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Note

Inclusion complexes of cyclohexaamylose with indole

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In recent years, there has been considerable interest in the inclusion compounds of cyclohexaamylose (cyclohexamaltose, α-cyclodextrin, α-CD, **1**) in solution[1]. Most studies involving organic guest molecules have shown 1:1 guest–host association complexes[2], although 2:1, 2:2, and 1:2 complexes have also been reported. The 2:1 complexes occur most frequently with cyclooctaamylose, where the cavity is large enough to accommodate the aromatic organic guests frequently used as probes. Thus 1-naphthyl acetate appears to form a 2:1 complex with **1** as evidenced by the excimer fluorescence[3]. Complexes in which two chemically different guests simultaneously occupy a cycloamylose cavity have also been reported[4]; also naphthalene and cycloheptaamylose have been postulated to form a 2:2 complex on the basis of the finding of the excimer fluorescence-band of naphthalene[5]. The existence of 1:2 complexes has been invoked to explain the spectral changes accompanying the addition of 6-(4-toluidino)-2-naphthalene sulfonic acid (TNS) to both cyclohepta- and cycloocta-amylose[6].

Organic inclusion-complexes of cyclohexaamylose (**1**) have been of the 1:1 variety[7], although there is no basic reason why multiple association should not also occur, except for the limitation imposed by the smaller size of the cavity of **1**. The binding forces responsible for these complexes may be characterized as weak intermolecular or "hydrophobic" interactions. Cycloamyloses resemble enzymes in having a polar exterior and hydrophobic interior. The formation of such complexes resembles, in some respects, the solubilization of hydrophobic molecules by micelles in aqueous solution and, likewise, may be studied effectively through use of fluorescence probes whose spectrum and fluorescence yield would be sensitive to the microenvironment of the cycloamylose cavity. Indole is an example of such a probe[8]. In passing from such highly polar solvents as water to nonpolar environments, indole exhibits a substantial blue-shift in its fluorescence spectrum. A major purpose of this work was to study the interaction between **1** and indole in order to shed more light on the guest–host interaction of cycloamyloses and clear up certain anomalies in the literature on the value of the association constant between **1** and indole[9].

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EXPERIMENTAL

Materials and procedures. — Indole and indole derivatives were purchased from Aldrich and purified by either sublimation, or recrystallization from ethanol. Cyclohexaamylose (α-CD, **1**, Aldrich) was used after recrystallization from alcohol and treatment with charcoal. Stock solutions of indole were made up with abs. ethanol and diluted with water. In no case was the concentration of ethanol in excess of 0.1%. All fluorescence studies were performed by measuring the fluorescence at right angles to the excitation beam in solution, where the fluorophore absorbance at the excitation wavelength was <0.05. Fluorescence spectra were not corrected. Stern–Volmer studies were performed by sequential addition of concentrated quencher to the fluorophore. The total volume change was <2%. Reproducibility of measurement was in general very good and results shown in this article are those of individual experiments. The one exception is the polarization results, which are reported as the average of three experiments obtained using the averaging routine provided in the Perkin–Elmer software package PECLS. The temperatures reported were within ±0.1°.

Fluorescence studies were performed with a Perkin–Elmer LS5 spectrofluorometer and absorbance studies with a Perkin–Elmer 559 u.v.–visible spectrophotometer. Both instruments were linked to a data station.

RESULTS AND DISCUSSION

In the absence of **1**, indole displays a broad, relatively structureless absorption band in which the ^1L_A and ^1L_B transitions are merged and indistinct[11]. The introduction of **1**, however, results in the appearance of a sharp band at 288 nm, as may be seen in Fig. 1 where the u.v.-absorption spectra of indole are shown as function of increasing concentrations of **1**. The most concentrated solutions of **1** employed (~0.1M) represent the solubility limit of **1**. The emergence of the peak at 288 nm is due to the appearance of the ^1L_B absorption band, which is a prominent feature of indole absorption in solvents of low dielectric-constant. Evidently, indole forms a guest–host association with **1** in which the indole probes the hydrophobic microenvironment of relatively low dielectric-constant in the cavity of **1**. Over the entire range of [**1**] investigated, no single isosbestic point exists. Increasing concentrations of **1** result in a red-shift in the leading edge of the absorption band until ~0.035M. Above this concentration, however, isosbestic points do appear at 283 and 287 nm. Fig. 2 shows the fluorescence spectra of indole at increasing concentrations of **1**. Excitation was at 260 nm. Increasing the concentration of **1** to 0.02M resulted in a general increase of fluorescence along the short-wavelength edge of the fluorescence band. Additional **1** resulted in a general shifting of the fluorescence band toward lower wavelengths, together with the appearance of several shoulders indicating the presence of vibrational structure in the fluorescence spectra. In keeping with the lack of an isosbestic point in the absorption spectra of indole, no iso-

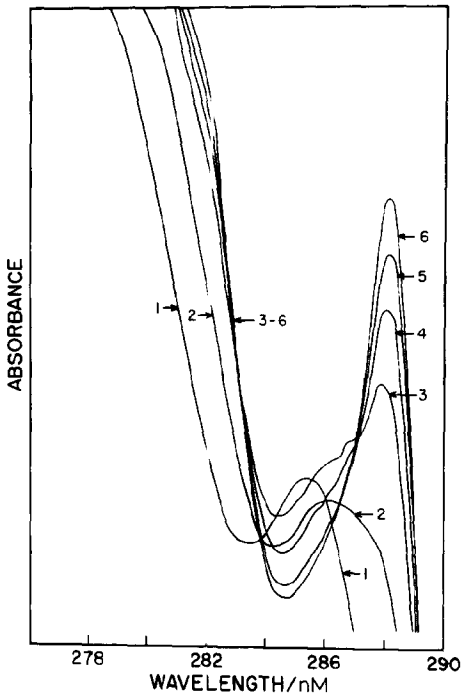

Fig. 1. Absorption spectra of aqueous indole in the presence of **1** (*1*) 0.0M **1**, (*2*) 0.037M **1**, (*3*) 0.055M **1**, (*4*) 0.074M **1**, (*5*) 0.093M **1**, and (*6*) 0.11M **1**. At concentrations of **1** <0.037M, isosbestic points appear at 283 and 287 nm.

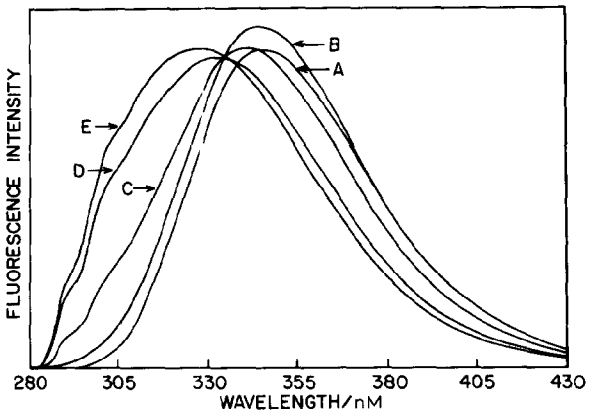

Fig. 2. Fluorescence spectra of aqueous indole (1×10^{-5}M) in the presence of **1**. *A*, 0.0M **1**, *B*, 0.01M **1**; *C*, 0.03M **1**; *D*, 0.067M **1**, and *E*, 0.10M **1**. $\lambda_{ex} = 260$ nm.

fluorescence point can be found that persists over the entire range of added **1**. The overall shift in the fluorescence band is in keeping with the changes observed in the absorption spectra, and may also be explained by an interaction of indole with **1** in which the indole occupies the environment of low dielectric-constant in the cavity

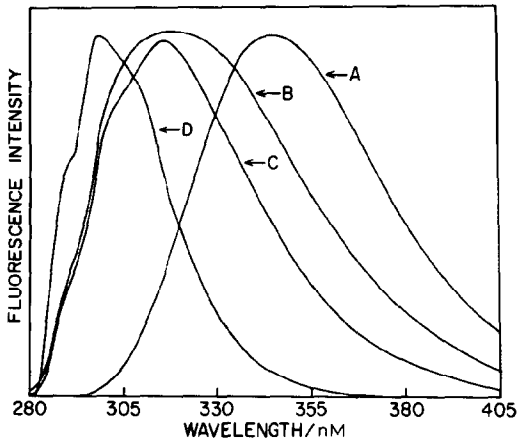

Fig. 3. Fluorescence spectra of indole. A, aqueous indole; B, indole–0.10M **1**; C, indole–0.10M **1**–7M CsCl; and D, indole dissolved in cyclohexane. λ_{ex} = 260 nm.

of **1**. The hypsochromic shift in the fluorescence band is similar to that occurring when indole fluoresces in solvents of low dielectric constant. In Fig. 3, the fluorescence spectrum of indole in water and also in cyclohexane is contrasted with that of indole in the presence of **1**. The intermediate position that the indole–**1** spectrum occupies would suggest that fluorescence is being observed from indole present in an environment whose dielectric constant lies between that of water and cyclohexane.

A 1:1 equilibrium between indole (In) and **1** may be represented by the following equation:

$$\mathrm{In} + \mathbf{1} \overset{K_1}{\rightleftarrows} \mathrm{In}\text{–}\mathbf{1} \qquad (1),$$

where In represents indole and K_1 the association constant for the formation of In–**1**. The Benesi-Hildebrand relationship[10] for such an equilibrium is:

$$\frac{1}{\Delta A_{288}} = \frac{1}{\Delta\varepsilon[\mathrm{In}_0]} + \frac{1}{\Delta\varepsilon\,\mathrm{In}_0 K_1}\,\frac{1}{[\mathbf{1}_0]} \qquad (2),$$

where ΔA_{288} is the change in absorbance accompanying the addition of **1**, $\Delta\varepsilon$ is the difference in molar absorptivity between In and In–**1**, and In_0 and $\mathbf{1}_0$ are the stoichiometric concentrations of In and **1**. A plot of ΔA_{288} *vs.* $(\mathbf{1})^{-1}$ is highly nonlinear, as may be seen in the upward curvature shown in Fig. 4. This fact, together with the lack of a single isosbestic point over the entire range of [**1**] studied, suggests that multiple equilibria involving additional indole–**1** species are present.

At least two mechanisms may be suggested to explain these results. In the first, indole, after combining with one molecule of **1** to form a 1:1 complex, reacts further with a second molecule of **1** to form a 1:2 complex:

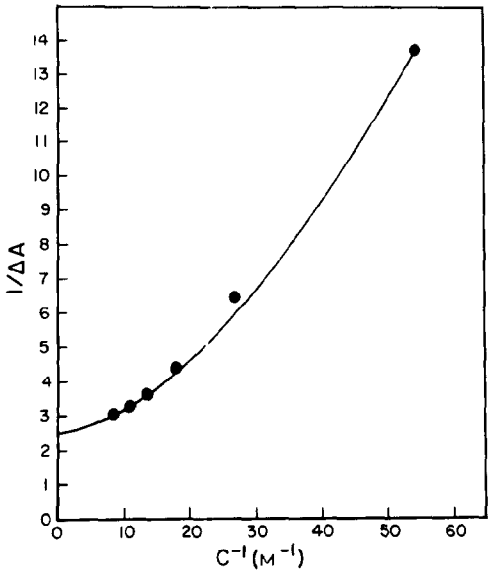

Fig. 4. Benesi–Hildebrand plot for changes in absorbance at 288 nm for indole solutions containing various concentrations of **1**. In = 1×10^{-5}M, T = 25°.

$$\text{In–}\mathbf{1} + \mathbf{1} \overset{K_2}{\rightleftarrows} \text{In}(\mathbf{1})_2 \qquad (3),$$

where $\text{In}(\mathbf{1})_2$ represents the 1:2 complex and K_2 the association constant for the formation of $\text{In}(\mathbf{1})_2$. Alternatively, the In–**1** complex formed in (*1*) may combine with a second to form a dimer of In–**1**:

$$2\text{In–}\mathbf{1} \overset{K_2}{\rightleftarrows} (\text{In–}\mathbf{1})_2 \qquad (4)$$

Additional mechanisms involving 2:1 guest–host complexes were rejected on steric grounds, that is, the cavity of **1** is simply too small to accommodate two indole molecules. Those examples in the literature where the mechanism of Eq. *4* was advanced as a possibility were made on the basis of the appearance of excimer bands in the fluorescence spectra[11]. The complete lack of excimer fluorescence in this study leads us to favor the mechanism of Eq. *3* although, of course, dimerization or simultaneous multiple-equilibria involving both mechanisms are also possible in this and similar instances in the literature.

Focusing on Eq. *1* and Eq. *3*, we may write:

$$K_1 = \frac{[\text{In–}\mathbf{1}]}{[\text{In}][\mathbf{1}]} \text{ and } K_2 = \frac{[\text{In}(\mathbf{1})_2]}{[\text{In–}\mathbf{1}][\mathbf{1}]} \qquad (5),$$

and as in this study, the concentration of **1** is large relative to indole **1** ($\approx \mathbf{1}_0$), the material balance condition requires

$$[\mathrm{In}_0] = [\mathrm{In}] + [\mathrm{In}\text{–}\mathbf{1}] + [\mathrm{In}(\mathbf{1})_2],$$

whereas

$$\Delta A_{288} = \varepsilon_{\mathrm{In}}[\mathrm{In}] + \varepsilon_{\mathrm{In}\text{–}\mathbf{1}}[\mathrm{In}\text{-}\mathbf{1}] + \varepsilon_{\mathrm{In}(\mathbf{1})_2}[\mathrm{In}(\mathbf{1})_2],$$

and then we find:

$$1/\Delta A_{288} = \frac{1 + K_1[\mathbf{1}_0] + K_1K_2[\mathbf{1}_0^2]}{\Delta\varepsilon[\mathrm{In}_0]K_1[\mathbf{1}_0] + \Delta\varepsilon[\mathrm{In}_0]K_1K_2[(\mathbf{1}_0)]^2} \qquad (6),$$

where $\Delta\varepsilon'$ is the difference in molar absorptivity between In and $\mathrm{In}(1)_2$. When $K_2 = 0$, Eq. (6) reduces to the normal Benesi–Hildebrand relationship.

An iteration technique designed to search for the parameters that best fit Eq. 6 to the experimental data was used to evaluate the equilibrium constants. The best fit was found when $K_1 = 2$ and $K_2 = 210\mathrm{M}^{-1}$.

Lewis and Hansen[9] reported a calorimetric technique for the binding constant of indole and **1** that allows for simultaneous measurement of both K and the enthalpy of complex formation. Their study assumed that a 1:1 complex was formed. In light of the spectroscopic evidence reported here, we conclude that their assumption and, therefore, the reported association constant, is incorrect. Lewis and Hansen pointed out that a plot of the enthalpy of binding *vs.* the entropy of binding of a number of organic molecules with **1** is linear, implying that changes in ΔH compensate for changes in ΔS. Not surprisingly, they found indole to be an exception to this general rule.

In the following discussion on quenching and polarization studies, the concentration of **1** was kept at 0.1M where, according to our calculations, the indole is distributed as ~70° $\mathrm{In}(\mathbf{1})_2$, 10% In–**1**, and 20% free indole.

The ability of compounds to quench the fluorescence of guest molecules is substantially altered in the presence of cycloamyloses. Such quenchers as iodide ion[5,9,11], O_2[11,12], and amines[11,13], are hampered in their ability to quench because of the protective nature of the cycloamylose. The exception occurs when the cavity is large enough to accommodate simultaneously both fluorophore and quencher. Enhanced static quenching is observed in these cases[11,13]. In this study, cesium ion was chosen as the quencher, as inorganic cations (unlike anions[14]) appear to have little affinity for **1**. Any change in fluorescence intensity may be attributed directly to quenching rather than to competitive equilibrium between indole, quencher, and **1**. The Stern–Volmer relationship (Eq. 7) was found adequate to describe the quenching of indole fluorescence by cesium ion:

$$I_0/I = 1 + k_q\tau_0\,[Q] \qquad (7),$$

where I_0 and I represent the fluorescence intensities in the absence and presence of quencher. The fluorescence lifetime[15] of indole in aqueous solution is $\tau_0 = 4.9 \times 10^{-9}$s. In the absence of **1**, cesium ion was found to quench indole fluorescence with a k_q value of $9.6 \times 10^8 M^{-1}s^{-1}$. Quenching studies in the presence of **1** revealed quenching constants that are wavelength-dependent, being equal to $8.1 \times 10^7 M^{-1}s^{-1}$ at 300 nm where only $In(\mathbf{1})_2$ fluoresces, and increasing gradually to 420 nm where $k_q = 4.7 \times 10^8 M^{-1}s^{-1}$. The greatly diminished quenching ability of cesium ion in the case of $In(\mathbf{1})_2$ is attributable, as in the cases already mentioned, to the protective nature of **1**, which effectively prevents close approach of the cesium ion. Even at 420 nm, where most of the fluorescence is due to uncomplexed indole, the quenching constant is halved. We attribute this result partially to the presence of In–**1**, which also fluoresces in this region and should offer at least partial protection from the quenching effects of cesium ion, and partially to the measured 30% increase in viscosity of a 0.1M solution of **1** as compared to aqueous indole alone. Increasing the Cs^+ concentration may lead to complete suppression of the free indole fluorescence, revealing primarily the fluorescence of the $In(\mathbf{1})_2$ complex. The spectrum in Fig. 3 indicated by (C) is the fluorescence from a 0.1M **1**–indole mixture dissolved in 7M CsCl. We consider that this fluorescence is due almost entirely to $In(\mathbf{1})_2$

Previous studies on **1**–guest complexation, and the present investigation, have been clarified through the use of Corey–Pauling–Koltum molecular models, which appear to be accurate in portraying the size of the cavity in **1** relative to the organic guest-compound. Indole fits snugly into the cavity of **1**, with the 5-membered ring entering first. The models also indicate that methyl substitution at C-2 of indole does not substantially hinder the introduction of indole into the cavity. In agreement with this, it was found experimentally that 2-methylindole behaves similarly to indole. On the other hand, addition of **1** to tryptophan and other such 3-substituted indole derivatives as indoleacetic acid showed little if any perturbation of either the absorption or fluorescence spectra. This result could indicate that the introduction of a bulky substituent at C-3 of indole sterically hinders its ability to enter the cavity. Certainly, this is true, judging from CPK models. On the other hand, Lewis and Hansen[9], using calorimetric measurements, reported that tryptophan forms a complex with **1** having an association of $K = 32M^{-1}$ (assuming a 1:1 complex). It is conceivable that, in the case of tryptophan, it is the alaninyl sidechain that forms a complex with **1** leaving the indole chromophore relatively free of the environment within the cavity that is responsible for changes in the absorption and fluorescence spectra.

Indole contained so securely within the cavity of one or two host molecules of **1** might be expected to be subject to a certain degree of rotational inhibition and in the extreme be so tightly wedged in the cavity as to acquire rotational characteristics of **1** itself. These questions may be studied by analysis of the polari-

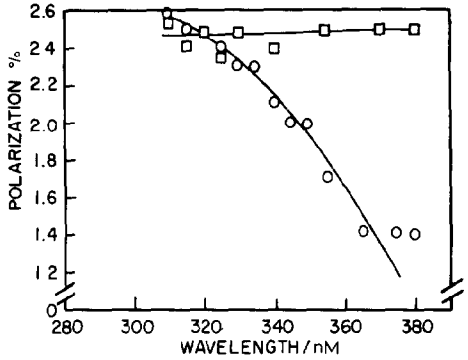

Fig. 5. Percent fluorescence polarization as a function of fluorescence wavelength for aqueous indole–0.1M **1** (○) and indole–0.1M**1**/7M CsCl (□).

zation of indole solutions. Indole itself, in aqueous solution at room temperature, has a negligible degree of fluorescence polarization. On the other hand solutions of indole containing 7M CsCl and 0.1M **1** — conditions where only $In(\mathbf{1})_2$ fluoresces — was found to be 2.5% polarized (see Fig. 5). In comparison, an indole–0.1M solution of **1** in the absence of the quenching effect of CsCl was found to exhibit increasing polarization with decreasing fluorescence wavelength, as would be anticipated, as the fraction of fluorescence from $In(\mathbf{1})_2$ increases with decreasing wavelength. These results suggest that indole, in fact, experiences rotational inhibition when associated with **1**.

At room temperature, uncomplexed indole can be made to exhibit fluorescence polarization by increasing the solvent viscosity. For example, indole dissolved in a saturated solution of sucrose was found to be 11% polarized. Addition of **1** to this solution resulted in the expected blue-shift in the fluorescence due to association, and the measured polarization of this solution at 325 nm (where the fluorescence is due to the complex) was only 7%. Now, if the indole were so restricted in its rotational motion within the cavity of **1** as to acquire the global rotation of the cycloamylose molecule itself, an increase, not a decrease, in polarization would have been expected. Evidently, the indole within the cavity of **1** must have a microviscosity greater than that of water but less than that of a saturated sucrose solution.

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