilibrium constant of the complex, $\Delta\epsilon$ is the

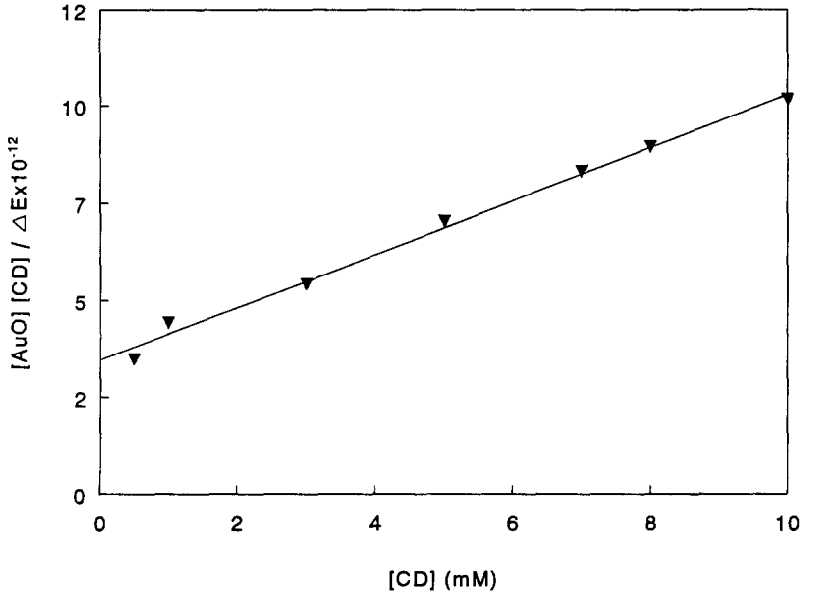

Fig. 2. Determination of the equilibrium constant of the AuO . β-CD complex according to Benesi and Hilderbrand.

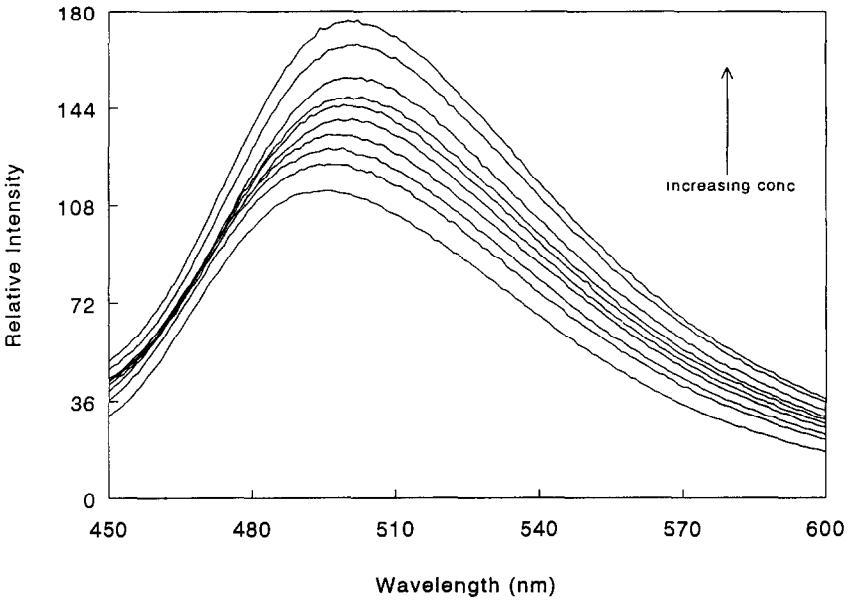

Fig. 3. Fluorescence spectra of AuO as a function of [α-CD].

difference in the molar extinction coefficients for free and complexed AuO, and ΔE is the change in the extinction of the AuO solution upon addition of β-CD. These values are plotted in Fig. 2, which exhibits a linear relationship. The equilibrium constant for the complex was estimated to be $197M^{-1}$. The effect of γ-CD on the absorption spectrum of AuO varied with the concentration of the CD. In general the absorbance values were higher than that of AuO in water, and the bands were slightly red shifted.

A slight decrease of absorbance was observed at low concentrations of SDS. However, further increase of the surfactant increased the absorbance of the dye. The increased absorbance of the bands was accompanied by a small red shift. As has been previously mentioned, this phenomenon suggests an interaction between the dye and the electron donating groups of the surfactant.[19] Similarly, the absorbance of AuO increased upon addition of Brij-78. However, unlike SDS, there was no shift in the absorption maxima of the dye. The equilibrium constants for α and γ-CD and the surfactants with AuO were not determined by the absorption method, owing to small and inconsistent changes in the absorption spectra.

Fluorescence measurements in CDs

The fluorescence spectra of AuO in the presence of α-CD are shown in Fig. 3. All three CDs enhanced the fluorescence of AuO. Fluorescence enhancement using several fluorescent dyes have been reported.[10,12,15,16,21] However, unlike most of these probes, the fluorescence of AuO depends to a large extent on the microviscosity.[1] The internal rotation of the diphenylaniline has been found to be responsible for the low quantum yield of this probe in water. In the CD cavity, these non-radiative rotational relaxations are apparently suppressed resulting in fluorescence enhancement of AuO in the presence of CDs. Furthermore, the enhanced fluorescence of the dye at higher concentrations of γ-CD was accompanied by a broad emission band at longer wavelengths. Among the three CDs, the highest enhancement of fluorescence was observed with γ-CD. The fluorescence intensity of AuO leveled off at a concentration of approximately 20mM for α-CD, 10mM for β-CD, and 80mM for γ-CD.

Stoichiometric ratio and binding strength between CDs and AuO

The interpretation of the results involving inclusion complexes varies significantly depending on the stoichiometry of the complex.[22] Most studies assume a 1:1 stoichiometry between CD and the guest, but different stoichiometries such as 1:2, 2:1, and 2:2 have been reported.[23–26] Usually the CPK models and Benesi-Hilderbrand plots provide the necessary information concerning the stoichiometry and the arrangement of the guest within or in association with

the CD molecule.[20] The equilibrium equation for a 1:1 complex between AuO and CD is given by equation (1). Then, the equilibrium constant for the complex is given by

$$K_1 = \frac{[\text{AuO} \cdot \text{CD}]}{[\text{AuO}][\text{CD}]} \quad (3)$$

By using $[\text{CD}] \gg [\text{AuO} \cdot \text{CD}]$ it can be assumed that $[\text{CD}]_0 = [\text{CD}]$ and the following equation can be derived

$$\frac{1}{[\text{AuO} \cdot \text{CD}]} = \frac{1 + K_1[\text{AuO}]_0}{K_1[\text{AuO}]_0[\text{CD}]_0} \quad (4)$$

where $[\text{AuO}]_0 = [\text{AuO}] + [\text{AuO} \cdot \text{CD}]$ and $[\text{CD}]_0 = [\text{CD}] + [\text{AuO} \cdot \text{CD}] \simeq [\text{CD}]$.

Since the fluorescence intensity of AuO in the absence (I_0) and presence (I_1) of CD is proportional to [AuO] and [AuO . CD], respectively, substitution of these values into equation 3 gives

$$\frac{1}{I - I_0} = \frac{1}{I_1 - I_0} + \frac{1}{K_1[\text{CD}]_0(I_1 - I_0)} \quad (5)$$

Thus a plot of $1/I_1 - I_0$ *vs.* 1/ [CD] should give a straight line for a 1:1 complex. Likewise for a

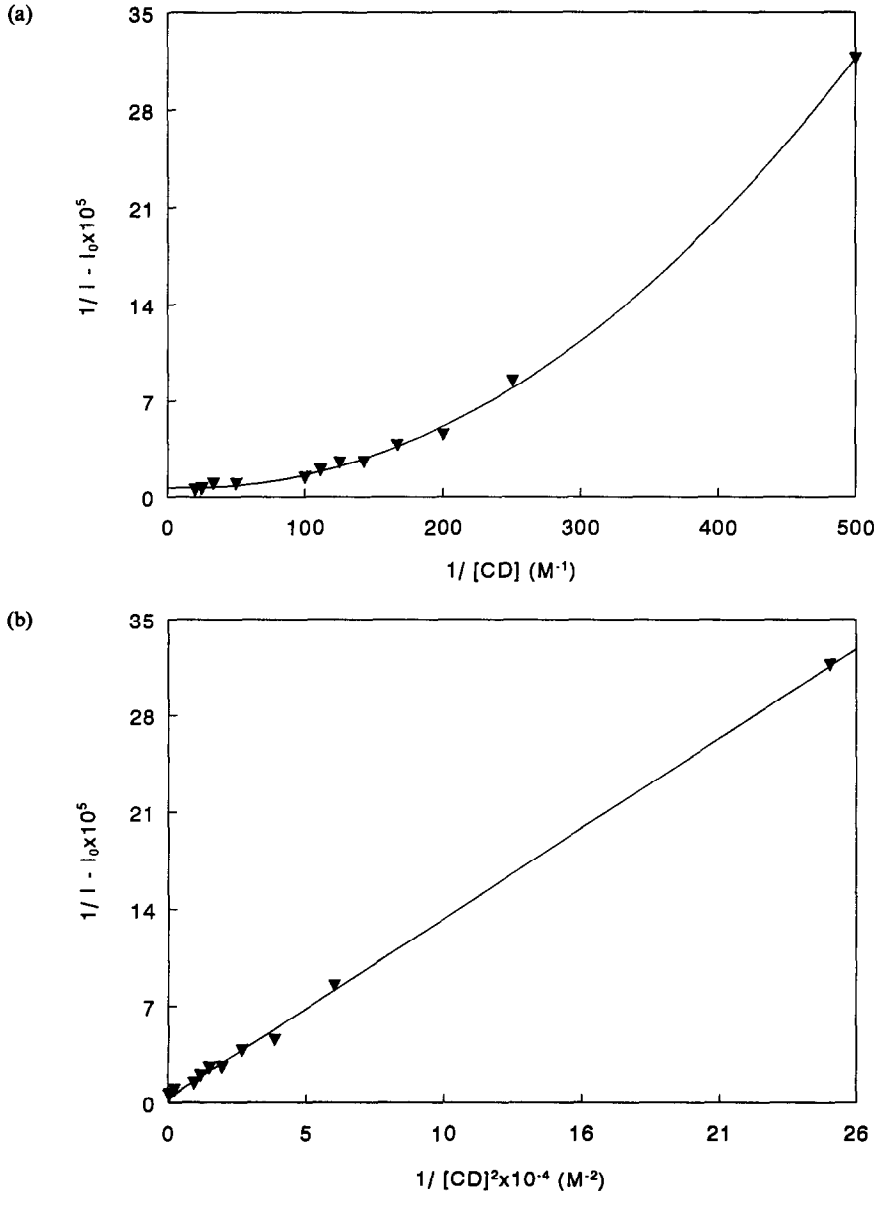

Fig. 4. Double reciprocal plots for the α-CD . AuO complex (a) $1/I - I_0$ *vs.* 1/[CD] (b) $1/I - I_0$ *vs.* 1/[CD]^2.

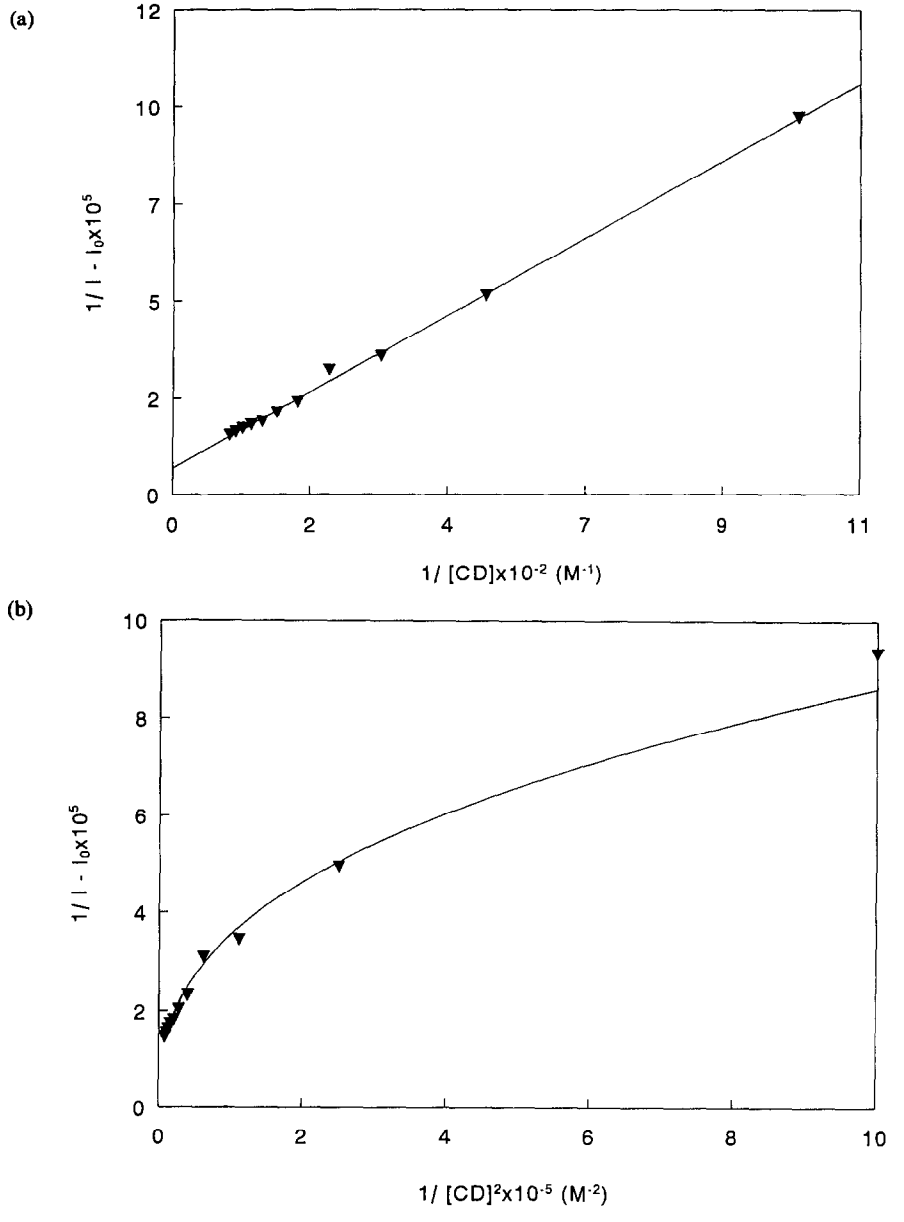

Fig. 5. Double reciprocal plots for the AuO . β-CD complex (a) $1/I - I_0$ *vs.* 1/[CD] (b) $1/I - I_0$ *vs.* $1/[CD]^2$.

2:1 complex, the overall equilibrium equation and constant K_2 is given by:

$$AuO + 2CD \rightleftharpoons AuO \,.\, CD_2 \qquad (6)$$

$$K_2 = \frac{[AuO \,.\, CD_2]}{[AuO][CD]^2} \qquad (7)$$

Table 1. Estimated formation constants and microviscosity values for AuO in organized media

Organized media	Log K	Microviscosity (cP)
α-CD	3.7	2.6
β-CD	1.9	2.3
Brij-78	3.0	17
SDS	3.1	5.7

If [CD] ≫ [AuO . CD_2] ≫ [AuO . CD] then an expression similar to equation 5 for 2:1 complex can be derived, *i.e.*,

$$\frac{1}{I - I_0} = \frac{1}{I_1 - I_0} + \frac{1}{K_2[CD]_0^2(I_1 - I_0)} \qquad (8)$$

In this case, a plot of $1/I - I_0$ vs $1/[CD]^2$ should be linear for a 2:1 complex. A typical double reciprocal plots for the α-CD . AuO complex are shown in Fig. 4(a) and (b). When $1/(I - I_0)$ is plotted *vs.* 1/[CD], an upward curvature is obtained. This suggests that the stoichiometry of the complex is not 1:1. However when $1/(I - I_0)$ is plotted *vs.* $1/[CD]^2$ a straight line

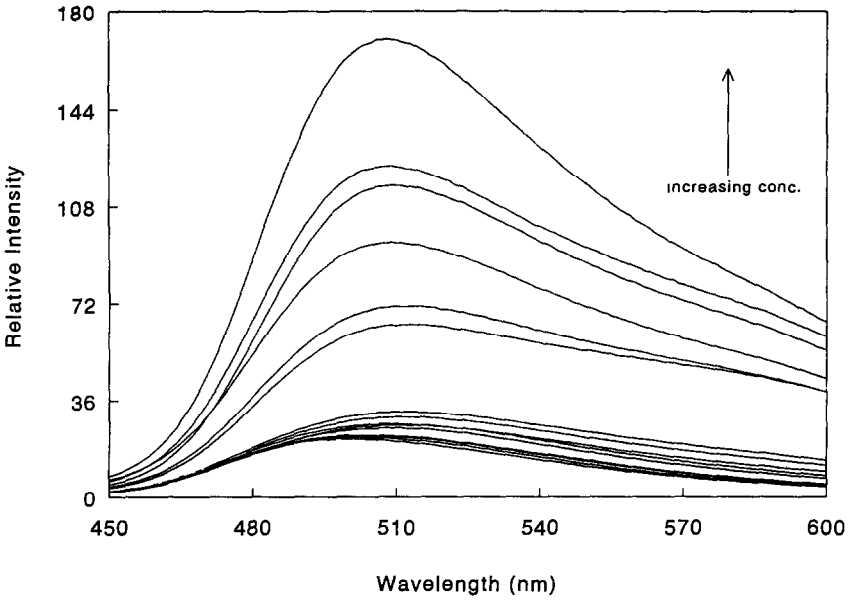

Fig. 6. Fluorescence spectra of AuO as a function of [γ-CD].

graph was obtained suggesting a predominant 2:1 stoichiometry for α-CD and AuO. Similarly, double reciprocal plots for β-CD . AuO shown in Fig. 5(a) and (b) suggest a predominant 1:1 complex. A downward curvature is obtained when the data is fitted into a 2:1 stoichiometry. This result indicates the absence or insignificant contribution of a 2:1 complex between β-CD and AuO. The equilibrium constants for the complexes can be easily calculated by dividing the slope to the ordinate-intercept of the linear line. However, the slope of the line used to calculate the equilibrium constant from the double reciprocal plots is very sensitive to the ordinate value of the points having small abscissa values.[22] This, in turn, tends to emphasize lower concentrations at the expense of higher concentrations. The use of non linear regression (NLR) has been proposed as an alternative approach to this graphical method.[27] This approach uses estimates of K_1 which have been determined by use of linear regression. The following equations were used to fit the data in a non linear regression method.

$$I = \frac{I_0 + I_1 K_1 [\mathrm{CD}]_0}{1 + K_1 [\mathrm{CD}]_0} \tag{9}$$

for β-CD . AuO complex, and

$$I = \frac{I_0 + I_2 [\mathrm{CD}]_0^2}{1 + K_2 [\mathrm{CD}]_0^2} \tag{10}$$

for α-CD . AuO complex. The estimated binding constants are summarized in Table 1.

Further characterization of the inclusion complexes of CDs and AuO were done using Corey-Pauling-Kolton (CPK) space filling models. It was found that AuO is a little too bulky to fill entirely in either the α or β-CD. In the case of a small α-CD with a cavity diameter of about 5.7 Å, at least two CDs may be needed to accommodate most of AuO inside the cavity. With β-CD, the small part of the AuO exposed to the aqueous environment is not enough to complex with a second CD molecule. Therefore, CPK models are in agreement with fluorescence measurements. The fluorescence enhancement of the dye at high concentrations of γ-CD was accompanied by the appearance of a broad emission band at longer wavelengths as shown in Fig. 6. The appearance of this band at higher concentrations suggests a stepwise formation of the 1:1 to 2:1 complex between AuO and CD. Since γ-CD has a large cavity, it is possible for two AuO to be included inside the CD cavity. Judging from CPK model, the 1:1 complex still has enough space in the CD cavity for a second molecule. Therefore, an additional guest molecule will certainly stabilize such an inclusion complex. Excimer formation in γ-CD have been reported for several compounds.[23–26,28] Computer simulations of the fluorescence intensities were carried out to estimate the binding constants associated with these kind of stoichiometries.

Fluorescence measurements in surfactants

The changes in the fluorescence intensity of the dye upon addition of the surfactant can be used to monitor the interaction between the dye and the surfactant. This method has been used to estimate the cmc of the surfactants, and recently AuO has been used to determine the microviscosity of the micelles.[5-7]

The anionic surfactant (SDS) appears to exert maximum influence on the fluorescence characteristic of the dye. The fluorescence intensity of the dye decreases at very low concentrations of the surfactant. In addition, in this concentration range solutions were turbid and the fluorescence maxima of the dye was accompanied by a red shift of about 15 nm. This behavior is consistent with the model which explains the interaction of cationic dyes with anionic surfactants in the premicellar region.[17] This model predicts a decrease in the fluorescence of the dye at low concentration of the surfactant due to the formation of aggregates between the dye and the surfactant. It has been reported that this kind of aggregation causes changes in the molecular geometry of the dye, which then non-radiatively deactivates the fluorescence.[9] The changed conformation appears susceptible to disaggregation on further addition of the surfactant. Figure 7(a) shows that the fluoresence intensity of the

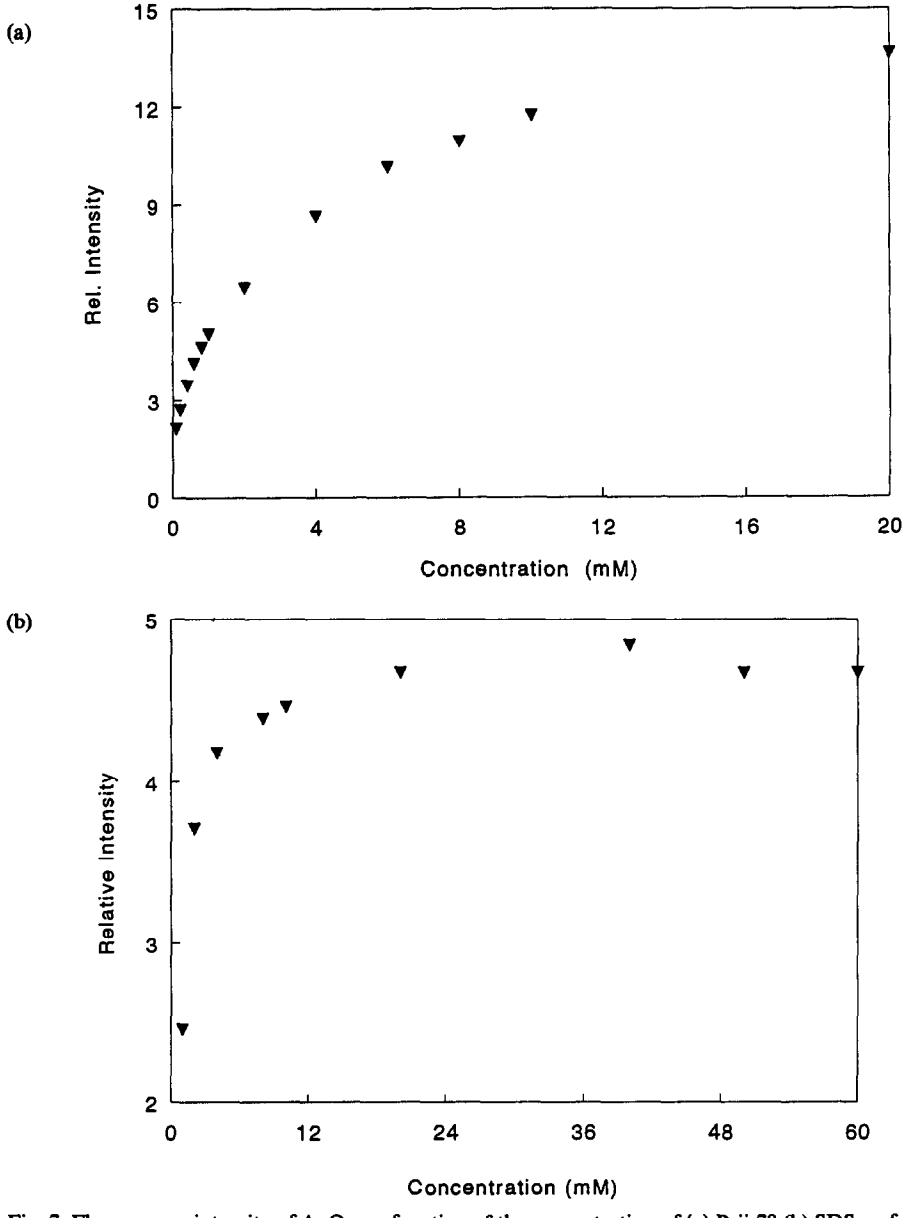

Fig. 7. Fluorescence intensity of AuO as a function of the concentration of (a) Brij-78 (b) SDS surfactant.

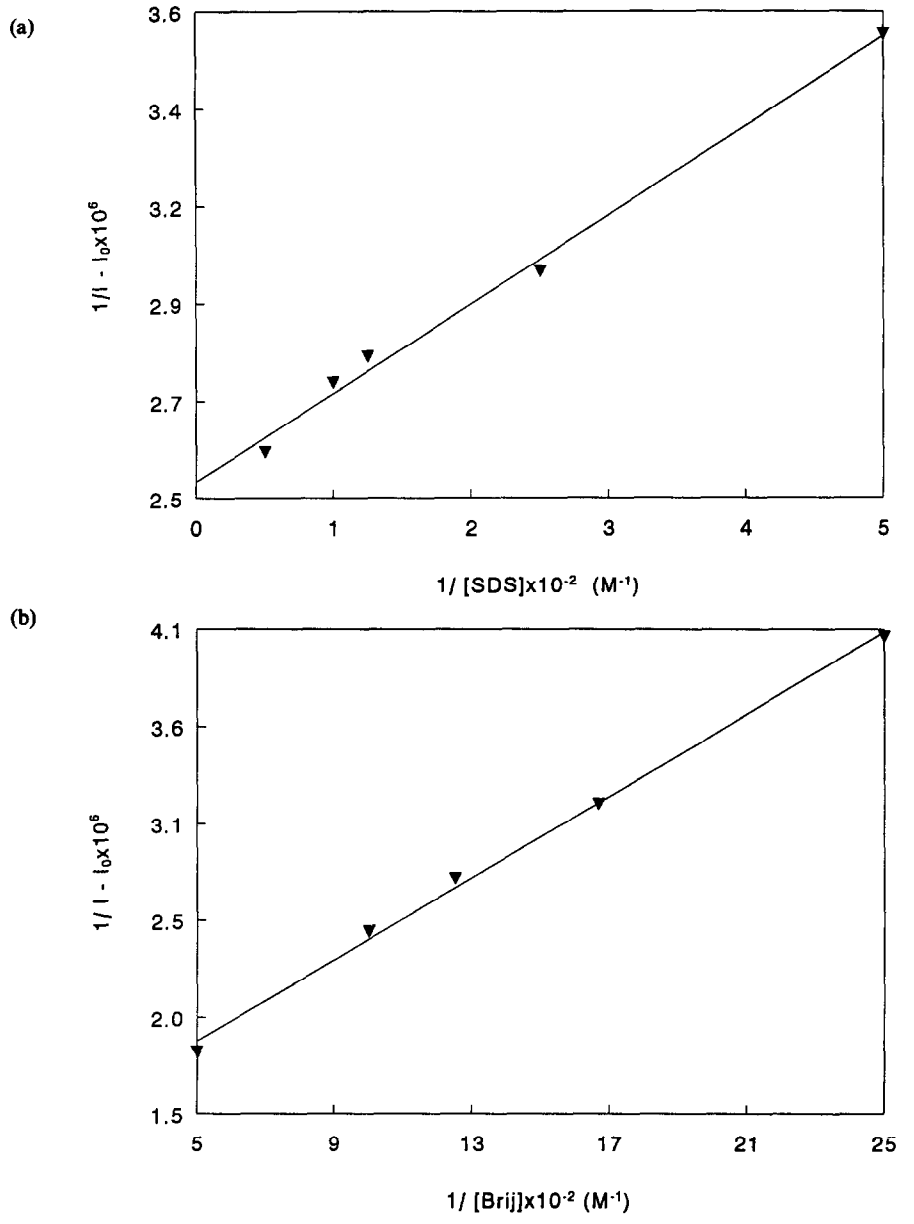

Fig. 8. Double reciprocal plots for the AuO and (a) SDS (b) Brij-78.

dye rises dramatically as the concentration of SDS approaches 8m*M*. This concentration is known as the cmc, and it is consistent with literature values.[11,29,30] At higher concentrations of SDS, the fluorescence of AuO levels off. Therefore, the AuO dye has no effect on the formation of SDS micelles. Since AuO is cationic, it is more likely that its orientation in the micelle is in close proximity to the polar group of the surfactant. Therefore, the fluorescence of the AuO will likely reflect the polar microenvironment of the surfactant rather than the hydrophobic region. Other fluorescent probes such as 2-*p*-toluidinylnaphthalene-6-sulfonate are known to bind with SDS micelles through hydrophobic interactions, and not charge neutralization.[11] These conclusions were based on the rationale that the binding involves a positive entropy change and that the salt increases the association constant only by increasing the entropy gain. Figure 7(b) shows the fluorescence of AuO in the presence of Brij-78 surfactant. The intensity of AuO showed a strong increase initially and levelled off at higher concentrations. The maximum intensity indicates a complete dye-surfactant interaction and subsequent disaggregation of the dye by the surfactant. In this case, the dye molecules are

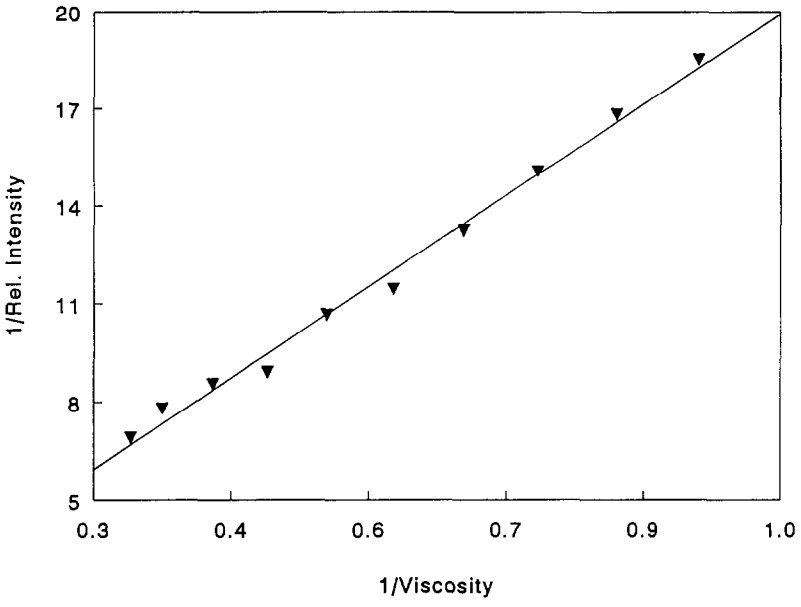

Fig. 9. Fluorescence intensity of AuO in glycerol-water solutions.

generally bound individually to the micelles. The equilibrium constants of AuO and micelles of Brij-78 or SDS were calculated using equation (5). Figure 8(a) and (b) show a typical double reciprocal plot of the surfactants with AuO. It is assumed that only one dye molecule is bound to the micelle. The equilibrium constants obtained from NLR method are summarized in Table 1.

Microviscosity in CDs and micelles

Fluorescence probes have been used to determine the microviscosity in micelles.[31,32] However, it is well understood that the microviscosity value strongly depends on the probe and the method used. For example, the microviscosity of SDS micelles was found to be 4 cP using intramolecular excimer formation of diphenylpropane and 19 cP using dipyrenylpropane.[31,32] Therefore, this method is more suitable for the estimation of a relative microviscosity, rather than obtaining absolute values. Since the correlation between the fluorescence intensity of AuO and the viscosity of the medium was discovered, AuO has been used to estimate the microviscosity of different micelles.[5,6] The working curve for AuO in glycerol-water mixtures is shown in Fig. 9. The fluorescence intensity of the dye increases linearly with the increased viscosity of the medium. Such dependence enabled us to estimate the microviscosity of the cavity in CDs and that of micelles. The maximum fluorescence intensities of the dye assuming complete complexation with the organized media were obtained from NLR method, and used to estimate the microviscosities. These values are summarized in Table 1. Much higher microviscosity values for CDs were obtained using fluorescence depolarization of a cationic detergent probe.[33] This probe was bound to the CD through hydrophobic interaction of the side chain and was too large to fit entirely into the CD cavity. The estimated microviscosities of the CDs in this work are meaningful if one assumes that only the CD cavity affects the rotational relaxations of AuO. Otherwise, these values only represent the effective microviscosity of the complexed AuO. The measured microviscosity in SDS micelles agrees well with other literature values.[5,31,32]

CONCLUSION

The results presented here demonstrate enhancement of the absorbance and fluorescence of AuO in the presence of cyclodextrins and surfactants. The enhancement in CDs is due to the interaction of the hydrophobic cavity with the hydrophobic moiety of the dye. As a result of such interactions the molecular interactions of the dye which are responsible for fluorescence quenching are restricted. The spectral changes of AuO dye by premicellar solutions of SDS is attributed to the change in the molecular geometry of the dye because of the attractive forces of the opposite charges. The observed

enhancement upon further addition of the surfactant can be attributed to a disaggregation process of the dye.

The maximum intensities of the dye assuming a complete complexation with the organized media in question have been estimated from NLR method. These values have been used to estimate the microviscosity in cyclodextrins and micelles.

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