PYRENE AND β-CYCLODEXTRIN COMPLEXES: Chiral Discrimination and Ternary Complex Formation

by

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Abstract

The aim of this project is to study whether chiral environments can lead to chiral discrimination for a photophysical process where only one of the partners is chiral. These studies will provide insights for biochemical processes by using simple chemical model molecules. The quenching of excited singlet pyrene complexed to ß-cyclodextrin (CD) by D- and L-tryptophan was studied in the absence and presence of alcohols or alkyl sulfates. Alcohols such as 1-butanol, 2-butanol and *tert*-butanol and alkyl sulfates such as 1-butylsulfate and 1-hexylsulfate were employed to enhance the equilibrium constant of pyrene with ß-CD. Steady-state fluorescence and time-correlated single photon counting showed that the quenching of pyrene inside the cavity by tryptophan occurs through a static mechanism. This mechanism requires the quencher to be in close proximity to pyrene within the ß-CD complex. Chiral discrimination was observed in the quenching by D- and L-tryptophan of excited singlet pyrene bound to ß-CD in the presence of alcohols and alkyl sulfates. The structure of the added alcohols or alkyl sulfates has a marked effect on the quenching efficiency of L-tryptophan. The association constant of tryptophan with ß-CD was determined to clarify the mechanism for the chiral discrimination. A 1:2:n stoichiometry for pyrene:ß-CD: tryptophan is suggested to be involved.

During the quenching studies of pyrene complexed to ß-CD we noted that tryptophan altered the equilibrium between pyrene and ß-CD. For this reason, a series of amino acids were employed to investigate the possibility of ternary complexation involving pyrene, ß-CD and amino acids. Among the nine amino acids tested, only leucine, phenylalanine and tryptophan formed ternary complexes.

This is the first example of zwitterionic compounds forming ternary complexes with pyrene and ß-CD. Temperature dependence studies on the pyrene fluorescence emission and lifetimes in the presence of ß-CD and leucine demonstrated the sequential pyrene ß-CD complexation with 1:1 and 1:2 stoichiometries. The conditional equilibrium constants between pyrene and ß-CD at a leucine concentration of 1.0×10^{-2} M were estimated by time correlated single photon counting (SPC) measurements. The values for complexes with 1:1 and 1:2 stoichiometries are $(3.8 \pm 1.2) \times 10^{2}$ M^{-1} and $(1.8 \pm 0.2) \times 10^{3}$ M^{-1} at 20 °C, respectively. This is the first time that the sequential equilibrium constants for pyrene and ß-CD ternary complexes were determined.

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To my parents

and in the memory of my grandmother

1. Introduction

1.1. Photophysics

1.1.1. Fluorescence

Some of the energy levels involved in the absorption and emission of light can be described by the Jablonski diagram.[1]

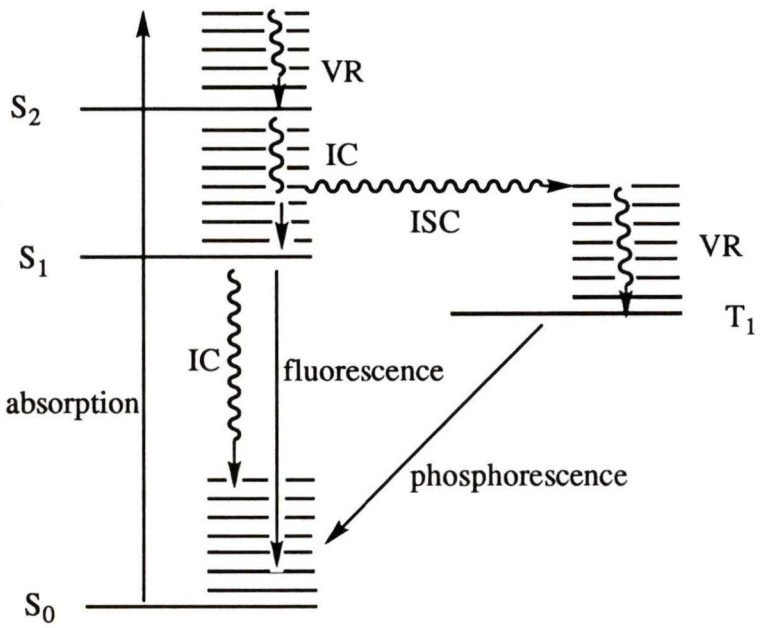


Figure 1.1: Jablonski energy-level diagram. The ground, first, second electronic singlet states and first electronic triplet state are depicted as S_0, S_1, S_2, and T_1, respectively. VR, IC, and ISC represent vibrational relaxation, internal conversion, and intersystem crossing, respectively.

For an electronically excited molecule, several processes can cause de-excitation.[2-4] Internal conversion (IC) refers to the nonradiative transition between states with the same spin multiplicity, such as $S_2 \rightarrow S_1$ and $S_1 \rightarrow S_0$, and intersystem crossing (ISC) is the nonradiative transition between states of

different spin multiplicity, such as $S_1 \rightarrow T_1$. In the solution phase, excess vibrational energy is rapidly removed by collisions with solvent molecules in a non-radiative process called vibrational relaxation (VR).

Excited states can also decay through two different radiative transitions, fluorescence and phosphorescence. Fluorescence is the radiative emission from an excited state to a lower state of the same multiplicity. The most common fluorescence emission involves the transition from first excited singlet state to the ground state, i.e. $S_1 \rightarrow S_0$ transition. If the spin multiplicity of the emitting state differs from the lower energy state, the emission is called phosphorescence. $T_1 \rightarrow S_0$ is the most common transition leading to phosphorescence. Most photochemical and photophysical processes come from S_1 and T_1 since internal conversion from higher excited states to S_1 and T_1 is very fast.

Fluorescence is a quantum mechanically "allowed" transition and the emissive rate constant is typically around 10^8 s^{-1} which results in fluorescence lifetimes shorter than 10 nanosecond (ns). The fluorescence lifetime or decay lifetime of a molecule can be defined in term of the rate of depopulation of the first excited singlet state (i.e. $S_1 \rightarrow S_0$):[5]

$$\frac{d[^1M^*]}{dt} = -\frac{[^1M^*]}{\tau_o} \qquad (1.1)$$

where τ_o is the molecular fluorescence lifetime. Upon integration, Eq. (1.1) gives a fluorescence response function of the form:

$$[^1M^*] = [^1M^*]_o \, e^{-t/\tau_o} \qquad (1.2)$$

where $[^1M^*]$ and $[^1M^*]_o$ represent the excited state concentration at time t and $t = 0$, respectively. From Eq. (1.2) it follows that the fluorescence lifetime, τ_o, is the time required for the concentration of excited states to fall to 1/e (0.368) of its initial value.

The fluorescence lifetime of $^1M^*$ is given by:

$$\tau_o = \frac{1}{k_r + k_{nr}} \tag{1.3}$$

where k_r and k_{nr} represent radiative and nonradiative rate constants, respectively. Furthermore, the fluorescence quantum yield (Φ_{fluo}), which corresponds to the ratio of the number of photons emitted to the number absorbed, is equal to the fraction of fluorophores which decay through emission and is given by:

$$\Phi_{fluo} = \frac{k_r}{k_r + k_{nr}} \tag{1.4}$$

or by combining Eq. (1.3) and Eq. (1.4)

$$\Phi_{fluo} = \tau_o \, k_r \tag{1.5}$$

Substances which display significant fluorescence generally possess delocalized electrons present in conjugated double bonds, such as perylene, pyrene, anthracene, dansyl chloride (DNS-Cl) and 1-anilino-8-naphthalene sulfonate (ANS). Since fluorescence detection is highly sensitive, very small

amounts of fluorescent probe molecules can be detected. For this reason fluorescence techniques have been exploited in many research areas.[6-12]

1.1.2. Techniques for the measurement of fluorescence

In this section, steady-state fluorescence spectroscopy and time-resolved single photon counting techniques are described.

Fluorescence spectroscopy. The fluorescence emission spectrum, or simply emission spectrum, is the wavelength distribution of the emission of an excited state. The spectrum is measured for a single constant excitation wavelength. Conversely, the fluorescence excitation spectrum is dependent on the emission intensity at the excitation wavelength.[4,13] These spectra are measured with a fluorescence spectrometer. The essential components are shown in **Figure 1.2.**

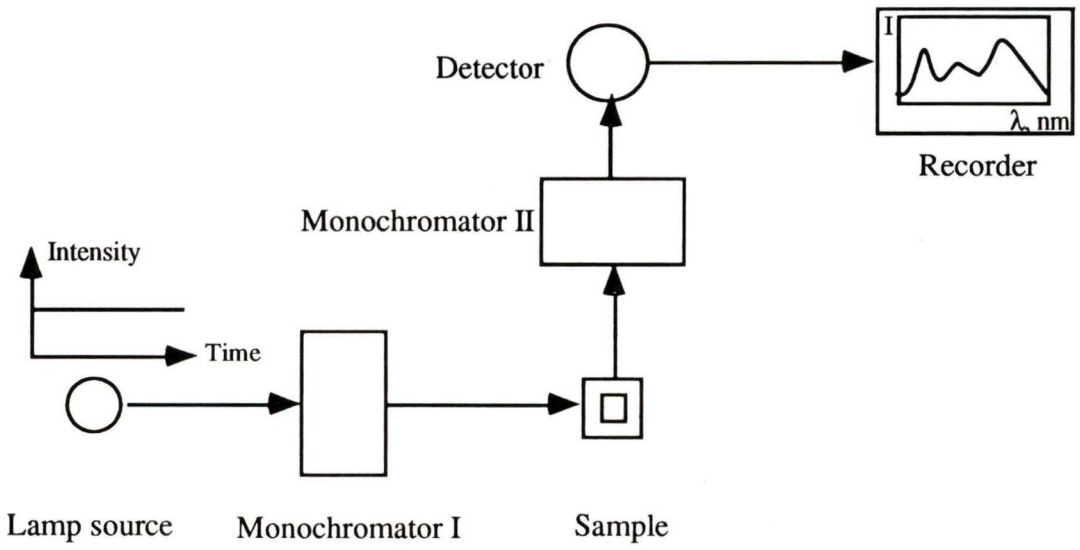


Figure 1.2: Simplified layout of a fluorescence spectrometer.

The fluorescence is observed at right-angles to the incident excitation light. Rayleigh and Raman scattered light have minimum interference in such a 90° arrangement. The lamp of the fluorometer is a Xenon arc lamp that is on continuously during the measurement.

The relationship between the transmitted light intensity of a dilute sample and its concentration follows the Beer-Lambert law:

$$I_{tr} = I_o \, 10^{-\varepsilon lc} \tag{1.6}$$

where I_{tr} and I_o are the intensities of transmitted and incident light, ε is the molar absorption coefficient (M^{-1} cm^{-1}), l is the pathlength (cm) and c is the molar concentration. Thus the intensity of the absorbed light, I_{abs}, is given by:

$$I_{abs} = I_o \, (1-10^{-\varepsilon lc}) \tag{1.7}$$

The fluorescence intensity I_{fluo} is given:

$$I_{fluo} = I_{abs} \, \Phi_{fluo} \tag{1.8}$$

by combining Eq. (1.7) and Eq. (1.8)

$$I_{fluo} = I_o \, \Phi_{fluo} \, (1-10^{-\varepsilon lc}) \tag{1.9}$$

If $\varepsilon lc \leq 0.05$ the above equation can be simplified to:

$$I_{fluo} = 2.303\ I_o\ \Phi_{fluo}\ \varepsilon\ lc \qquad (1.10)$$

Systematic errors occur by using Eq. (1.10) even with very dilute solutions. A solution with an absorbance of 0.01 will generate about 1% error in the fluorescence intensity by using Eq. (1.10) when compared with the value obtained by using Eq. (1.9). A solution with absorbance 0.05 will give an error in fluorescence intensity of about 5%. In addition, optical artifacts such as inner filter effects can also lead to a nonlinear dependence of I_{fluo} with I_o. This artifact occurs when a molecule that is different from that for which the fluorescence is being measured either absorbs the fluorescence emission or the excitation light.

Time-correlated single photon counting (SPC). SPC is also known as time-correlated single photon timing or time-resolved SPC. It is a technique in which the fluorescence is measured in the time domain.[2,5,14] A greater amount of information about rates and kinetics of intra- and intermolecular processes is gathered with SPC when compared to steady-state fluorescence spectroscopy. A typical single photon counting apparatus is shown in **Figure 1.3**.

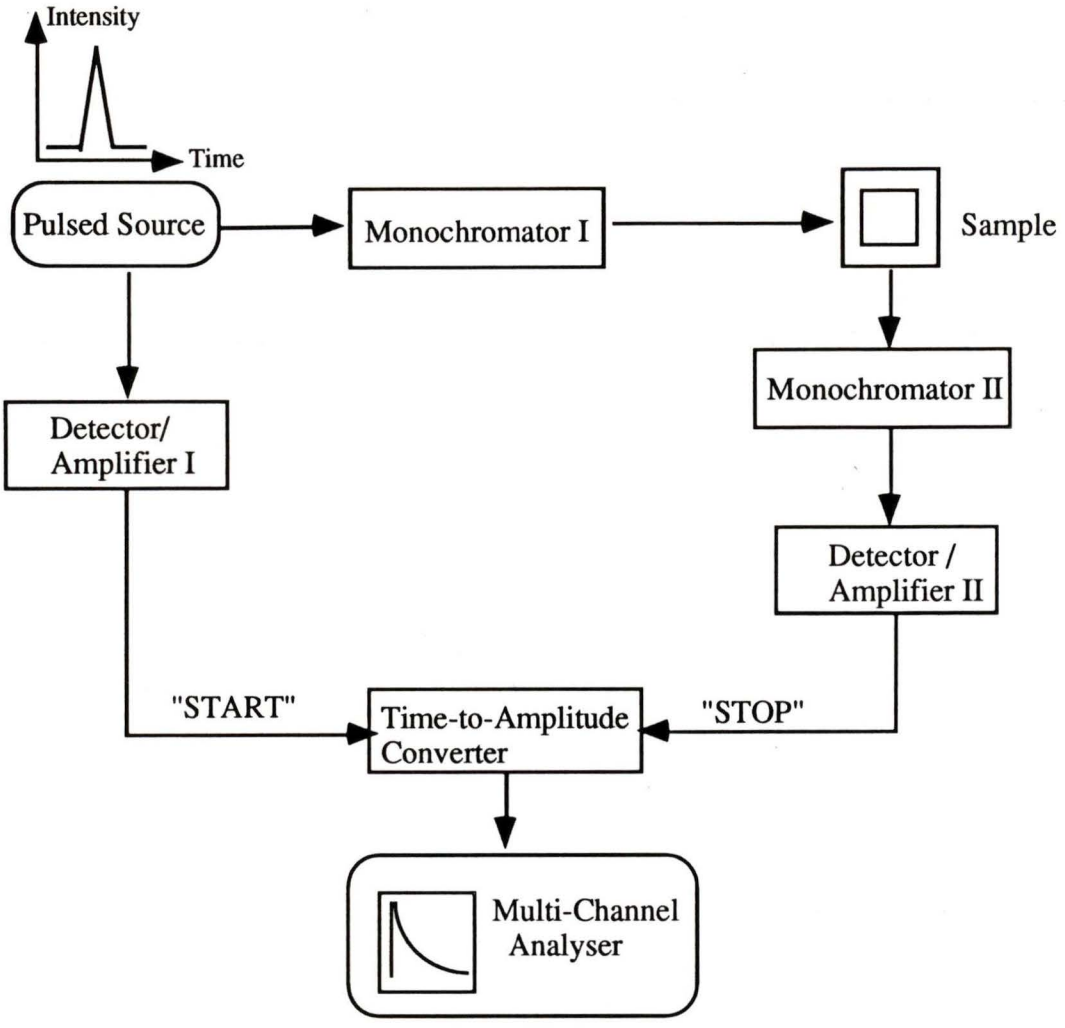


Figure 1.3: Simplified time-correlated single-photon counting diagram.

The system contains two light detectors. Detector/amplifier I is used to trigger the START channel for each pulse from the excitation source. Detector/Amplifier II is employed to trigger the STOP channel. The STOP channel detects the arrival of the first photon emitted from the sample. The voltage ramp of the time-to-amplitude converter (TAC) is triggered by the START channel. Simultaneously, molecules in the sample are excited by the lamp pulse and

undergo radiative decay. Detector/Amplifier II detects the first photon from this emission and the voltage ramp is halted when the TAC is triggered from the STOP channel. The amplitude of the TAC output is proportional to the time delay between the START and STOP channel (**Figure 1.4**).

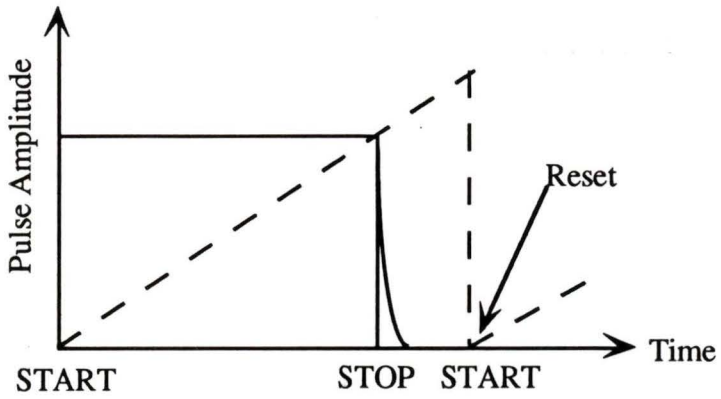


Figure 1.4: Voltage ramp generation by TAC.

For each stop pulse the voltage readout is stored in one channel of the multichannel analyzer (MCA). The MCA is calibrated and each of the channels of the MCA corresponds to an certain time interval. After sufficient cycles a decay curve is obtained which corresponds to the distribution of counted photons per channel. The channel contents of the MCA are independent and strictly Poisson distributed. From this histogram of the fluorescence emission lifetimes are obtained by iterative reconvolution procedures. 10,000 counts were accumulated in the channel with maximum counts since this number was considered to be sufficiently high for the data to be statistically meaningful.[15]

In practice, the judgment of the goodness of the statistical fit is an important issue, since it allows the comparison and examination of experimental

data by other research groups. The commonly used statistical tests for the fitting quality include the chi squared value, plots of calculated residual and the autocorrelation function, the Durbin-Watson parameter, and the Z-test.[8,15] The criteria for goodness of fit are: 1). Chi squared (χ^2). Acceptable values are between 0.8 and 1.3 for "ideal" Poisson distributed data. Low χ^2 values (≤ 0.75) mean that the data set is insufficient for a meaningful fit, while high values (> 1.5) indicate significant deviation from the equation to which the data were fitted. 2). Residuals. Good fits should yield randomly distributed residuals centered about zero. 3). Autocorrelation. Random distribution around the zero point is desired. 4). Durbin-Watson (DW) parameter. This parameter is used to judge the fitting quality for multilinear models. For decays, DW values higher than 1.7, 1.75 and 1.8 indicate acceptable quality for fits (256 or 512 data channels) for single, double or triple exponential models, respectively. Lower DW values are symptoms of an incorrect fitting function or skewed data.[8] 5). Z-test. The residuals are generally considered to be random for $|Z| < 3$. When the Z value is within the [-2, 2] interval there is a 95.44% probability that the residuals to the fit are random.[16]

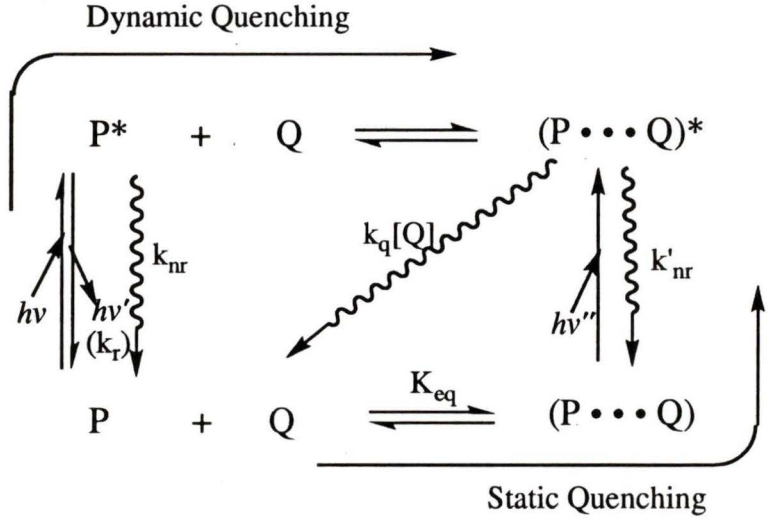
1.1.3. Fluorescence Quenching

Fluorescence quenching is the acceleration of the deactivation process of an excited state by a quencher.[4,17] The most common case is bimolecular deactivation of an excited-state by a quencher molecule. Self-quenching or concentration quenching is a process in which the excited-state is quenched by its ground-state. This process also involves a bimolecular reaction.

Fluorescence quenching can proceed by different mechanisms, such as energy transfer, charge transfer or electron transfer. These reactions can occur

after the collision between the excited state and the quencher, at long-ranges[18-20] when no contact between the reaction partners occurs or within complexes between the molecule and the quencher formed prior to the excitation process. Thus, quenching has wide applications in the study of different fundamental phenomena. In this section only the quenching mechanisms which require close contact will be addressed.

The quenching of an excited singlet state probe, P*, by a solute quencher, Q, can be described by the following reaction scheme:



Scheme 1.1: Static and dynamic quenching.

There are two quenching mechanisms to be considered. One requires collisional encounters between quencher and the fluorescence probe, which is called dynamic or collisional quenching. Since probe and quencher need to diffuse to a space close enough for dynamic quenching to occur, the diffusion rate of the molecules in the solution is the maximum quenching rate constant that can

achieved. Some typical values for diffusion limited rate constants are given in **Table 1.1.**

Table 1.1: Diffusion limited rate constants (k) for several solvents at 25 °C.[a]

Solvent	Viscosity η (cp)	k (10^{10} M^{-1} s^{-1})
isopentane	0.215	4.6
benzene	0.603	1.6
decane	0.861	1.2
water	0.890	1.1
dodecane	1.35	0.76

[a] Data from reference 21[21] as predicted by the Smoluchowski equation k = 8RT/ 3000 η. This equation underestimates the diffusion coefficient of small molecules such as oxygen.

The other quenching mechanism, static quenching, results from the formation of a ground state complex, (P • • • Q). When this complex absorbs light deactivation of the excited state is normally fast and no emission is observed. In some instances, this excited complex (exciplex) has a high enough emission quantum yield that fluorescence is observed. However, this fluorescence is generally shifted to longer wavelengths and does not interfere with the emission of P*.

The dynamic quenching of fluorescence is described by the Stern-Volmer equation:[4,22]

$$\frac{\tau_o}{\tau} = \frac{I_o}{I} = 1 + k_q\tau_o [Q] = 1 + K_{sv} [Q] \qquad (1.11)$$

where τ_o and τ are the lifetimes, I_o and I are the fluorescence intensities in the absence and presence of quencher, respectively, k_q is the quenching constant, [Q] is the quencher concentration and K_{sv} is the Stern-Volmer constant ($K_{sv} = k_q\tau_o$).

For static quenching, a decrease of the steady-state fluorescence intensity is observed. However, the fluorescence lifetime remains constant, since the observed fluorescence comes only from uncomplexed excited probe molecules. This quenching mechanism is described by the following equations:[4]

$$\frac{I_o}{I} = 1 + K_{eq} [Q] \qquad (1.12)$$

$$\frac{\tau_o}{\tau} = 1 \qquad (1.13)$$

where K_{eq} is the equilibrium constant for the ground state complex.

In some cases, both dynamic and static quenching happen simultaneously. In this case, the quenching equation is given by:[4]

$$\frac{I_o}{I} = (1 + K_{eq} [Q]) (1 + K_{sv} [Q]) \qquad (1.14)$$

1.2. Determination of equilibrium constants

Two common spectroscopic methods can be employed to determine equilibrium constants of complexes. One of these methods is the Benesi-

Hildebrand treatment. For a solution containing substrate or guest molecules in the presence of ligand or host molecules, the change of a property, such as absorption, fluorescence emission, ellipticity, chemical shift, relaxation time and retention time, as a function of total ligand or host concentration is measured. The second method relies on time resolved fluorescence measurements. The pre-exponential factor of each lifetime component is proportional to the concentration of one species, bound or free guest, in solution. This method is only applicable if the fluorescence lifetimes for the free and bound guest are different.

1.2.1. Benesi-Hildebrand treatment[23]

This classical treatment was introduced by Benesi and Hildebrand when they studied the complex formation between iodine and aromatic hydrocarbon by measuring the iodine complex UV absorption.[24]

The equilibrium between a guest molecule and host molecules is given by:

$$G + nH \underset{}{\overset{K_{1n}}{\rightleftharpoons}} G{\cdot}H_n \qquad (1.15)$$

where K_{1n} is the association constant for a guest:host complex with 1:n stoichiometry. Benesi and Hildebrand assumed a 1:1 stoichiometry (n=1) and in this case the equilibrium constant is defined by the following equation:

$$K_{11} = \frac{[G{\cdot}H]}{[G]\,[H]} \qquad (1.16)$$

The relation between the observed absorbance change (ΔI) and host concentration is given by:

$$\Delta I = \frac{[G]_t K_{11} \Delta\varepsilon [H]}{1 + K_{11} [H]} \qquad (1.17)$$

where $\Delta\varepsilon$ is the difference in the molar absorption coefficients between complexed and free guest, $[G]_t$ is the total concentration of guest and [H] is the free host concentration (in a typical experiment, [H] is much bigger than $[G]_t$ thus $[H] \approx [H]_t$).

Eq. (1.17) can be rearranged into a double-reciprocal linear relationship which corresponds to the equation originally employed by Benesi and Hildebrand:

$$\frac{1}{\Delta I} = \frac{1}{[G]_t K_{11} \Delta\varepsilon [H]_t} + \frac{1}{[G]_t \Delta\varepsilon} \qquad (1.18)$$

It is worthwhile to mention that although Eq. (1.17) and Eq. (1.18) are equivalent from the mathematical point of view the fit to the data in the form of a linear double reciprocal plot (Eq. (1.18)) gives a higher weight for data points with larger errors (smaller ΔI values). In the non linear fit to Eq. (1.17) the experimental points are equally weighted and this analysis is more accurate. A further advantage of the non linear fit is that deviations from the assumed stoichiometry can be more easily identified.[25]

The Benesi-Hildebrand approach can be generalized for host systems with a 1:n stoichiometry assuming that this stoichiometry corresponds to the complex

formed predominantly. Under these conditions Eq. (1.17) and Eq. (1.18) are modified to:

$$\Delta I = \frac{[G]_t K_{1n} \Delta\varepsilon [H]_t^n}{1 + K_{1n} [H]_t^n} \qquad (1.19)$$

$$\frac{1}{\Delta I} = \frac{1}{[G]_t K_{1n} \Delta\varepsilon [H]_t^n} + \frac{1}{[G]_t \Delta\varepsilon} \qquad (1.20)$$

For systems involving several equilibria, the Benesi Hildebrand approach is not very useful as the equations get more complex and the data have to be fitted to a greater number of unknown parameters. For example, the sequential formation of guest:host complexes with 1:1 (K_1) and 1:2 (K_2) stoichiometries the equation for ΔI is given by:

$$\Delta I = \frac{[G]_t (K_1 \Delta\varepsilon_{11} [H]_t + K_1K_2 \Delta\varepsilon_{12} [H]_t^2)}{1 + K_1 [H]_t + K_2 [H]_t^2} \qquad (1.21)$$

where $\Delta\varepsilon_{11}$ and $\Delta\varepsilon_{12}$ are the differences for the molar absorption coefficients between 1:1 and 1:2 complexes and the free guest, respectively.[23,26]

1.2.2. Determination of equilibrium constants using time-correlated single photon counting

Association constants can also be obtained from the pre-exponential factors of decay curves measured by time-correlated SPC. If the lifetimes of the

bound (τ_b) and free (τ_f) probe are different, the decay curve can be fitted to the sum of two exponentials:

$$I_{fluo}(t) = N\ (k_b\ \varepsilon_b[GH^*](t) + k_f\ \varepsilon_f[G^*](t))$$
$$= N\ (k_b\ \varepsilon_b[GH^*](t=0)\ \exp(-t/\tau_b) + k_f\ \varepsilon_f[G^*](t=0)\ \exp(-t/\tau_f)) \quad (1.22)$$

where k_b and k_f are fluorescence emission rate constants, ε_b and ε_f are the molar absorption coefficients at the excitation wavelength, [GH*] and [G*] are excited state concentrations of bound and free probe molecules. $I_{fluo}(t)$ is the fluorescence emission intensity at a specific time t. *N* is an equipment parameter depending on the experimental conditions.[27,28] For many cases including pyrene/CD systems, the excited state and ground state equilibrium constants can be consider equal. Thus Eq. (1.22) can be rewritten in terms of ground state concentrations:

$$I_{fluo}(t) = N\ (k_b\ \varepsilon_b[GH]\ \exp(-t/\tau_b) + k_f\ \varepsilon_f[G]\ \exp(-t/\tau_f)) \quad (1.23)$$

The experimental data are fitted to the sum of two exponentials:

$$I_{fluo}(t) = A_b\ \exp(-t/\tau_b) + A_f\ \exp(-t/\tau_f) \quad (1.24)$$

where A_b and A_f are the pre-exponential factors corresponding to the emission of bound and free probe, respectively. By combining Eq. (1.23) and Eq. (1.24) we obtain:

$$A_b/A_f = (k_b\ \varepsilon_b[GH])\ /\ (k_f\ \varepsilon_f[G]) \quad (1.25)$$

$$[GH]/[G] = (A_b/A_f)\ (k_f/k_b)\ (\varepsilon_f/\varepsilon_b) \qquad (1.26)$$

Substituting $k = \Phi/\tau$ into Eq. (1.26), the concentration ratio of bound to free probe can be given by:

$$\frac{[GH]}{[G]} = \frac{A_b}{A_f}\ \frac{\tau_b}{\tau_f}\ \frac{\varepsilon_f}{\varepsilon_b}\ \frac{\Phi_f}{\Phi_b} \qquad (1.27)$$

By substituting Eq. (1.26) into Eq. (1.16) and assuming that the host concentration is in excess over the guest concentration we obtain:

$$K_{11} = \frac{A_b}{A_f}\ \frac{\varepsilon_b}{\varepsilon_f}\ \frac{k_f}{k_b}\ \frac{1}{[H]_t} \qquad (1.28)$$

It is important to note that Eq. (1.28) is derived based on the assumption that the lifetimes for [GH] and [G] are distinguishable. Thus, this method can be employed for complexation phenomena where two or more species are present as long as the lifetimes for the different species are distinguished. This method is very useful for the determination of association constants for CD inclusion complexes because CDs frequently alter the photophysics of probe molecules.

The two methods described above, i.e. Benesi-Hildebrand and lifetime treatment, are often employed concurrently, particularly when complexes with 1:1 stoichiometries are studied. Several examples for complexation with β-CD are shown in **Table 1.2**.

Table 1.2: Association constants (K_a) for 1:1 ß-CD complexes.[a]

Probe	Method		Reference
	Lifetime treatment	Benesi-Hildebrand	
2,4,6-trimethylphenol	47	56	29
3,4,5-trimethylphenol	54	60	29
4-aminophthalimide	208	185	30

[a] Unit of K_a is M^{-1}.

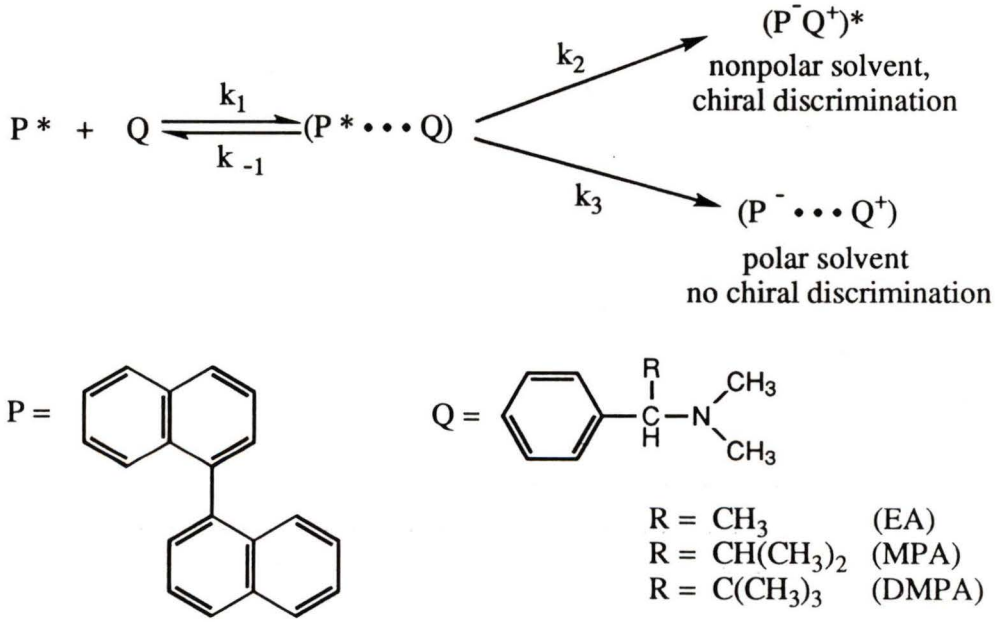
1.3. Chiral discrimination

Chiral discrimination is a well-known phenomenon, specially in biological system. D-sugars and L-amino acids are the enantiomeric forms found in living systems. The interaction between biologically active compounds and receptor proteins often shows a high or complete stereo specificity. For example, D-enantiomers of some amino acids such as histidine, leucine, isoleucine, tryptophan and tyrosine have a sweet taste while their L-enantiomers have either a bitter taste or no taste at all; (R)-(+)-limonene has an orange odor while (S)-(-)-limonene has a lemon odor. It is also documented that (R)-N-phthalyglutamic acid imide is a non-teratogen which functions as a sedative and sleeping-inducing drug whereas its (S)-enantiomer is a teratogen.[31]

In chemistry, chirality has been the subject of numerous studies employing a variety of techniques. In this chapter, we will confine our discussion to chiral recognition in photophysical processes and for systems involving CDs.

In the late 70s and early 80s, Irie et al[32,33] reported chiral discrimination in the fluorescence quenching of (R)-1,1'-binaphthyl by (S)-(-)- and (R)-(+)-

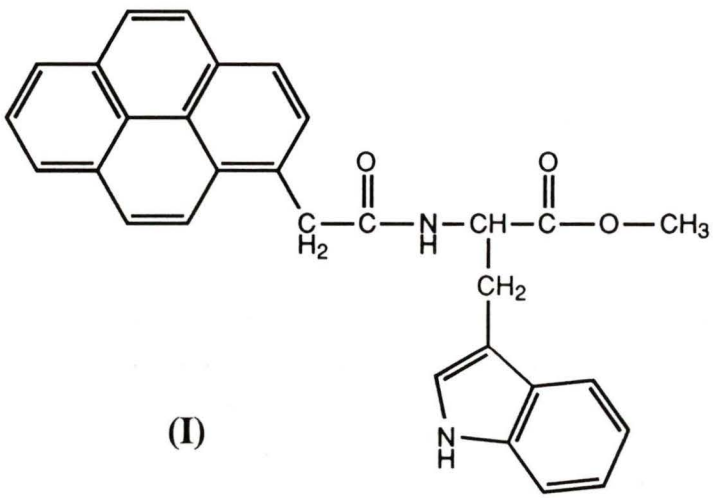
N,N-dimethyl-1-benzylamine (DMPA) (**Scheme 1.2**) in several solvents. The highest k_q(S-amine)/k_q(R-amine) observed was 4.0 at 22.0 °C and 7.9 at -10.0 °C in *n*-hexane. Furthermore, they found that the chiral discrimination depends on solvent polarity with no chiral discrimination occurring in polar solvents such as acetonitrile. These phenomena were explained by exciplex [$(P^-Q^+)^*$, **Scheme 1.2**] formation in nonpolar solvents. Only in the exciplex is a specific geometry attained that leads to chiral discrimination. Further, a 30% difference was observed for the Stern-Volmer constants of the quenching of singlet excited (R)-(-)-1,1-binaphthyl-2,2'-dihydrogen phosphate bound to silica surfaces by (R)-(+)- and (S)-(-)-DMPA ($K_{sv}(S) > K_{sv}(R)$).[34] A similar quenching mechanism as for the solution studies was suggested.



Scheme 1.2: Chiral discrimination in the quenching of 1,1'-binaphthyl by chiral amines.

Gafni[35] reported the first fluorescence study of chiral discrimination in which he showed the direct observation of stereoselectivity in the interaction between an enzyme, liver alcohol dehydrogenase (LADH) and chiral molecules. He studied the fluorescence quenching of εNAD$^+$ (nicotinamide 1,N^6-ethenoadenine dinucleotide) -pyrazole bound to LADH by D-, L- and DL-methionine. The ratio between the quenching rate constants by the D- and L-enantiomers was 3. Quenching occurred through a dynamic mechanism, which implied that the quenchers diffused to the adenine binding site on the LADH during the excited-state lifetime of the εNAD$^+$.

Chiral discrimination was also observed by means of steady-state and time-resolved spectroscopy in the excimer formation between *N*-[4-(1-pyrene)-butanoyl]-D-tryptophan methyl ester (py-D-Trp) or *N*-[4-(1-pyrene)butanoyl]-L-tryptophan methyl ester(py-L-Trp) and their racemate, py-DL-Trp **(I)**.[36]

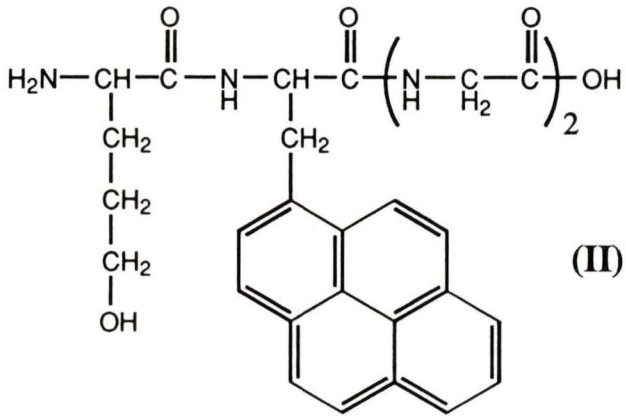


(I)

The rate constants and quantum efficiencies of excimer formation for the enantiomers of **I** and racemic **I** in methanol are $(4.0 \pm 0.7) \times 10^9$ M^{-1} s^{-1} and

$(6.9 \pm 0.5) \times 10^9$ M^{-1} s^{-1}, (1.1± 0.2) and (0.7 ± 0.1), respectively. The ratio for rate constants and quantum efficiencies between pure enantiomer and racemate can be calculated to be approximately 0.58 and 1.6, respectively. Chiral discrimination was suggested to originate from differential electrostatic, dispersion and resonance interactions. For pyrene, a parallel sandwich configuration has to be achieved for efficient excimer formation. Thus, optimization of configurational and conformational interactions is required. Excimer formation is affected by chiral solvents such as (R)-(-)- and (S)-(+)-2-octanol. These solvent-induced chiral interactions come from selective solvation, differential complex formation and hydrogen bonding between **I** and the enantiomers of the alcohols.

Recently, chiral discrimination was observed in the fluorescence quenching of the tryptophyl moiety in a monoclonal antibody. The quencher was a tetrapeptide containing L-1-pyrenylalanine **II**.[37] The antibody bound the L-enantiomer of **II** but did not bind the D-enantiomer.



(II)

1.4. Cyclodextrins

Cyclodextrins (CDs), also known as Schradiger dextrins, cycloamyloses, and cyclo glucoamyloses, are a family of cyclic oligosaccharides that contain six to twelve ß-1,4-linked D-glucose units. α–, β–, and γ–CD are the three smallest rings with six, seven and eight glucose units, respectively. The torus-shaped cavities have an internal diameter between 4.7 to 8.3 Å.[10,38,39]

CDs exhibit some advantageous properties for their use in supramolecular chemistry.[40] 1). CDs are stable over a wide range of pHs and temperature (pH > 3.5 including alkaline solution and temperature < 60 °C).[41] 2). They have little or no absorption above 250 nm. 3). They are natural-occurring and nontoxic. 4). CD solutions do not foam when purged. 5). The most important property of CDs is their ability to form complexes with a broad range of molecules. This is in part due to the fact that CDs with different cavity sizes can be employed but is also due to the presence of hydroxyl groups on both edges of the hydrophobic cavities as well as a "flexible" skeleton of the CD framework.[42] This flexibility can lead to guest-induced conformational adjustments of the ring.[43,44] 6) The last important property of CDs is that they are *chiral*. This distinguishes CDs from other synthetic host supramolecules, such as podands, coronands, and cryptands.

Table 1.3: Characteristics of α-, ß- and γ-Cyclodextrins.

Parameter	α	ß	γ
Number of glucose units	6	7	8
Molecular weight (g mol^{-1})	972	1135	1297
Cavity diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3
Height of torus (Å)	7.9	7.9	7.9
Cavity volume ($Å^3$)	176	346	510
Solubility in water (g/100 ml at 25 °C)	14.5	1.85	23.2
pK_a values	12.33	12.20	12.08

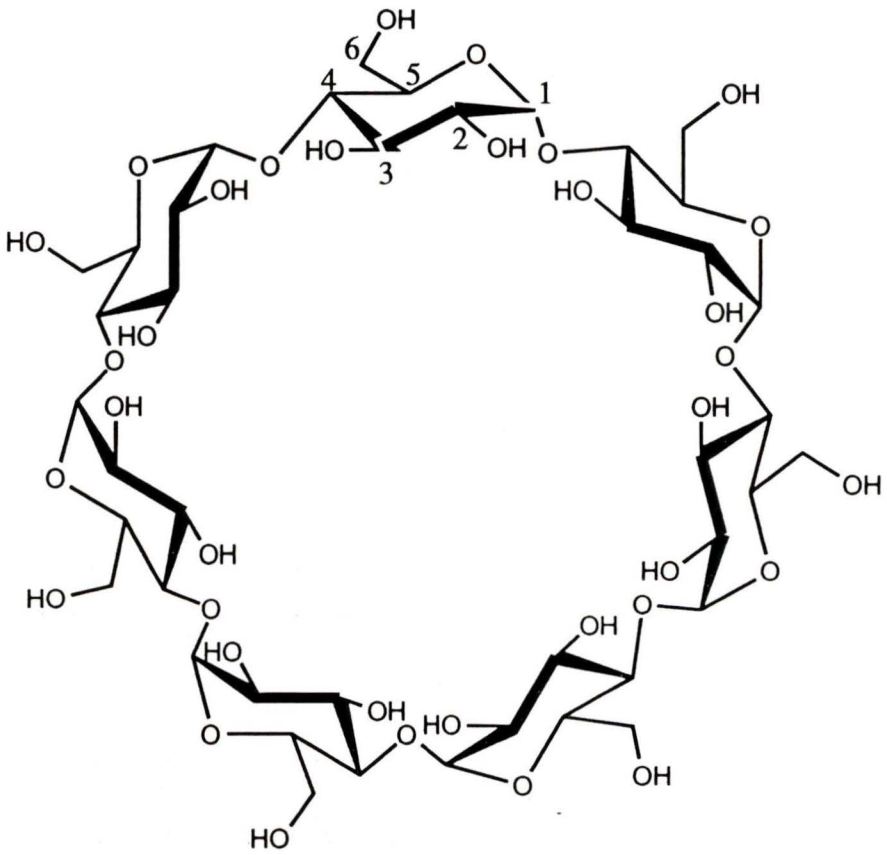


Figure 1.5: Structure of β-cyclodextrin.

An example of the high complexation ability is the formation of ß-CD tris(*tert*-butylphenyl)hexa-amide hexaazatriphenylene 3:1 complex.[45]

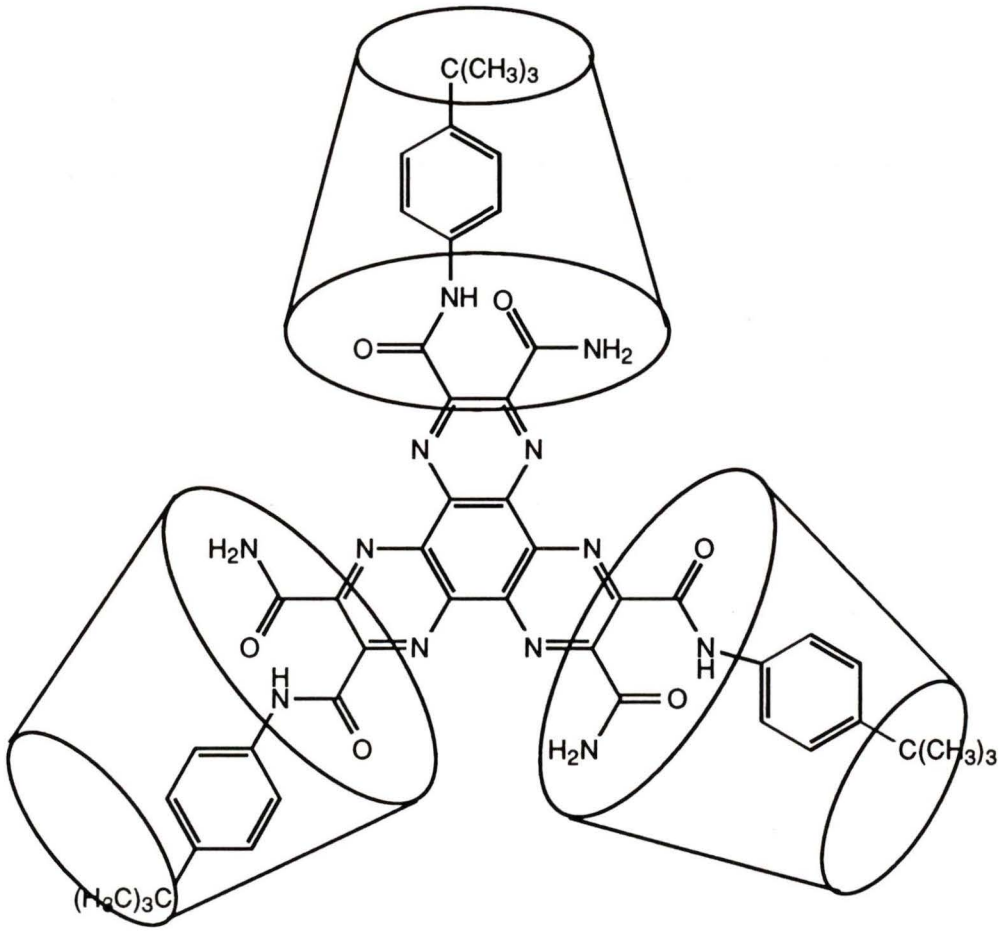


Figure 1.6: Suggested structure of ß-CD tris(*tert*-butylphenyl)hexa-amide hexaazatriphenylene 3:1 complex.

Because of these unique characteristics, CDs have been used in different areas of chemistry,[8,38,39,41,46-56] such as enzymes models[57-59] and chiral separations.[31,60] In this chapter, only the aspects involving CDs which are relevant to our experimental approach will be described.

1.4.1. CD in chiral discrimination in photophysical processes

Chiral discrimination in photophysical processes involving CDs have been known for some time. Tran and Fendler[61] observed chiral discrimination in the fluorescence excitation and emission spectra as well as singlet lifetimes for (-)- and (+)-α-(1-naphthyl)ethylamine (NEA) in the 60:40 dimethylsulfoxide: H_2O, in the presence of ß-CD. The largest difference was observed for the fluorescence lifetimes. The lifetime for (-)-NEA/CD is 27% longer than that of (+)-NEA in 60:40 Me_2SO: H_2O when the chromophores are excited at 375 nm.

Kano et al.[62] demonstrated by measuring the fluorescence intensity enhancement in presence of permethylated-ß-CD that the (S)-enantiomers of binaphthyl derivatives, such as 1,1'-bi-2-naphthol, 2,2'-dimethoxy-1,1'-binaphthyl, and 1,1'-binaphthyl-2,2'-diyl hydrogen phosphate formed a stronger complex with heptakis-(2,3,6-tri-O-methyl)-ß-CD (TMe-ß-CD) than the (R)-enantiomers. The complexation was analyzed for a 1:1 guest: host stoichiometry and the ratio of $K_a(S)/K_a(R)$ is 8 for TMe-ß-CD complexes but much lower for β-CD complexes (**Table 1.4**).

Table 1.4: Association constant (K_a) values for 1:1 complexes between (S)- or (R)-1,1'-bi-2-naphthol and CDs.[a]

CD type	$K_a(S)$	$K_a(R)$	$K_a(S)/K_a(R)$
ß-CD	280	230	1.2
TMe-ß-CD	400	50	8

[a] Unit of K_a is M^{-1}.

Recently, p-(dimethylamino)benzoyl labeled CDs were employed as fluorescent sensors to study chiral recognition.[63-65] Proton NMR and induced circular dichroism were used to study the formation of inclusion complexes. By measuring the change of fluorescence intensity of the labeled CD in presence of *d*- and *l*-menthol, the binding constants of menthol were measured. The K_a values are 18000 and 10000 M^{-1} for the *l*- and *d*-enantiomers, respectively ($K_a(l)/K_a(d)=1.8$).

1.4.2. Induced optical activity of guests by CD complexation

Induced optical activity (IOA) is the induction of optical activity on an achiral compound (chromophore) when it is in contact with a chiral compound (inducer).[66] IOA of the electronic transitions of achiral chromophores comes from the asymmetric perturbation by the inducer. A sufficient condition for IOA to occur is an ordered supramolecular organization where the rotational and transitional degrees of freedom of the chromophore are restricted and a chiral conformation arises. A unique aspect of IOA is that from induced circular dichroism (ICD) spectra the geometry of the complex and chiral conformations can be inferred.[66,67]

IOA has been observed for many CD complexes.[52] Guest chromophores include aromatic hydrocarbons, such as benzene and naphthalene, heteroaromatic compounds (including nucleic acids), organometallic molecules; metal complexes and acyclic carbonyl compounds.

Kobayashi et al[68] observed the induced circular dichroism of the pyrene dimer in the presence of γ-CD suggesting that this ground state dimer in the γ-CD is asymmetric. Upon excitation of pyrene in γ-CD, an extremely intense circularly polarized fluorescence signal was measured in the excimer region of the

spectrum.[69,70] The re-orientation of the two pyrene molecules was suggested to occur in the CD cavity to form a thermodynamically meta-stable chiral excimer.

1.4.3. CDs used for chiral separation in analytical methods

During the past two decades, the use of CDs as chiral separation agents in analytical chemistry has increased dramatically.[31,41,60] Different CD-bound chiral stationary phases that can be used for HPLC or GC are commercial available. These include α-, ß- and acetylated ß-CD for HPLC stationary chiral phases (Cyclobond I, II, III by Advanced Separations Technologies, Inc. (USA) and Serva (FRG)).[31,71] CDs are also used in chiral mobile phases for HPLC and TLC and as chiral sensors in electrochromatography.[72-76]

Different binding constants have been observed for the complexation of dansyl-D- and dansyl-L-amino acids with ß-CD. This property has been frequently used for the chromatographic separation of amino acids.[60] However, only those D- and L-amino acids with large side chain groups such as tryptophan, phenylalanine and tyrosine can be separated directly by CD stationary phases (**Table 1.5**).[77] It is important to note that the D isomer of tryptophan is eluted first which suggest a better binding of this enantiomer with CD than the binding of L-tryptophan.

Table 1.5: Resolution of racemic amino acids on α-CD bonded column.[a]

Amino acid	Capacity factor[b] (k')	Selectivity factor[c] (α)	Resolution[d] (R_S)
tryptophan	2.7	1.20	1.90
phenylalanine	1.1	1.09	0.85
tyrosine	0.1	1.40	0.90

[a] The mobile phase is H_2O with 1% triethylammonium acetate at pH 5.1.
[b] Capacity factor, k'= (retention volume of enantiomer-void volume)/void volume.
[c] Selectivity factor, $\alpha = k'_D/k'_L$.
[d] Resolution, R_S =2×(distance between two peaks)/sum of the bandwidths of the two peaks.

The theory of chiral separation by chromatography has yet to be developed. The well-known three-point interaction model for chiral recognition has been postulated by Dalgleish.[71,78] According to this postulate, chiral recognition requires a minimum of three simultaneous interactions between a chiral stationary phase and an enantiomer (**Figure 1.7**). An interaction can be any non-covalent attachment such as hydrogen bonding, ionic or dipole attraction as well as hydrophobic interactions in aqueous media.[74,79,80]

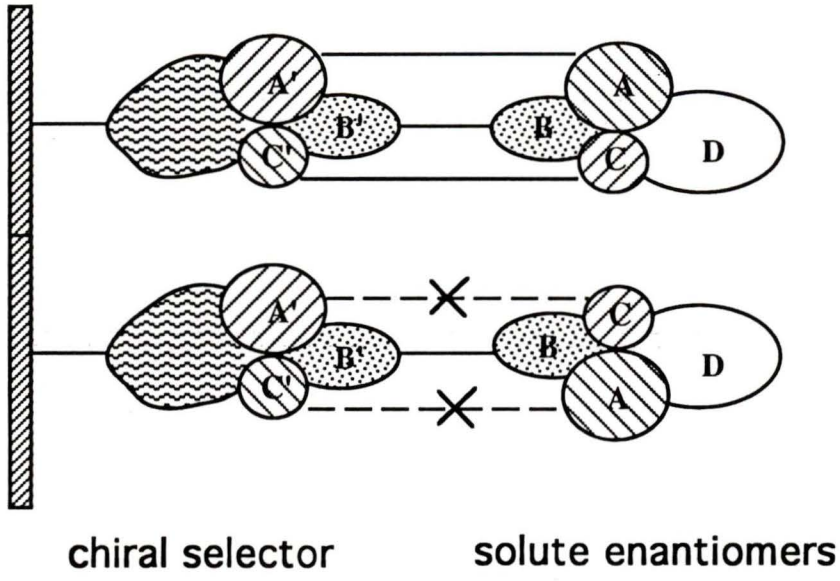


Figure 1.7: Dalgleish's three-point interaction model for chiral recognition.

Armstrong et al demonstrated the primary requirements for CD induced chiral recognition involving the formation of inclusion complexes by studying the propranolol/ß-CD complex.[81] They concluded that a number of requirements are needed for chiral recognition by CD to occur: A). An inclusion complex must be formed, and there must be a relatively tight fit between the complexed moiety and CD. B). The stereocenter or one substituent of the stereocenter must interact with the atoms at the entry of the cavity. C). The unidirectional 2- and 3-hydroxyl groups of the glucose units located at the entry of the cavity are particularly important for chiral recognition. D). Guest compounds have to have at least one aromatic ring.

1.5. Pyrene

Excited pyrene has a very long singlet lifetime with a high fluorescence quantum yield (**Table 1.6**). Besides its long lifetime, pyrene has other very useful properties: 1). The shape of pyrene fluorescence spectrum is extremely sensitive to solvent polarity. 2). Excited state pyrene can form excimers with ground state molecules at high pyrene concentrations or when confined to constrained micro-environments. 3). Pyrene is a very stable compound and a series of derivatives are commercially available.

Table 1.6: Fluorescence lifetimes and quantum yields of pyrene in several solvents.[a]

Solvent	lifetime (τ), ns	quantum yield (Φ_{fluo})
cyclohexane	450	0.65
ethanol	475[b]	0.65
95% ethanol	290	—
water	205[c]	—

[a] Data measured at 25 °C.[82]
[b] Lifetime also reported as 410 ns and 530 ns.
[c] Datum from reference 21.

Because of the above characteristics, pyrene is a popular fluorescence probe in photophysics and biophysics, specially in organized media, and has been extensively studied as a guest molecule.[6,83,84] For example, pyrene and its derivatives have been extensively used to characterize micelle and vesicle properties such as probe distribution, movement within the organized system or between phases and conformational restrains.[47] Pyrene probes have also been

exploited to characterize biochemically important structures such as bile salt micelles which are biological amphiphiles,[85] enzymes,[86] adduct formation with DNA,[87,88]and the binding dynamics with RNA.[89] In a different area, pyrene-labeled polymers have been used to study different aspects of polymer behavior and an overview of the field can be found in a recent review.[90]

The two aspects of pyrene photochemistry that are relevant to our research project and will be described in detail are: A). Dependence of pyrene fluorescence spectra on the polarity of the solvent or microenvironments. B). Quenching of pyrene by tryptophan.

1.5.1. Relation between pyrene fluorescence spectra and solvent polarity

At room temperature the pyrene fluorescence emission in solution shows five principal vibronic bands (**Figure 1.8**). The usual numbering of the vibronic bands (I-V) is shown in **Figure 1.8**. Band I at 372.4 nm shows significant intensity enhancement in polar solvents, while the band III located at 383.0 nm shows minimal variation of its intensity with the change of solvent polarity.

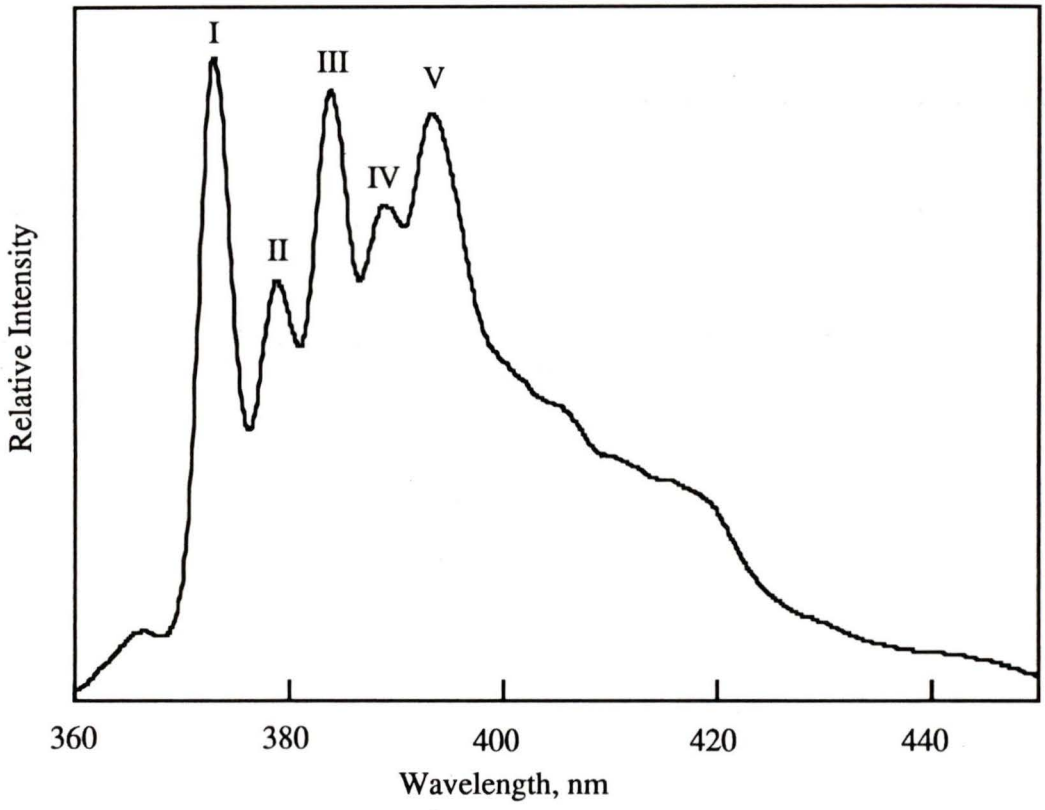


Figure 1.8: Pyrene (5×10^{-7} M) fluorescence emission spectrum in 2-butanol.

A general explanation for this "medium effect" on the pyrene fluorescence spectra comes from the Ham effect.[91-93] For aromatic molecules with a minimum D_{2h} symmetry the absorption and fluorescence spectra show mixed polarization due to the vibronic coupling between the first (S_1) and second (S_2) singlet excited states. According to the Ham effect, band I, which is symmetry forbidden, shows big intensity enhancement with increasing solvent polarity. The dispersion forces between the aromatic compounds and solvent molecules are important for the enhancement of coupling.

The strong dependence of the intensities of different bands makes it possible to use pyrene monomer fluorescence as an "indicator" of solvent polarity (**Figure 1.9**). Kalyanasundaram and Thomas[93,94] have systematically studied the changes of vibronic band intensities in different solvents and micellar environments. Dong and Winnik[95,96] first introduced the pyrene scale of solvent polarity. The ratio of intensities of the first to third vibronic bands R(I/III), also called *Py* values, was used to study the environmental changes in different solvent. In their study, over 90 solvents were employed and the relationship between R(I/III) scale and other solvent polarity parameters was found.

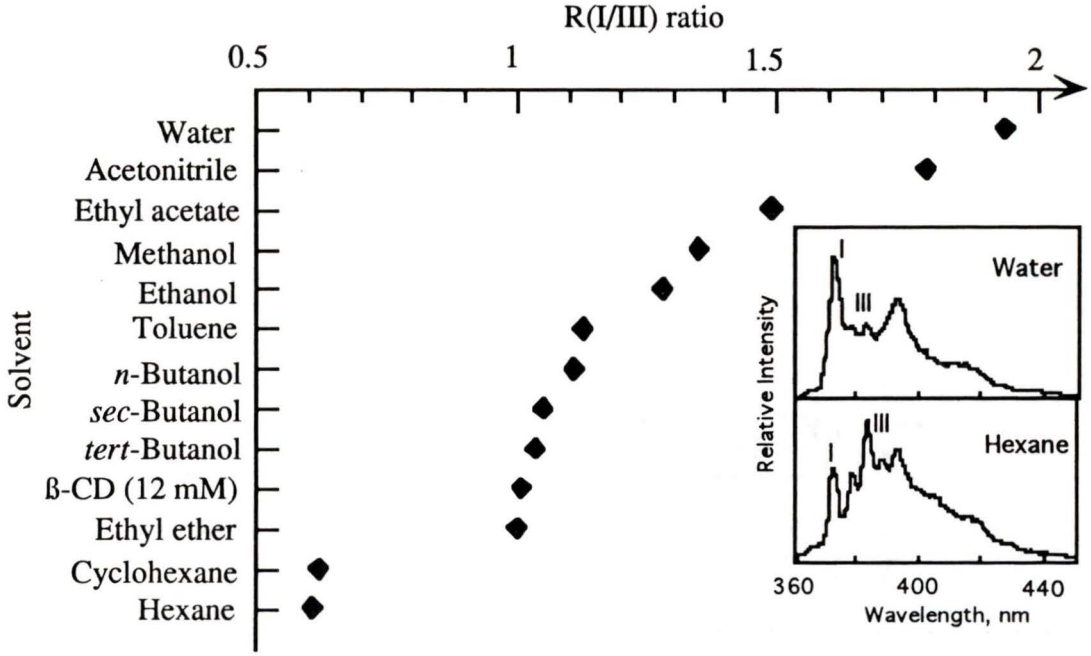
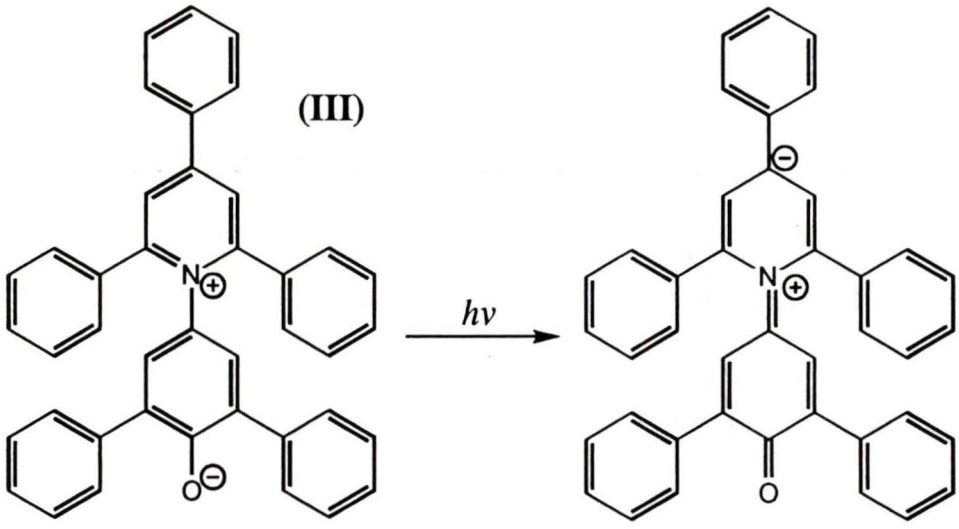


Figure 1.9: Dependence of pyrene R(I/III) ratio on solvent polarity.

One of such relations is the correlation between the R(I/III) ratio and another well-recognized empirical parameter, $E_T(30)$. $E_T(30)$[23,97,98] is the

transition energy (in kcal mol^{-1}) of the intramolecular charge transfer band for 2, 6-diphenyl-4-(2, 4, 6-triphenylpyridinio) phenolate (**III**).



The correlation between R(I/III) and $E_T(30)$ for aprotic solvents is:

$$R(I/III) = 0.080\ E_T - 1.74 \qquad \text{corr. } 0.924 \qquad (1.29)$$

for protic solvents, the correlation is given by:

$$R(I/III) = 0.0584\ E_T - 1.750 \qquad \text{corr. } 0.892 \qquad (1.30)$$

It is worthwhile to mention that accurate R(I/III) determinations require the elimination of instrumental artifacts.[99] Temperature control, narrow slits, low pyrene concentration, and adequate blank correction are required for obtaining precise R(I/III) values.

1.5.2. Quenching of pyrene fluorescence by tryptophan

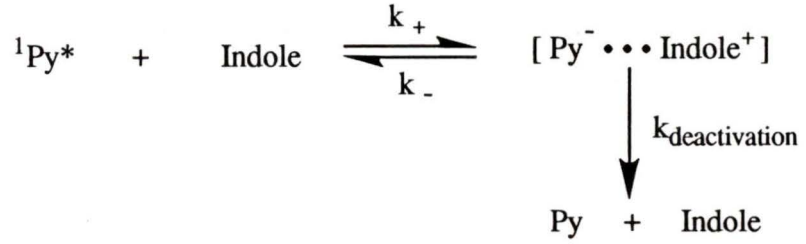
Singlet pyrene in homogeneous solutions and organized systems can be quenched by a variety of molecules.[47] The quenchers can be neutral molecules such as olefins, alkyl iodides, amines, indole and its derivatives and O_2; anions like I^-, Br^-, $S_2O_3^{2-}$ and C_nH_{2n+1}-Nitroxyl or cations such as Cu^{2+}, Ti^+, Ag^+, Ni^+, Hg^{2+}, Eu^{3+} and Cs^+.[8]

For our experiments the chiral quencher employed was tryptophan, which is an indole derivative. Encinas and Lissi studied the quenching of pyrene fluorescence by tryptophan and indole in homogeneous solutions and micelles.[100,101] The k_q of tryptophan quenching excited singlet pyrene in water was determined to be 2.2×10^9 M^{-1} s^{-1} and this value was independent of pH, i.e. the quenching rate constants were the same for tryptophan in its anionic, zwitterionic and cationic forms (pK values of 2.38 and 9.39). The quenching constant only changed slightly in water/ethanol (up to 48% (v/v)) mixtures (1.7×10^9, 1.5×10^9 and 1.2×10^9 M^{-1} s^{-1} at pH 11, 7 and ≤2, respectively). The quenching was efficient however it was below the diffusion controlled limit (1.1×10^{10} M^{-1} s^{-1} in water at 25 °C [21]).

The quenching rate constant of singlet pyrene by O_2 in water or at pH =7.4 at 25 °C is 1.04×10^{10} M^{-1} s^{-1} indicating that this reaction is diffusion controlled. This is relevant as several of the experiments described in following chapters were performed in aerated aqueous samples.

Encinas and Lissi did not observe any significant photoreaction between pyrene and tryptophan. They also observed that the rate constants for quenching of pyrene fluorescence by tryptophan depended strongly on solvent polarity. Thus,

a charge transfer mechanism similar to the quenching of pyrene fluorescence by indole[101] (**Scheme 1.3**) was suggested.



Scheme 1.3: Suggested mechanism of quenching of excited singlet pyrene by indole.

The charge transfer mechanism is also supported by work from De Schryver's group which showed the intermolecular exciplex formation between pyrene or its derivatives and indole moieties.[102] These exciplexes are known to have partial charge-transfer character. This study also showed that in solvents with higher polarity the quantum yield of exciplex formation decreases sharply and ion formation becomes important. Recent studies on intramolecular exciplex formation between pyrene and indole moieties of 1-pyrenyl-3-indolylpropanes suggested that the relative position and orientation of these two chromophores is important.[103]

The above studies suggest that a close contact between pyrene and tryptophan is necessary for deactivation of the excited state. This is relevant since such a quenching process does not involve long-range deactivation mechanism. For this latter mechanism, no chiral discrimination is expected.

1.5.3. Complexation with β-CD

Complexation of pyrene with ß-CD has been extensively studied. Several different spectroscopic techniques, such as absorption, steady-state and time-resolved fluorescence were employed to determine the values for the equilibrium constants between pyrene and β-CD.

In the presence of β-CD, a shoulder at longer wavelengths was observed at 339 nm in the UV-Vis absorption and fluorescence excitation spectra. The isosbestic points between free and complexed pyrene were 320, 327, and 335 nm.[104] Fluorescence spectra of pyrene/β-CD excited at the isosbestic points had different fluorescence intensities and the R(I/III) values when compared to free pyrene in water. The pyrene fluorescence decay became double exponential in the presence of β-CD. In addition, the rate constants for the association of pyrene with ß-CD or dissociation of pyrene from ß-CD are slow[105] compared to the decay of singlet excited pyrene. Thus, both fluorescence and absorption methods measure the equilibrium constant for ground-state pyrene.

The reported values for the equilibrium constant and stoichiometry of the pyrene β-CD complex have not been consistent.[104-112] A sequential 1:1 and 1:2 pyrene: β-CD complex formation[110-112] is the best analysis currently available.

$$\text{Py} + \beta\text{-CD} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{Py·CD} + \text{CD} \overset{K_2}{\rightleftharpoons} \text{CD·Py·CD} \qquad (1.30)$$

$$\text{Py} \xrightarrow{h\nu} \text{Py}^* \longrightarrow \text{Py} + h\nu \text{ or } \Delta \qquad (1.31)$$

$$\text{Py·CD} \xrightarrow{h\nu} \text{Py·CD*} \longrightarrow \text{Py·CD} + h\nu \text{ or } \Delta \qquad (1.32)$$

$$\text{CD·Py·CD} \xrightarrow{h\nu} \text{CD·Py·CD*} \longrightarrow \text{CD·Py·CD} + h\nu \text{ or } \Delta \qquad (1.33)$$

In these studies the fluorescence intensities of the first and third bands or the R(I/III) ratio in the presence of CD were measured. When the R(I/III) ratio was used,[110,111] the authors assumed that the observed R(I/III) value was the weighted average for the R(I/III) values of free pyrene and pyrene CD complexes with 1:1 and 1:2 stoichiometries. The non linear equation (Eq. (1.34)) or double reciprocal plots (Eq. (1.35)) were employed:

$$R = \frac{R_o + R_{11}K_1[CD]_t + R_{12}K_1K_2[CD]_t^2}{1 + K_1[CD]_t + K_1K_2[CD]_t^2} \qquad (1.34)$$

$$\frac{1}{R_o - R} = \frac{1}{(R_o - R_{12})K_1K_2[CD]_t^2} + \frac{1}{R_o - R_{12}} \qquad (1.35)$$

where R_o, R_{11}, R_{12}, and R are the R(I/III) values for the fluorescence of free pyrene, pyrene complexes with a 1:1 or 1:2 stoichiometries and observed value, respectively.

From this analysis a K_1K_2 value was obtained. Furthermore, Kusumoto et al[110] assumed that the pyrene:ß-CD 1:1 complex was dominant at ß-CD concentrations below 3×10^{-3} M and a 1:2 complex was the main species at higher

ß-CD concentrations . The K_1 value was estimated from Benesi-Hildebrand double-reciprocal plots for 1:1 binding at β-CD concentrations below 3×10^{-3} M.

A more comprehensive analysis of the pyrene/ß-CD system was recently reported by Xu et al.[112] They used global analysis by correlating the fluorescence intensities of the first and third fluorescence bands of pyrene. When comparing the different mechanistic models, the sequential binding described in Eq. (1.30) gave the best fit to their data. All the results for sequential binding of pyrene to β-CD are summarized in **Table 1.7**.

Table 1.7: Parameters for pyrene ß-CD complexes.[a]

K_1	K_2	$K_1 \cdot K_2$	R_{Py}	$R_{Py \cdot CD}$	$R_{CD \cdot Py \cdot CD}$	Reference
128	79	1.2×10^4	1.84	1.01	0.55	110
277[b]	3100[c]	8.5×10^5				111
140	210	2.9×10^4	1.85	0.83	0.58	112

[a] The units of K_1, K_2 and $K_1 \cdot K_2$ are M^{-1}, M^{-1} and M^{-2}, respectively. Excitation wavelength were at 337 nm[110,112] or 335 nm[111]. The first band was measured at 373 nm for all studies. The third band was measured at 384 nm[110,112] and at 385 nm[111] .

[b] Data from reference 113.

[c] Data calculated from reported K_1 and $K_1 \cdot K_2$ values.

1.5.4. Ternary complex formation

The association constant of the pyrene β-CD complex can be enhanced in the presence of alcohols or the sodium salts of short-chain alkyl sulfates or short-chain alkylammonium chlorides. The enhancement is due to co-inclusion of the alcohols or alkyl sulfates with pyrene into the CD cavity.[104,105,114] Different

stoichiometries have been reported for these ternary complexes. A 1:1:1 stoichiometry was suggested for the pyrene ß-CD complex in the presence of *n*-alkylsulfates and *n*-alkylammonium chlorides.[105] From the lifetime measurements the K_a values for the proposed 1:1 pyrene: ß-CD complex were obtained. A remarkable binding enhancement was found in the presence of 1-butyl-sulfate, 1-hexylsulfate, and 1-hexylammonium chloride with K_a values of 3000 M^{-1}, 1400 M^{-1} and 1300 M^{-1}, respectively. In the presence of these third component molecules, a single exponential decay of pyrene fluorescence, with a strikingly long lifetime of approximately 450 ns, was observed. The lifetime enhancement effect of surfactants has been suggested to be due to the exclusion of water molecules originally in the CD cavity, thereby creating a more hydrophobic environment. The interaction between the hydrophobic chain of the surfactant and pyrene have been suggested to lead to the higher binding constant for pyrene with ß-CD.

Two different stoichiometries (1:1:1 and 1:2:2 pyrene: β-CD: alcohol) were proposed for the complexes in the presence of alcohols. The 1:1:1 stoichiometry of pyrene/ß-CD/alcohol system was suggested by Hamai.[104] Their experimental evidence for this ratio was twofold. At a low concentration range of 1-propanol, the pyrene absorbance or fluorescence excitation intensity at 339 nm was proportional to the concentration of 1-propanol added. The continuous variation method,[115 116] (Job's method), which is commonly used for binary system, was used to determine the ratio of ß-CD and 1-pentanol. A 1:1 stoichiometry between ß-CD and 1-pentanol was established. The effect of the alcohol structure on the pyrene singlet lifetime in these ternary systems was also investigated. Among the eight primary alcohols ($C_nH_{2n+1}OH$ with n =1- 8) measured, 1-propanol had

the strongest effect on lifetime of pyrene when complexed to ß-CD. Cyclic alcohols such as cyclobutanol and cyclopentanol have approximately the identical molecular length as 1-propanol and 1-butanol, respectively. The effect of cyclic alcohols on the pyrene lifetime was very similar to the effect observed for 1-propanol. The authors suggested that the enhancement of the pyrene lifetime could be correlated mainly with the lengths rather than with the bulkiness of the alcohols.

Muñoz de la Peña et al[114] proposed a 1:2:2 stoichiometry for the pyrene/ß-CD/alcohol system. They followed the same methodology as Hamai[104] and observed a 1:1 ß-CD/alcohol ratio. Their claim for a 1:2:2 stoichiometry for the ternary system was based on their previous observation of 1:2 pyrene: ß-CD complexes.[117] Estimated association constants of pyrene with ß-CD in the presence of 1% (v/v) alcohols were obtained by using the R(I/III) values for double reciprocal plots. The trends observed for different alcohols parallels Hamai's observation.

Ternary complexation with ß-CD was also involved in the quenching of pyrene fluorescence by co-included molecules such as aniline, *N, N*-dimethylaniline and alkylamine. The stoichiometries reported were 1:1:1 for pyrene/ß-CD/alkylamine[118] and both 1:1:1 and 1:2:2 complexes for pyrene/ß-CD/aniline.[108] For the quenching by alkylamines it was suggested that the specific positioning of the lone pair of electrons on the nitrogen was critical. In the case of diethylamine, molecular modeling showed that the trans-trans conformer was sterically restricted in the CD cavity, thereby leading to an unfavorable interaction between pyrene and amine.

1.6. Tryptophan

As mentioned in the previous section, we chose tryptophan as a quencher because it is chiral and the quenching of pyrene fluorescence in homogeneous solutions had been previously studied. For our interest, the complexes between tryptophan and CDs and their chiral discrimination are relevant and are discussed below. In general, the complexation between tryptophan and CDs is weak. However, chiral discrimination is observed in several systems involving tryptophan or tryptophyl groups in organized media.

1.6.1. Complex formation with ß-CD

Lewis and Hansen[119] studied the complex formation (K_a) between L-tryptophan and α-CD. A value of K_a of 29 M^{-1} at 25 °C was observed. Matsuyama et al[120] obtained a similar K_a value for L-tryptophan and α-CD complex, 21.2 M^{-1} at 25 °C by using flow microcalorimeter. No complexes between tryptophan and γ-CD complex were observed.

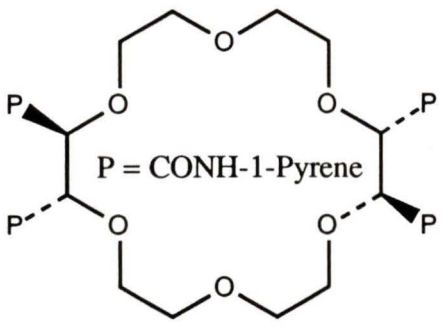
The literature values for the equilibrium constants for tryptophan ß-CD complexes are not consistent. Matsuyama et al.[120] reported a K_a for the L-tryptophan/ß-CD complex of 213.7 M^{-1} at 25 °C, almost ten-fold higher than the value for the α-CD complex. The competitive binding of ANS (borate buffer (pH 8.9) at 12 °C) and tryptophan to β-CD was measured by fluorescence spectroscopy.[121,122] The measured K_a values for D- and L-tryptophan ß-CD complexes were 9.0 ± 1.2 and 7.9 ± 1.3 M^{-1}, respectively. Brown et al[123] measured the K_a for ß-CD and tryptophan *anion* complexes in aqueous solution at 25 °C and ion strength 0.10 ($NaClO_4$). A value of 210 M^{-1} was determined for both enantiomers.

The dynamics of ß-CD and its inclusion complex with *N*-acetyl-L-tryptophan was studied by deuterium NMR.[53] A 1:1 complex was formed with a K_a of 136.1 M^{-1} at 25 °C. The three important observations were: A). On the NMR time-scale, ß-CD appears as a monomer in water, even at concentrations close to saturation. This suggests that there is no pre-aggregation of CD molecules. B). The formation of inclusion complexes induces no change in the dynamics of CD molecules. C). The guest molecule retains a local mobility in the cavity of the host.

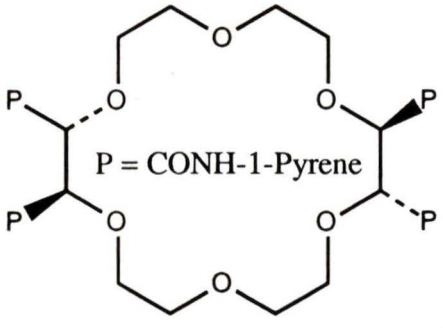
1.6.2. Chiral discrimination involving tryptophan and tryptophyl groups in organized media

Besides the outstanding examples of direct chiral separation of tryptophan by CDs,[77] examples of chiral discrimination involving tryptophan and tryptophyl moieties in different organized systems have been found.

Tundo and Fendler[124] reported the first use of a photophysical technique, fluorescence quenching, to study the chiral discrimination in crown ethers. Fluorescence quenching of glycine-L-tryptophan by pyrene labeled 1,2,10,11-(S,S,S,S)-(-)- and 1,2,10,11-(R,R,R,R)-(+)- tetracarbo-(N,N)-dimethylpyrene-3,6,9,12,15,18-hexaoxocyclooctadecane (D- and L-crown-Py) was studied.



L-crown-py



D-crown-py

The K_a values for glycine-L-tryptophan with D- and L-crown-Py were $(1.2 \pm 0.2) \times 10^5$ M^{-1} and $(5.0 \pm 0.3) \times 10^4$ M^{-1}, respectively. Proper alignment of the host and guest moieties was suggested as a requirement for maximizing chiral recognition in these crown ethers.

Several different functionalized-CDs showed chiral discrimination when complexing tryptophan. Ternary complex formation of Cu(II)/6-deoxy-6-(n-histamino)/ß-CD and tryptophan were studied by potentiometric and calorimetric measurements as well as fluorescent quenching.[125] The K_a (D-Trp) /K_a (L-Trp) ratios for the 1:1:1 complexation were 2.34, 1.17 and 1.07 for the anionic, zwitterionic, and cationic forms of tryptophan, respectively. D-tryptophan had a higher affinity than the L-enantiomer and consequently D-tryptophan was quenched more efficiently by the complexed Cu(II). Intramolecular aromatic ring stacking of histamine and tryptophan was reported for the ternary copper(II) complexes [Cu(II) (histamine)(amino acid)].[126]

A recent paper reported the enantioselective complexation of tryptophan anions with metallo-6^A-(3-aminopropylamino)-6^A-deoxy-ß-CDs.

The K_a(L-Trp$^-$)/ K_a(D-Trp$^-$) for CD/tryptophan anions/Ni^{2+}, Co^{2+} or Cu^{2+} are 10, 1.9 and 1.7, respectively.

For unmodified CD tryptophan complexes, Lipkowitz et al.[127] showed by ^1H and ^{13}C NMR the chiral discrimination in the tryptophan/α-CD complex formation. These studies were complemented by molecular modeling simulations. The general observation was that D-tryptophan formed more hydrogen bonds with the guest molecule than the L-enantiomer and the orientations of the included indole moieties of the D- and L-enantiomers were different.

1.7. Project proposal

Structured organized systems can significantly alter the chemical reactivity of a variety of processes. Some general mechanisms, such as the increase in local concentration of reactants at interfaces or higher reactivity through spatial confinement of the reaction have been firmly established in the literature. However, the importance of specific interactions has received much less attention. The primary objective of the present study is to assess the possibility of chiral discrimination when a chiral host provides the chiral environment that influences the reaction between a complexed *achiral* guest and a chiral quencher molecule. Several examples of CDs as host molecules for chiral discrimination were described in the introduction. Pyrene was chosen as the achiral probe molecule. The induced optical activity observed for pyrene and other polynuclear aromatic hydrocarbons such as benzo(a)pyrene in the presence of CDs suggests that achiral pyrene molecules are in a chiral environment when complexed to CDs. In addition, pyrene is a very good fluorescent probe molecule since its excited singlet state has a long lifetime, the fluorescence spectrum is sensitive to the

environment's polarity and efficient quenching of excited singlet pyrene by tryptophan had been observed.

Besides quenching, we also established that tryptophan could form ternary complexes with pyrene and β-CD. For this reason, we investigated the possibility of ternary complex formation with other amino acids. Previous studies on the complexation of pyrene to CDs showed co-inclusion of third components such as alcohols, amines, analine, alkylsulfates (anion) and alkylammonium cations. The general property of these third components is that they all bear a polar head group which can form hydrogen bonds with the hydroxyl groups of CDs and a hydrocarbon side chain which is included into the hydrophobic cavity of the CD. Therefore, amino acids which have both N and O binding sites, are potentially good third components for the pyrene ß-CD system.

2. Experimental

2.1. Materials

β–CD was a generous gift from American Maize Products and was used as received. No fluorescence impurities were detected when the β-CD was excited at the wavelengths employed for the fluorescent probes.

Pyrene was purchased from either Polysciences Inc. (high purity; used as received) or Aldrich (99%, recrystallized at least twice from ethanol). No impurities were detected by gas chromatography. The pyrene fluorescence decay in water was monoexponential ($\tau \geq 193$ ns).

8-Anilino-1-naphthalenesulfonic acid (97% Aldrich) was converted to its magnesium salt (ANS) by using magnesium sulphate. The salt was recrystallized four times from water[128] and the green crystals were dried at 100°C for 3 hours. In the course of purification, aluminum foil was used to minimize the exposure of ANS to light. Purity was checked by thin-layer chromatography (TLC) (0.25 mm silica gel Polygram Sil G/UV_{254} plate and aluminum plate). The TLC plates were run with either chloroform-methanol-water (65:25:4 (v/v)) or 1-butanol saturated with 21% (v/v) acetic acid aqueous solution.[129,130] All runs gave one round spot. The R_f values were 0.73 and 0.38 for chloroform-methanol-water (65:25:4 (v/v)) and 1-butanol saturated with 21% (v/v) acetic acid aqueous solution, respectively. The absorbance maximum of UV-Vis spectrum for ANS was at 350 nm ($\varepsilon = 5.9 \times 10^3$ M^{-1} cm^{-1}) .

2-Butanol (2-BuOH) (99% Aldrich), *tert* -butanol (*tert* -BuOH) (98%, BDH), 1-butanol (1-BuOH) (>99.4% Anachemia), sodium 1-butylsulfate (C_4-SO_4) (Lancaster), sodium 1-hexylsulfate (C_6-SO_4) (Lancaster) L-leucine (L-Leu) (99%

Aldrich), D-leucine (D-Leu) (99% Aldrich) L-Alanine (L-Ala) (99% Aldrich), glycine (Gly) (+99% Aldrich), L-histidine (L-His) (99% Aldrich), DL-lysine monohydrochloride (DL-Lys) (99% Aldrich), L-phenylalanine (L-Phe) (99% Aldrich), L-serine (L-Ser) (99% Aldrich) DL-valine (DL-Val) (United States Biochemical Corporation), D-Tryptophan (99+ % Aldrich, 99% ICN), L-tryptophan (99% Aldrich, 99+% ICN, BDH), DL-tryptophan (99+% Aldrich) and magnesium sulphate (anhydrous reagent, CALEDON) were used as received. Deuterium Oxide (D_2O, D 99.9%) was purchased from Cambridge Isotope Laboratories (CIL) and was used as received.

Water was organic pure, i.e. distilled water passed through a SYBRON Barntead system for deionization, followed by irradiating with UV light for ca. 0.5 hour. Methanol (99.9% spectroscopic grade), hexane (95+% spectroscopic grade) and acetonitrile (99.9+% HPLC grade) were purchased from Aldrich and used as received. Ethyl acetate (≥ 99.4%) was purchased from Anachemia and distilled before used.

Several different purification methods were used for tryptophan as a weak emission different from tryptophan fluorescence (excitation at 280 nm) was observed in the same spectral region as pyrene fluorescence when this amino acid was excited between 330 and 340 nm. The magnitude of this emission was small when compared to pyrene in water but was significant when compared to the small intensity difference in some of the quenching studies. The emission intensity was dependent of the source of tryptophan and attributed to an impurity. This impurity could not be detected by reversed-phase HPLC using UV detection (280, 300, 320 or 340 nm) on a Varian 5000 liquid chromatograph with a 2 mm 25 × 0.46 cm CSC-SODS2 column (Eluants at pH 2.51 and gradients from 50% to 30% of B in a A-B system (A is an aqueous solution of 25 mM sodium hexasulfonate and 25 mM triethylamine hydrochloride. B is a water

methanol mixture (1:9 (v/v)) solution of 25 mM sodium hexasulfonate and 25 mM triethylamine hydrochloride. TLC plates (0.25 mm silica gel Polygram Sil G/UV_{254} plate) run with the solvent mixtures suggested in the literature[131] for simple indole derivatives showed one spot. The components of the solvent mixture were: n-butanol-acetic acid-water (65:13:22), acetone-chloroform-acetic acid-water (40:40:20:5), isobutanol-methanol-water (80:5:15) or butanol-ethanol-water (76:19:5). Finally, recrystallization from ethanol/water was also performed. As all these purification methods failed the tryptophan emission measured in blank experiments was subtracted from the pyrene emission.

2.2. Sample preparation

Py/ß-CD samples for quenching study. The aqueous solution of pyrene ($\leq$ 0.5 μM) was prepared by injecting a pyrene methanolic stock solution (1 - 4 mM) into water. Pyrene ß-CD complexes were prepared by adding the appropriately weighted amount of CD to the pyrene aqueous solution. The concentration of methanol in the solution is $\leq$ 0.08% (v/v) ($\leq$ 20 mM). To ensure a constant pyrene concentration, tryptophan solutions were prepared by dissolving the solid in the same pyrene CD solution employed to measure the fluorescence intensity in the absence of quencher. Tryptophan blank solutions were prepared by solubilization in aqueous CD solutions with the same CD concentrations as for the pyrene solutions. The tryptophan concentration was checked for several experiments by measuring the ε value at 279 nm. The standard deviation for the average ε at this wavelength (5600 M^{-1} cm^{-1}, 15 determinations) was below 2%.

All solutions for the pyrene/β-CD/alcohol(or alkyl sulfate) system were prepared from aqueous stock solutions containing β-CD and alcohols or alkyl sulfates

(third components). The following solutions were prepared: (i) Solution **A** was prepared by injecting the appropriate volume of a pyrene methanolic stock solution into the CD/third component solution. The final pyrene concentration was 0.97 μM. This solution was employed to measure the emission intensity in the absence of quencher. (ii) A solution at the highest tryptophan concentration employed was prepared by dissolving the solid with solution **A**. Several solutions at lower tryptophan concentrations were prepared by dilution. (iii) Blank solutions containing all components but pyrene were prepared by dissolving tryptophan into the β-CD/third component stock solution. All samples were left to equilibrate for at least 2 hours in the dark.

Two different quenching methods were used. (i) In the "addition method" small aliquots of a concentrated tryptophan solution were added to the pyrene-CD solution. The decrease of the fluorescence intensity (integrated area between 365 and 400 nm or intensity value at 400 nm) was measured for each tryptophan concentration. (ii) For high tryptophan concentrations the "dilution method" was employed as tryptophan is not very soluble in water. The pyrene emission was measured at different tryptophan concentrations for samples prepared by dilution. The emission for the blank containing tryptophan and all other components but pyrene was measured at each tryptophan concentration and was subtracted from the integrated pyrene emission intensity (365-400 nm). The tryptophan emission was always smaller than 15% of the total pyrene emission. The quenching experiments for pyrene in water and pyrene-CD complexes were performed in solutions deaerated by bubbling nitrogen for 15 minutes, whereas in the presence of third components, aerated solutions were employed. A standard was used to correct for drift in the excitation intensity of the fluorimeter.

Pyrene/ß-CD/amino acid ternary complexes. Solution containing 5×10^{-7} M pyrene and β-CD concentrations between 0.1 and 1.2×10^{-2} M were prepared as described above for the quenching experiments. From this pyrene/ß-CD stock solution, a solution at high amino acid concentration was prepared by dissolving the solid. Lower concentrations were prepared by dilution. No fluorescence emission was observed for all amino acids but tryptophan when excited between 330 and 340 nm. Some samples were prepared in 0.1 M phosphate buffer solution (0.05 M Na_2HPO_4 and 0.05 M $NaH_2PO_4 \cdot H_2O$) at pH 6.74.

ANS/ß-CD complexes for competitive binding studies. All samples were prepared in 0.1M phosphate buffer solution at pH 6.74. For the determination of the association constants between ANS (1.4×10^{-5} M) and ß-CD the CD concentration was varied between 0.12-1.2×10^{-2} M. Samples were prepared by diluting the solution at the highest ß-CD concentration with aqueous ANS.

The equilibrium constant of amino acids with ß-CD were determined by measuring the decrease of the fluorescence intensity of ANS/ß-CD with increasing amino acid concentrations. The concentrations of ANS and ß-CD were fixed at 1.4×10^{-5} M and 1.1×10^{-3} M, respectively. Samples were prepared by diluting the solution at the highest amino acid concentration with the ANS/ß-CD solution. The concentration employed varied from 7.8×10^{-3} M to 8.5×10^{-2} M for leucine and from 6.0×10^{-3} M to 3.0×10^{-2} M for tryptophan.

2.3. Correction for inner filter effect

Since steady-state fluorescence is an absolute measurement, a decrease of the excitation intensity will decrease the fluorescence emission intensity. The absorption of the fluorescence emission by chromophores on the pathlength between the

molecules being excited and the detector will also decrease the observed fluorescence intensity. Both these effects are called inner filter effects. In measurements of fluorescence quenching "inner-filter " absorption by the quencher of the excitation light can cause errors in the evaluation of quenching efficiency.[4,99]

The absorption of pyrene at the excitation wavelength (340 nm) was smaller than 0.06. Tryptophan absorbed at the same wavelength and at high tryptophan concentrations the amount of light absorbed by pyrene decreased due to the inner filter effect. This artifact was corrected for by calculating the decrease of the excitation light intensity absorbed by pyrene in the presence of tryptophan.

The excitation and detection monochromators in the fluorimeter are at a 90° angle and the sample is irradiated at the center of the cell. With an excitation slit of 2.0 nm the width of the irradiated area on the cell was 0.2 cm (**Figure 2.1**).

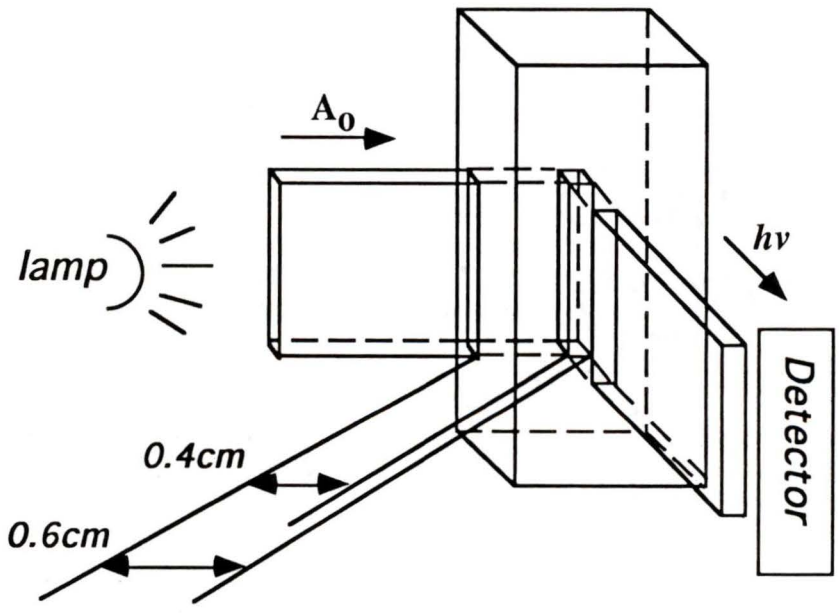


Figure 2.1 Geometric arrangement for the observation of fluorescence

By using Eq. (1.6), the fraction of absorbed light between 0.4 cm and 0.6 cm was calculated for pyrene in the absence $(f_{Py}^{abs})_o$ and presence (f_{Py}^{abs}) of the highest tryptophan concentration employed from the following equations:

$$(f_{Py}^{abs})_o = \exp(-2.303 \times 0.4 \times A_{Py}) - \exp(-2.303 \times 0.6 \times A_{Py}) \quad (2.2)$$

$$f_{total}^{abs} = \exp(-2.303 \times 0.4 \times A_{total}) - \exp(-2.303 \times 0.6 \times A_{total}) \quad (2.3)$$

$$f_{Py}^{abs} = (A_{Py} / A_{total}) \times f_{total}^{abs} \quad (2.4)$$

where A correspond to the absorbances in a 1 cm cell and A_{total} is defined as:

$$A_{total} = A_{Py} + A_{Trp} \quad (2.5)$$

where A_{Py} and A_{Trp} were the absorbances for pyrene and tryptophan, respectively. The fractional decrease due to the inner filter effect was:

$$\Delta f_{Py} = [(f_{Py}^{abs})_o - f_{Py}^{abs}] / f_{Py}^{abs} \quad (2.6)$$

The Δf_{Py} value normalized for the tryptophan concentration, $\Delta f_{Py}/[Trp]$, corresponds to the contribution of the inner filter effect to the value of the slope in the quenching plot.

A simulation of the inner filter effect by placing a second 1 cm cell containing tryptophan between the excitation beam and the cell (1 cm) containing pyrene was performed. The decrease of the pyrene emission intensity observed should ideally be

twice of the calculated value, since the correction was for the fraction of the absorbed light between 0.4 cm and 0.6 cm, i.e. centered at a pathlength of 0.5 cm. The observed value of was within 10% of the calculated value.

The contribution of $\Delta f_{Py}/[Trp]$ to the measured slope was more prominent for low quenching efficiencies and was generally smaller than 30%. The values for the Stern-Volmer constants were obtained by subtracting the normalized fraction from the slope of the quenching plot.[132]

$$K_{sv} = K_{sv}(obs) - \Delta f_{Py}/[Trp] \qquad (2.7)$$

2.4. Pyrene R(I/III) scale

The R(I/III) ratio of the pyrene fluorescence has been shown to be sensitive to solvent polarity and, in practice, dependent on experimental conditions, e.g. the width of emission and excitation slits and temperature. As slightly different values were reported in the literature we determined the R(I/III) ratios for pyrene (5×10^{-7} M) in several solvents under our experimental conditions (**Table 2.1**).

2.5. Fluorescence measurements

Steady-state fluorescence. Steady-state fluorescence was acquired with a Perkin Elmer MPF 66 fluorimeter. Unless otherwise stated, a standard was used to correct for drifting in the excitation intensity of the fluorimeter. The temperature of the sample holder was controlled by a Haake F 3 circulating bath. Unless otherwise stated, samples were kept at 20.0 ± 0.5 °C. The excitation and emission slits were set at 2.0 nm for pyrene samples and 5.0 nm for ANS samples. When necessary, the

fluorescence intensity was decreased by attenuating the excitation beam with neutral density filters.

The following excitation wavelength were employed: 334 nm for pyrene excitation in the tryptophan quenching experiments; 337 nm for pyrene excitation in the pyrene/amino acid/ß-CD ternary complex, 340 nm for pyrene excitation in the pyrene/ß-CD/alkyl sulfates (or alcohol) complexes and 350 nm for the excitation of ANS.

Table 2.1: R(I/III) scale of pyrene in different solvents.[a]

Solvent	This work	Dong et al[d]	Kalyanasundaram et al[e]
Hexane	0.61	0.58	0.61
Cyclohexane	0.62	0.58	0.59
Ethyl ether	1.00[c]	1.02	0.98
tert-BuOH	1.04	1.02	
sec- BuOH	1.05	1.03	
n- BuOH	1.11	1.06	1.02
Toluene	1.13[b]	1.04	1.11
Ethanol	1.28	1.18	1.14
Methanol	1.35[b,c]	1.35	1.33
Ethyl acetate	1.49	1.37	1.45
DMF	1.77[c]	1.81	1.82
Acetonitrile	1.90	1.79	1.75
H_2O	1.92	1.87	1.84

[a] The excitation wavelengths were set to the maximum of their excitation spectra. Excitation (Ex) and emission (Em) slits =2.0 nm. T= 20.0 °C.

[b] Deaerated sample.

[c] Ex/Em slits = 1.0/1.0 nm.

[d] Deaerated samples. Ex/Em slits = 0.5 nm. Ex wavelength = 338 nm. Room temperature. See reference 95.

[e] Data from reference 93.

Time-correlated single photon counting (SPC). Lifetime measurements were performed on a PTI LS-1 time-correlated single photon counter at room temperature (20 ± 4 °C) for samples with pyrene in water and in the presence of β-CD and 20.0 ± 0.5 °C (RM6 Lauda circulating bath) for all other samples. A 10,000 maximum count was used for all experiments. Visual analysis of residuals and the autocorrelation, χ^2 values, in some cases Z, and DW (Durbin-Watson) parameters were used as criteria for fitting and to differentiate single, double and triple exponential decays. Typical values of χ^2 were between 1.0 and 1.2, Z values were bigger than -1.96 and DW values were bigger than 1.70, 1.75 and 1.80 for single, double and triple exponential fits, respectively.[16] A detailed description of this equipment can be found in reference 133.

Excitation wavelengths were set at 334 nm for samples of pyrene in water, 334 nm or 336 nm for samples of pyrene in the presence of β-CD, 340 nm for samples of pyrene/ß-CD/alcohol (or alkyl sulfate) in presence or absence of tryptophan and 337 nm for samples of pyrene/ß-CD/amino acids.

2.6. UV-Vis spectra

UV-Vis spectra were recorded on a Varian Cary 5 or Cary 1 or on a Philips PU8740 spectrophotometer at room temperature. The Cary 5 was employed for differential spectra and equilibrium constant determinations. Typical scan rates and slits were 200 nm/min and 2 nm, respectively. Baseline correction was always employed. The blank contained all components other than that whose absorption spectrum was being determined.

2.7. Circular dichroism spectra

Circular dichroism spectra were measured on a JASCO J-720 spectropolarimeter at room temperature (20 ± 4 °C). Typical scan rate and step resolution were 200 nm/min and 0.5 nm, respectively.

2.8. NMR spectra

Proton NMR spectra were recorded on a Bruker AMX 360 NMR spectrometer in the ^{2}H lock mode. Spectral width of 4000 Hz in 32 K memory size was used. 30° pulses with overall delay of 10 μs between pulses were employed. The DOH peak was set to 4.65 ppm and no water suppression was used.

3. Chiral discrimination in the quenching of singlet pyrene complexed to β-cyclodextrin by tryptophan

3.1. Quenching of excited singlet pyrene in water

Previous studies on the quenching of singlet pyrene in water by tryptophan showed no complex formation between pyrene and tryptophan in the ground state in aqueous solution.[100] The rate constant for quenching of excited singlet pyrene in water by D-, L- and DL-tryptophan were measured. The equal intensity (I_0/I) and lifetime ratios (τ_0/τ) indicate that quenching occurs through a dynamic mechanism (**Figure 3.1**).

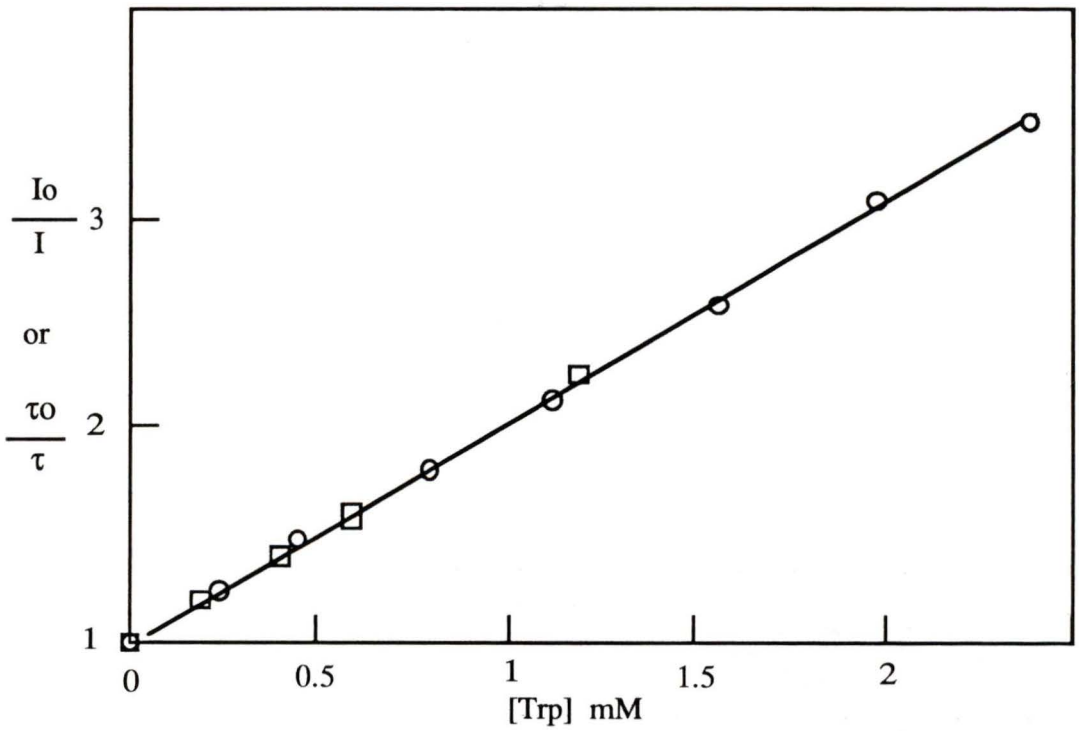


Figure 3.1: Stern-Volmer plots (I_0/I (□) and τ_0/τ (○)) for the quenching by tryptophan of pyrene (5×10^{-7} M) fluorescence in water.

The fluorescence decay monitored at 400 nm had a fast component when tryptophan was added to the pyrene solution; the magnitude of this contribution increased at higher quencher concentrations. Blank experiments with only tryptophan showed that this emission came from the quencher. Thus, the fast component was disregarded when fitting the data, i.e. the intense fast decay in the SPC trace was not included into the range of data being fitted.

Since pyrene and the solvent are achiral, no difference was expected for the quenching by the enantiomers of tryptophan. The average k_q values obtained were $(4.9 \pm 0.4) \times 10^9$ M^{-1} s^{-1} (13 determinations), $(4.9 \pm 0.6) \times 10^9$ M^{-1} s^{-1} (5 determinations) and $(5.1 \pm 0.5) \times 10^9$ M^{-1} s^{-1} (3 determinations) for L-, D- and DL-tryptophan, respectively. A constant R(I/III) ratio for the pyrene emission (1.94 ± 0.02) was observed in the presence of all tryptophan concentrations.

Our k_q values are higher by a factor of 2 when compared to those previously published.[100] The experiments were carefully repeated with tryptophan from different sources including the source of the previous report. The quencher concentration was checked by UV-Vis absorption. In all instances we obtained higher k_q values and we can not explain this discrepancy.

3.2. Quenching of excited singlet pyrene in the presence of ß-CD

3.2.1. Complex formation between pyrene and ß-CD

The equilibrium constant between pyrene and β-CD was determined from the non-linear Benesi-Hildebrand fit and the lifetime treatment. Both ground and excited singlet state pyrene were employed to probe the complexation with ß-CD.

The changes of the pyrene absorption spectrum and fluorescence spectrum in the presence of ß-CD are shown in **Figure 3.2.**

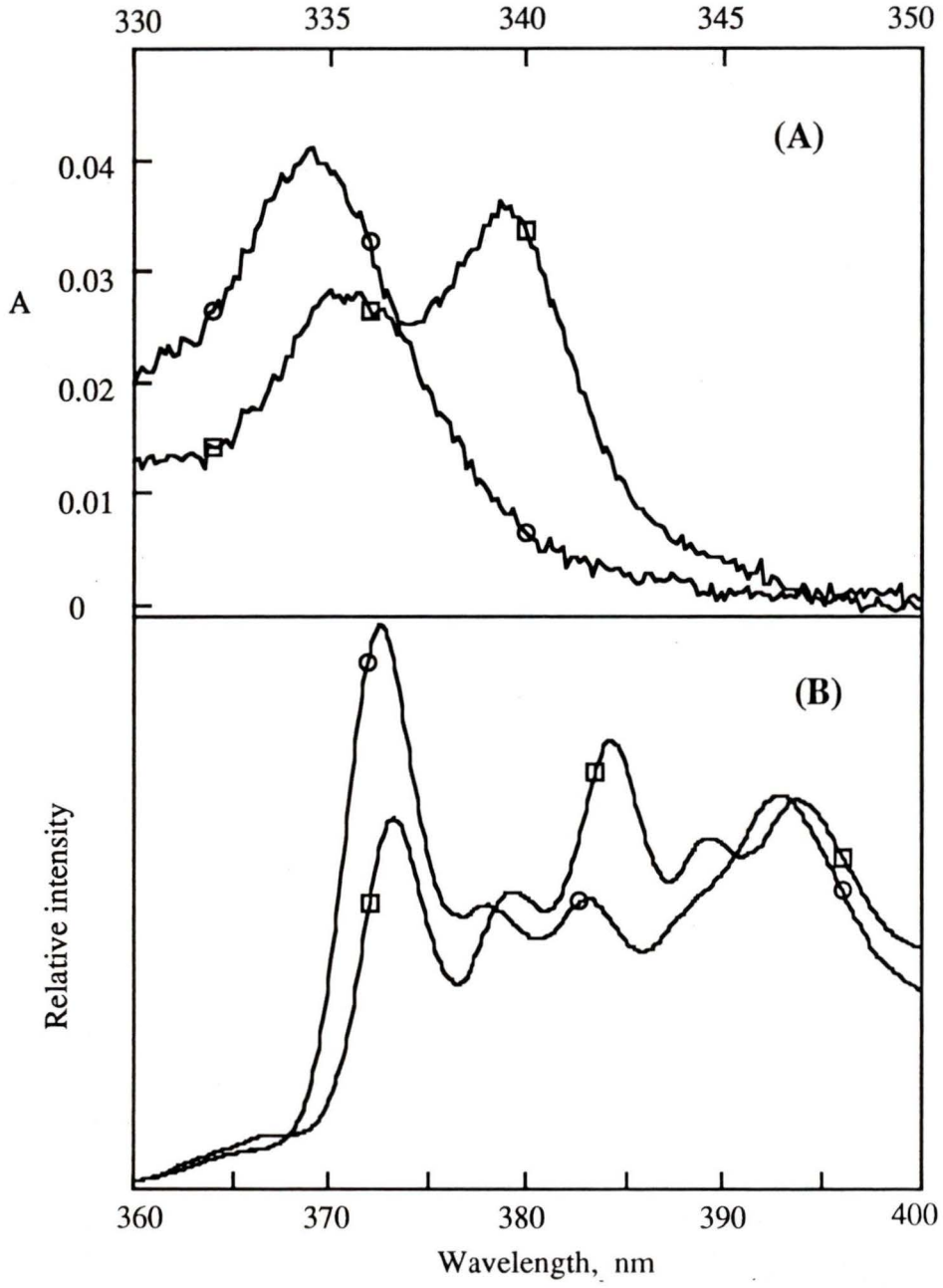


Figure 3.2: Absorption (A) and fluorescence emission (B) spectra of pyrene (5×10^{-7} M) in the presence (□) and absence (○) of ß-CD (1.3×10^{-2} M). Excitation wavelength was set to 337 nm.

Although the sequential 1:1 and 1:2 complexation stoichiometries have recently been shown to satisfactorily explain the pyrene/ß-CD complexation, we fitted the data to a 1:1 stoichiometry to compare our values with those in the literature. The K_1 values were determined from nonlinear fits of the absorbance ($(6 \pm 2) \times 10\ M^{-1}$) or fluorescence intensity change ($(8 \pm 2) \times 10\ M^{-1}$) with increasing β-CD concentrations (**Figure 3.3**). These values are within the broad range (7.6 - 277 M^{-1}) reported in the literature.[105-109]

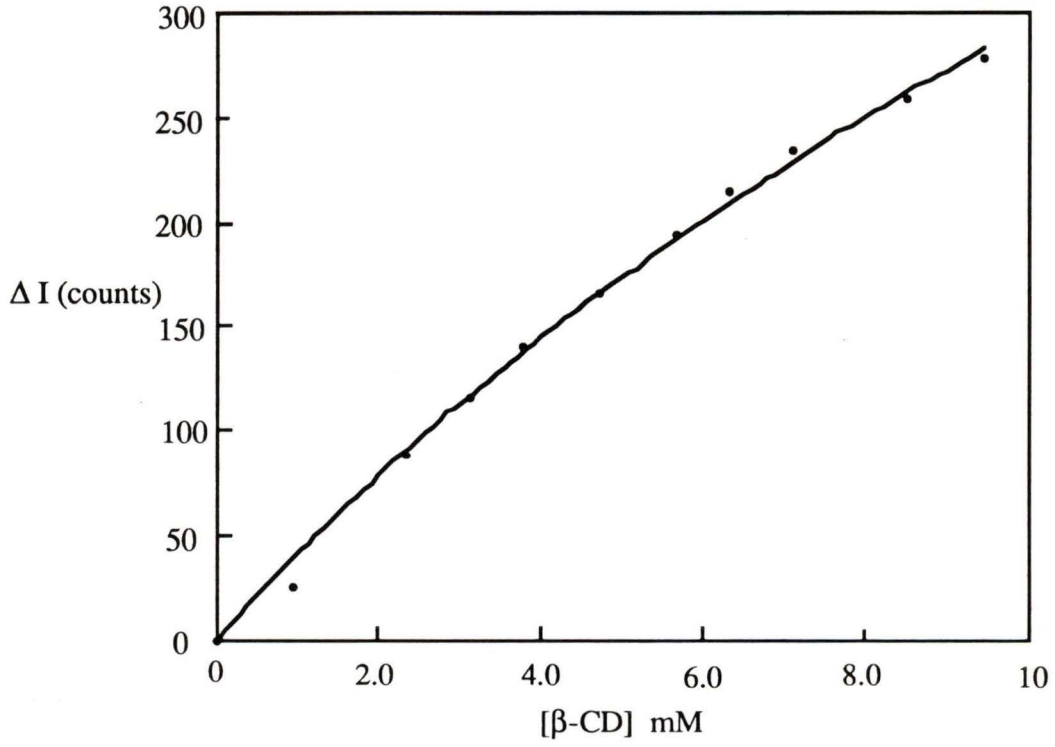


Figure 3.3: Non-linear fit of the pyrene fluorescence enhancement at 385 nm with increasing ß-CD concentration. The non-linear fit corresponds to a 1:1 stoichiometry for pyrene:ß-CD.

The fluorescence decay of pyrene in the presence of ß-CD was fitted to the sum of two exponentials (**Figure 3.4**). As previously reported,[112] the short lived component corresponds to the lifetime of pyrene in water and complexed to ß-CD with a 1:1 stoichiometry, whereas the long lived component was assigned to pyrene complexed to β-CD with a 1:2 stoichiometry. Our lifetime values (128 and 288 ns) are very close to those reported earlier (130 and 300 ns).[112]

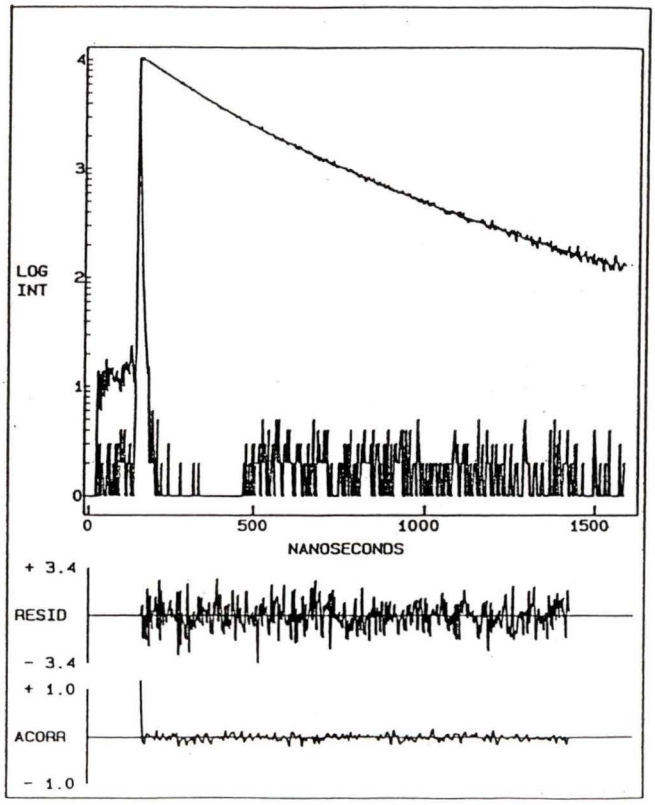


Figure 3.4: Pyrene (5×10^{-7} M) fluorescence decay in the presence of ß-CD (1.25×10^{-2} M). The fit to the experimental data corresponds to the sum of two exponentials.

The pre-exponential factors of the fit to the fluorescence decay can be related to free and bound pyrene concentrations by Eq. (1.27) or (1.28). The sample was excited at the isosbestic point and therefore the ratio of absorption coefficients was unity. As the lifetimes for pyrene free in water and bound to ß-CD in a 1:1 complex can not be differentiated we only determined the degree of pyrene bound in a 1:2 complex. The ratio of concentrations can be expressed by Eq. (3.1) assuming that the lifetimes are inversely proportional to the fluorescence lifetime.

$$\frac{[\text{pyrene}\text{-}(\beta\text{-CD})_2]}{[\text{pyrene}] + [\text{pyrene}\text{-}\beta\text{-CD}]} = \frac{A_2}{A_1}\,\frac{\tau_2}{\tau_1} \qquad (3.1)$$

In the presence of 12.5 mM β-CD the fluorescence decay was fitted to the sum of two exponentials ($A_1 = 0.31$, $\tau_1 = 128$ ns, $A_2 = 0.69$, $\tau_2 = 288$ ns). Under these conditions, 78% of the pyrene was complexed to β-CD with a 1:2 stoichiometry, whereas 22% of the pyrene was free in solution or bound as a 1:1 complex. These values were within the range of the recently published equilibrium constants.[112]

3.2.2. Quenching of Pyrene in the presence of CD

Pyrene in the presence of β-CD was excited at the isosbestic point (337 nm) to ensure equal excitation efficiencies for free and CD-complexed pyrene. The decrease of the fluorescence emission intensity is shown in **Figure 3.5**.

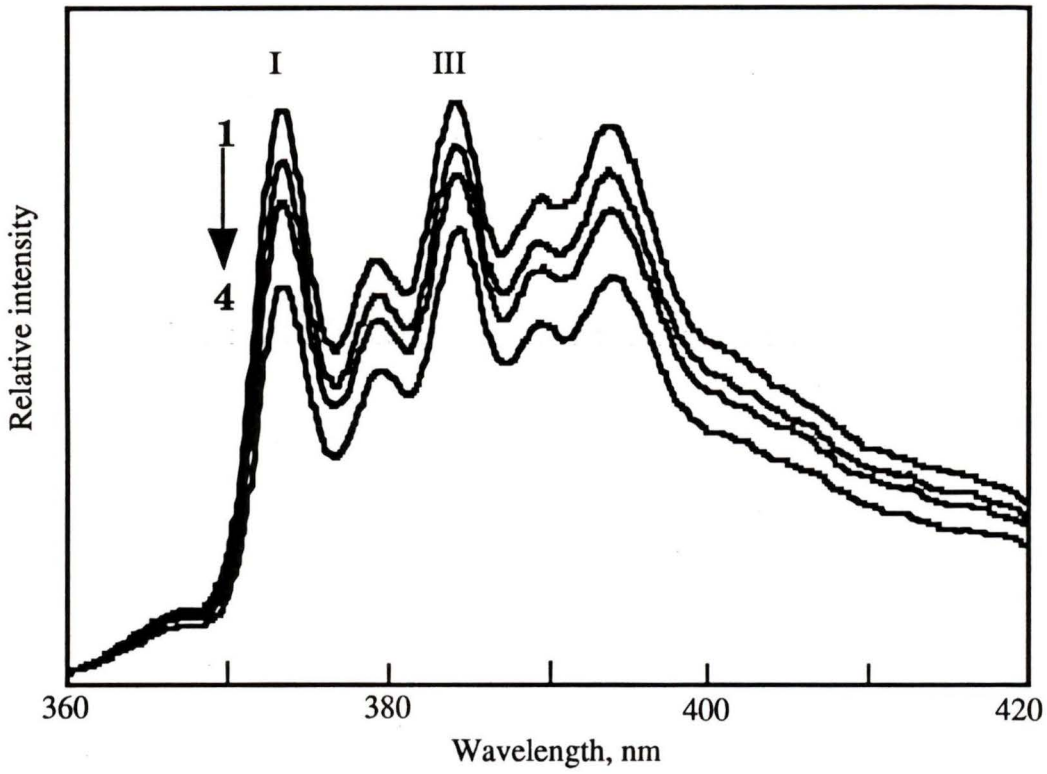


Figure 3.5: Fluorescence spectra of pyrene (5×10^{-7} M) in the presence of 1.0×10^{-2} M ß-CD. The tryptophan concentrations were **1**, 0 M; **2**, 3×10^{-4} M: **3**, 5×10^{-4} M and **4**, 1×10^{-3} M.

The I_o/I plot for tryptophan quenching of singlet pyrene in the presence of 1.0×10^{-2} M β-CD (**Figure 3.6**) was initially linear but curved downwards at higher quencher concentrations. This behavior suggested that pyrene in at least two different environments was being quenched. The decrease of the quenching efficiency at high quencher concentrations indicated that the deactivation of excited state pyrene by tryptophan was substantially decreased once pyrene is bound to CD. We assigned the process at high tryptophan concentrations to the quenching of pyrene-β-CD complexes with a 1:2 stoichiometry. At low tryptophan concentrations, pyrene in water and probably pyrene complexed to β-CD with a

1:1 stoichiometry were quenched. No chiral discrimination was observed for the quenching processes at low tryptophan concentrations (**Figure 3.6**).

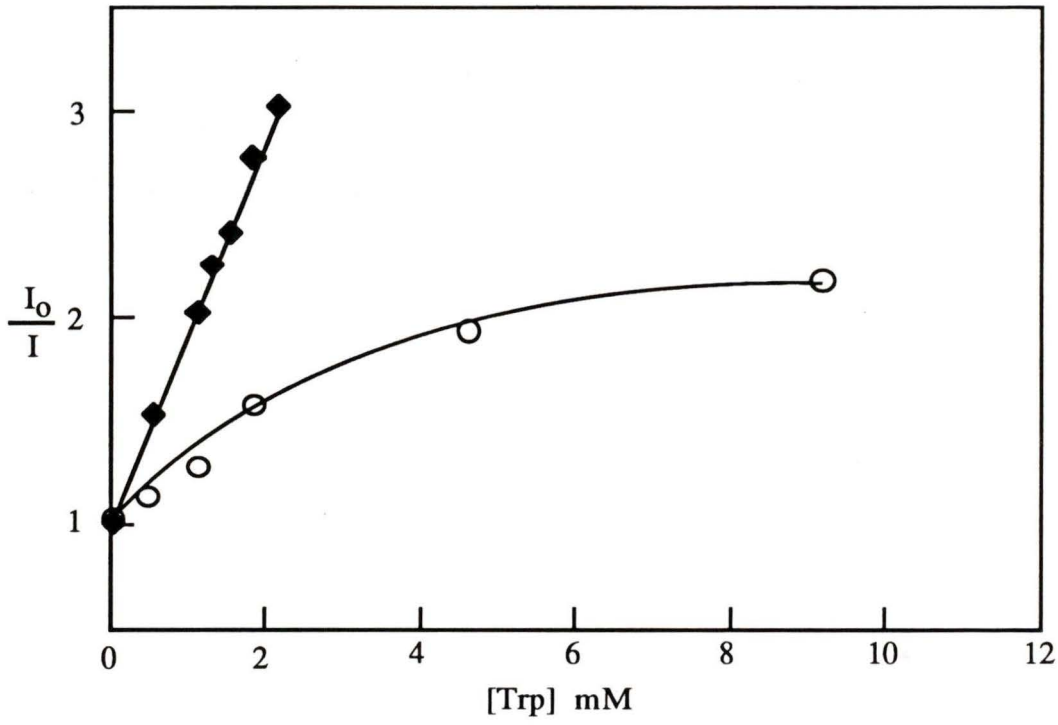


Figure 3.6: Quenching plot of pyrene (5×10^{-7} M) in the absence (◆) and presence (O) of ß -CD (1.0×10^{-2} M).

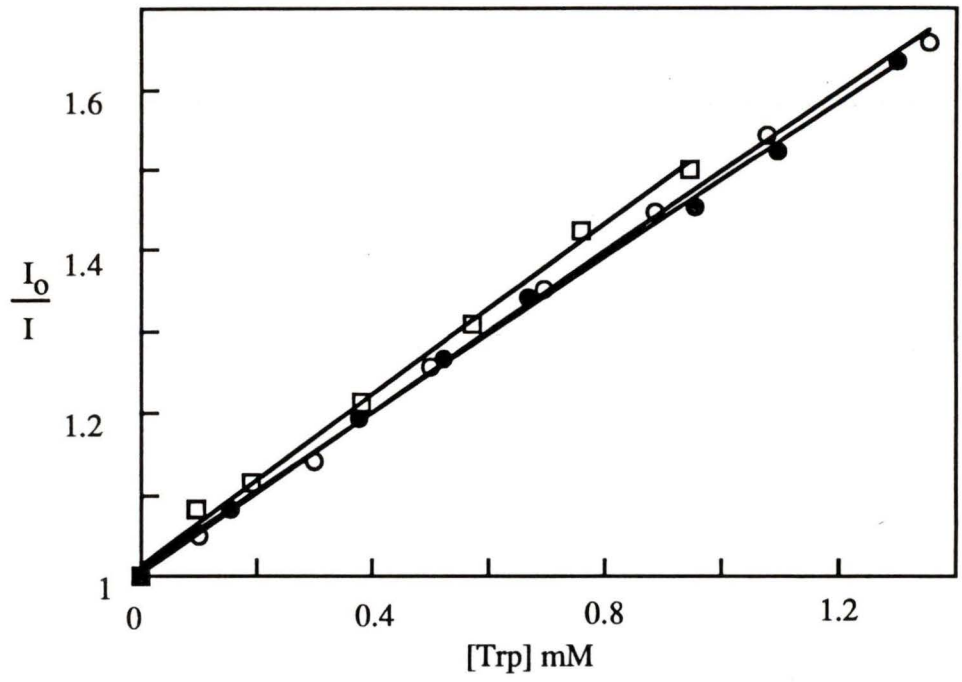


Figure 3.7: Quenching of pyrene (5×10^{-7} M) in the presence of ß-CD (1.0×10^{-2} M) by (O) D-, (□)L- and (●) DL-tryptophan.

A careful analysis of the I/III intensity ratios in the presence of tryptophan substantiates the qualitative picture drawn above. The value for R(I/III) of pyrene in the presence of 12.5 mM ß-CD is 1.01. This indicates that the average polarity sensed by pyrene is much lower than in water (R(I/III) =1.92). However, this R(I/III) value in the presence of CD corresponds to the combinations of R(I/III) values for pyrene free in water and complexed to ß-CD. At low quencher concentrations, tryptophan primarily quenched those pyrene molecules that were involved in processes with high quenching efficiency, such as free pyrene in water and possibly pyrene complexed to β-CD with a 1:1 stoichiometry. Thus, by increasing the tryptophan concentration the relative contribution to the R(I/III) ratio of the pyrene complexed to β-CD in a 1:2 stoichiometry which has a lower

value of R(I/III) ratio increases. For this reason a decrease of the R(I/III) ratio was observed when the tryptophan concentration increased (**Figure 3.8**).

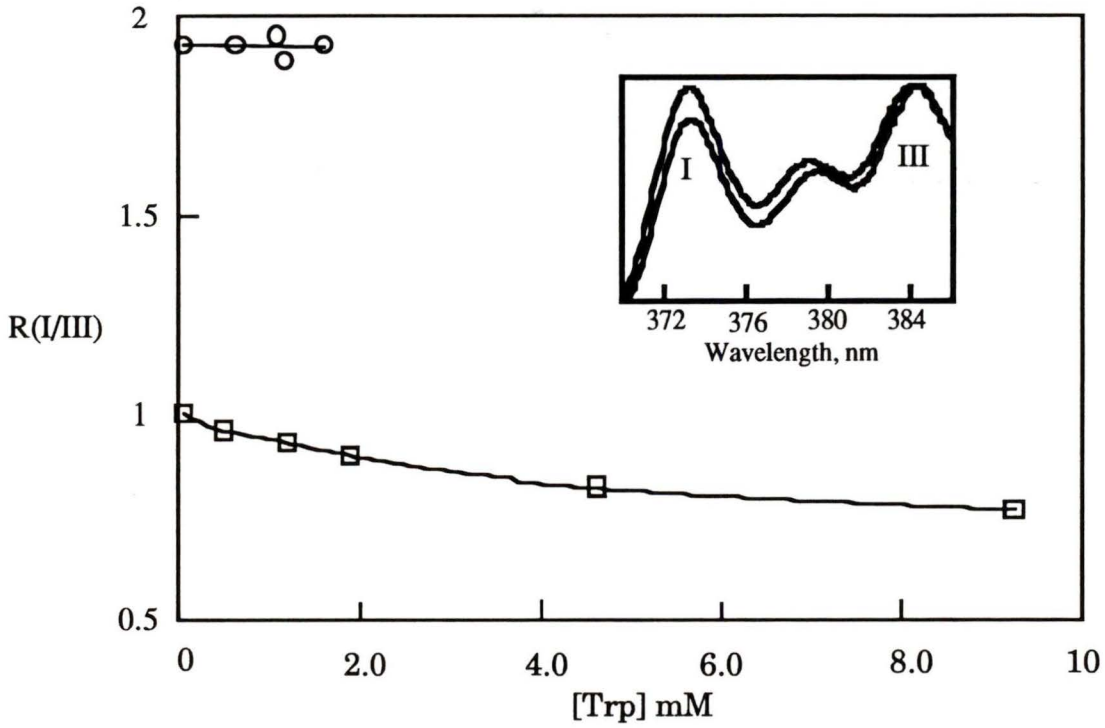


Figure 3.8: R(I/III) values at different tryptophan concentrations for pyrene in water (O) or in the presence of ß-CD (1.0×10^{-2} M)/H_2O (□). The inset shows the vibronic bands of pyrene (5×10^{-7} M) in the presence of 1×10^{-2} M ß-CD and 0 M (upper curve), 1.0×10^{-3} M (lower curve) tryptophan. The spectra were normalized for the intensity of band III.

In the presence of β-CD, three pyrene species were present and most of the pyrene quenching was due to pyrene in water and possibly for the pyrene β-CD as a 1:1 complex. A further complication of this system was the observation that the pyrene emission intensity increased at low tryptophan concentrations when pyrene was excited at 340 nm. At this wavelength complexed pyrene has a much higher absorption coefficient than aqueous pyrene. This indicated that the

equilibrium constant between pyrene and β-CD increased in the presence of tryptophan. Indeed, tryptophan and other amino acids have been shown to act as zwitterionic ternary complexation agents. A detailed study of this effect will be described in the next chapter. Unfortunately, no quantitative analysis was possible for the quenching studies as they require the quencher not to affect the equilibrium constant between pyrene and β-CD.

3.3. Quenching of excited singlet pyrene complexed to β–CD in the presence of alcohols and alkyl sulfates

To avoid the complexity of having to analyze for the quenching of several excited pyrene species, we studied the quenching of pyrene complexed to ß-CD in the presence of alcohols or alkyl sulfates. These third components were shown to dramatically increase the equilibrium constant for pyrene complexation to CD.[114,105]

3.3.1. Pyrene ß-CD and third component complexation

In this study, three alcohols and two alkyl sulfates were chosen as third components. In the presence of 20 mM alcohol or alkyl sulfate and 13 mM ß-CD, the SPC trace for pyrene fluorescence corresponded to a single exponential (**Figure 3.9**). This suggested that in these ternary complexes only one pyrene species, that has a lifetime twice as long as that of free pyrene, was predominant.

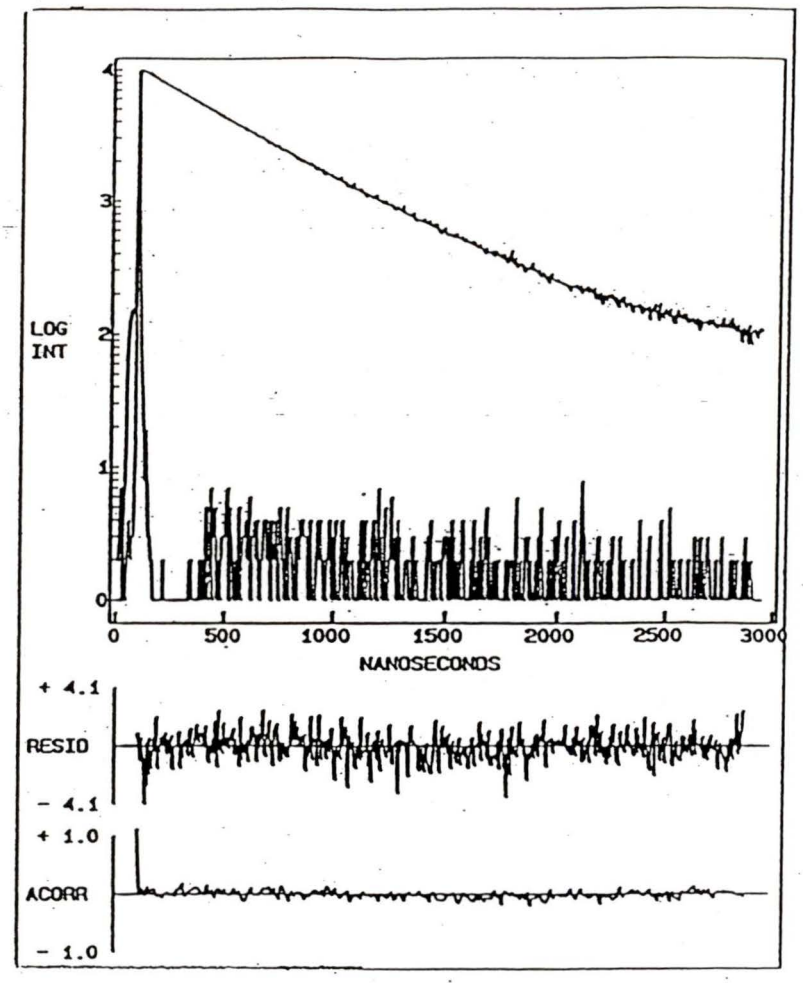


Figure 3.9: Pyrene fluorescence decay curve for pyrene (1.0×10^{-6} M)/ß-CD (1.3×10^{-2} M) / *tert*-butanol (2.0×10^{-2} M). The fit to the experimental data corresponds to a single exponential.

As only one pyrene species is present, the R(I/III) ratio corresponds to the polarity sensed by pyrene within the CD cavity. The fluorescence lifetime and R(I/III) of pyrene in these ternary systems together with the values in H_2O and ß-CD are summarized in **Table 3.1**.

Table 3.1: Excited singlet pyrene lifetimes and R(I/III) ratios in water and when complexed to β-CD in the absence and presence of alcohols or alkyl sulfates.

System	R(I/III)	τ (ns)	
		N_2 bubbling	aerated
Water[a]	1.92	193	128
β-CD[a-c]	1.01	311[c]	288[c]
β-CD-1-butanol[d]	0.63	456	448
β-CD-2-butanol[d]	0.72	452	447
β-CD-*tert*-butanol[d]	0.81	453	447
β-CD-C_4-SO_4[d]	0.72	457	447
β-CD-C_6-SO_4[d]	0.59	468	457

[a] [Pyrene] = 5×10^{-7} M.
[b] [β-CD] = 1.25×10^{-2} M.
[c] Double exponential decay; the short lifetime was fixed at 193 ns and 128 ns, in the presence of nitrogen and air, respectively; the lifetime shown corresponds to the long lifetime component.
[d] [Pyrene] = 1.0×10^{-6} M, [β-CD] = 1.25×10^{-2} M and [alcohol or alkyl sulfate] = 2.0×10^{-2} M.

The R(I/III) ratios for ternary-complexed pyrene was similar to that of pyrene in non polar solvents. This suggests that water is excluded from the surroundings of pyrene which therefore indicates that pyrene was located in the nonpolar environment within the CD cavity. Our R(I/III) ratios for complexes with alkyl sulfates are in full agreement with those in the literature[105] but our values are higher than those published in the presence of alcohols.[114] I/III intensity ratios are very dependent on instrumental settings and temperature, and this may be the cause for the difference observed.

3.3.2. Quenching of excited singlet pyrene β-CD complexes in the presence of alcohols or alkyl sulfates.

The tryptophan quenching efficiency for β-CD complexed pyrene in the presence of alcohols or alkyl sulfates decreased dramatically when compared to quenching in the presence of only β-CD (**Figure 3.10**).

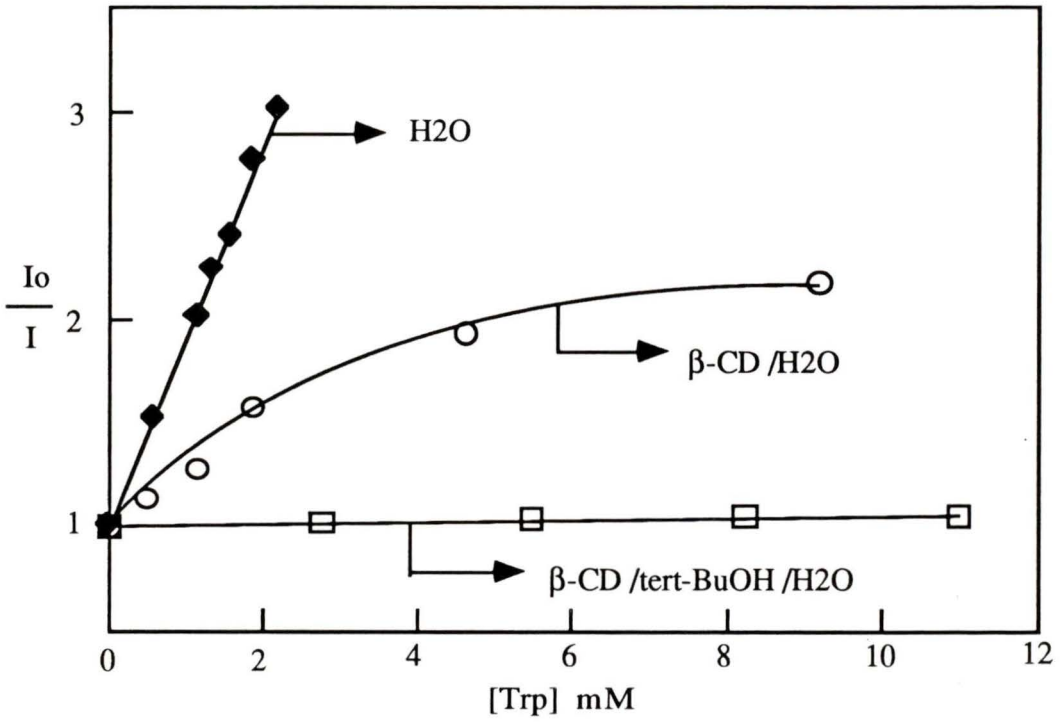


Figure 3.10: Quenching of singlet pyrene by tryptophan in water (◆) and when complexed to ß-CD (1.0×10^{-2} M) (○) and ß-CD(1.3×10^{-2} M)/tert-butanol (2.0×10^{-2} M) (□).

The low quenching efficiency required the use of high tryptophan concentrations and therefore corrections for tryptophan impurity emission and inner

filter effects had to be performed. Typical Stern-Volmer plots are shown in **Figure 3.11** and the corrected K_{SV} values are given in **Table 3.2**.

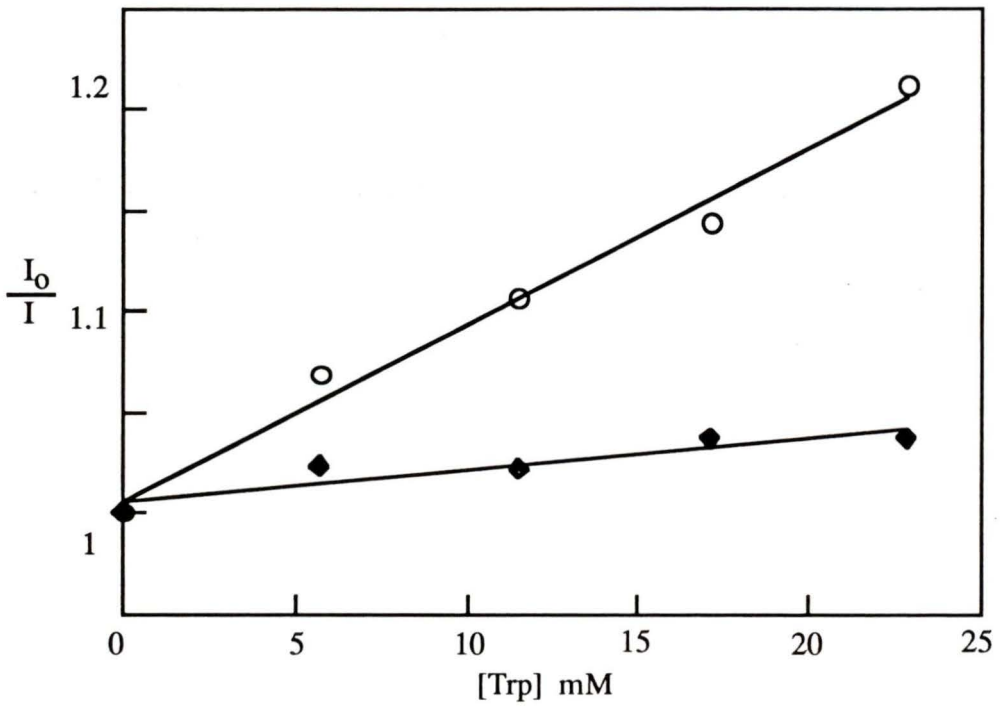


Figure 3.11: Chiral discrimination for the quenching of singlet pyrene (1.0×10^{-6} M) in the presence of β-CD (1.3×10^{-2} M) and *tert* -butanol (2.0×10^{-2} M) by D- (O) and L- (◆) tryptophan

Table 3.2: Corrected Stern-Volmer constants (M^{-1}) and ratio of constants for the quenching of pyrene complexed to β-CD in the presence of alcohols or alkyl sulfates by D- and L-tryptophan.[a]

Third Component	D-Trp	L-Trp	$\frac{K_{SV}\ (D\text{-}Trp)}{K_{SV}\ (L\text{-}Trp)}$
tert -butanol	5 ± 1 (6)	1.4 ± 0.7 (4)	3.6 ± 1.9
2 -butanol	4.0 ± 0.4 (3)	2.3 ± 0.3 (3)	1.7 ± 0.3
1 -butanol	5 ± 1 (3)	2.6 ± 0.1 (3)	1.9 ± 0.4
C_4-SO_4	5 ± 2 (4)	4 ± 1 (7)	1.3 ± 0.6
C_6-SO_4	3.2 ± 0.5 (3)	1.6 ± 0.3 (3)	2.0 ± 0.5

[a] [pyrene] = 1.0×10^{-6} M, [β-CD] = 1.3×10^{-2} M and [third component] = 2.0×10^{-2} M. The number in parentheses indicates the number of independent experiments performed.

Chiral discrimination was fairly small in the presence of linear alcohols or alkyl sulfates but was significantly larger for bulky molecules such as *tert*-butanol. The magnitude of the chiral discrimination observed was similar to that observed in quenching studies involving crown ethers[124] or enzymes.[35] Our relatively large errors reflect the small intensity decreases being measured. The smallest I_o/I value that we could reliably measure after corrections was 1.02. The Stern-Volmer constant for the quenching in the presence of *tert*-butanol and C_6-SO_4 by L-tryptophan were 1.4 M^{-1} and 1.6 M^{-1}, respectively. These values were very close to our detection limit as they correspond to I_o/I values at the highest tryptophan concentration (30 mM) employed of 1.04 and 1.05 for *tert*-butanol and C_6-SO_4, respectively. Most of our quenching experiments were performed in pairs with samples for D- and L-tryptophan being prepared in parallel from the same stock solutions. In *all instances* we observed a higher quenching efficiency for

D-tryptophan than for L-tryptophan, although the absolute values for K_{sv} varied between experiments. This fact gives us confidence that even in cases (pyrene/ β-CD/C_4-SO_4) where the K_{sv} values for both enantiomers are the same within the experimental error, chiral discrimination occurred. Quenching of pyrene/β-CD/*tert*-butanol by D/L-tryptophan was also studied, but this experiment has intrinsically much higher errors because of the lower solubility of the racemate. For this reason we can not determine if the value obtained (4.5 M^{-1}) is higher than the expected average (3.2 M^{-1}) for the quenching by D- and L-tryptophan.

Contrary to the experiments in the presence of only β-CD, the R(I/III) ratio stayed constant with the addition of tryptophan (**Figure 3.12**), suggesting that pyrene inside the CD cavity was quenched.

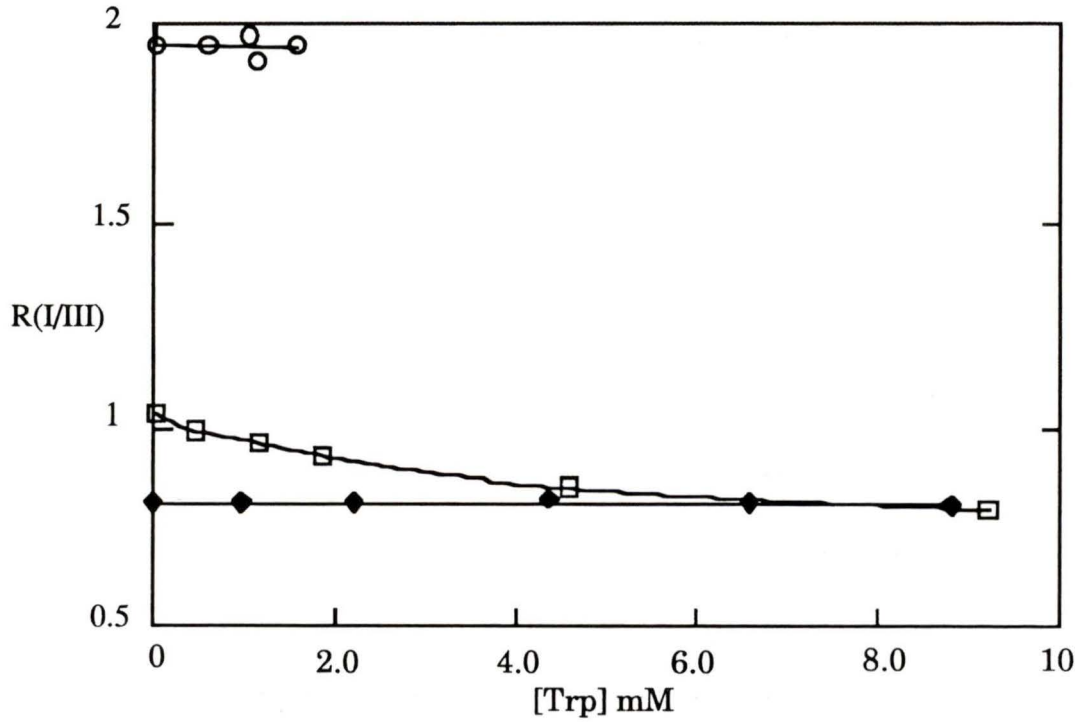


Figure 3.12: Pyrene R(I/III) values at increasing tryptophan concentrations in H_2O (○) and in the presence of ß-CD (1.0×10^{-2} M)/H_2O (□), or ß-CD (1.3×10^{-2} M)/*tert*-butanol(2.0×10^{-2} M)/H_2O (◆).

The constant value of the R(I/III) ratio also indicates that proper corrections for the emission of tryptophan were performed. A systematic error in the correction would lead to systematic changes in the R(I/III) ratio due to the contribution of the broad tryptophan emission to the well resolved pyrene fluorescence.

The same lifetime for pyrene in the absence and presence of tryptophan ($\tau_o/\tau = 1$, **Figure 3.13**) was obtained, suggesting that static quenching occurred.

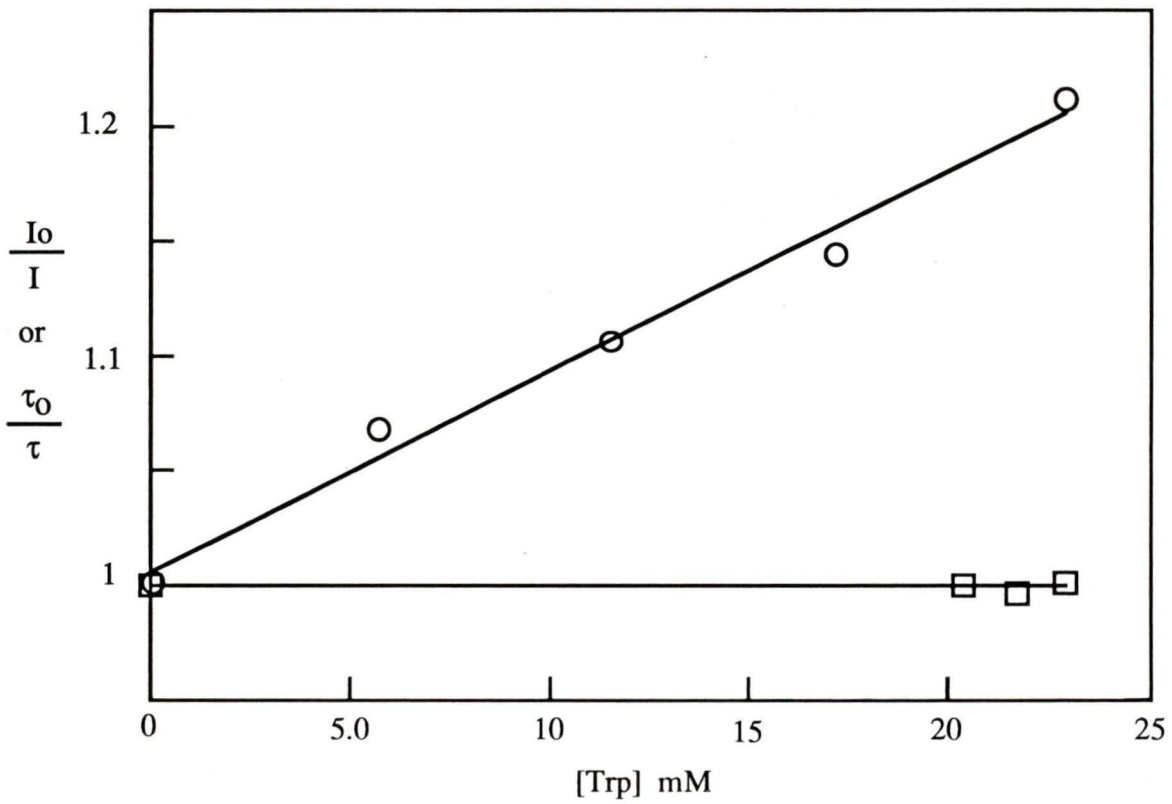


Figure 3.13: Quenching of pyrene (1×10^{-6} M) fluorescence by tryptophan (I_o/I (O) and τ_o/τ (□)) in the presence of 1.25×10^{-2} M ß-CD and 2.0×10^{-2} M *tert*-butanol.

This result eliminates any dynamic quenching involving tryptophan from the aqueous phase. Thus, pyrene and tryptophan have to form a complex within the cyclodextrin cavity prior to excitation of pyrene. However, we cannot rule out the relocation of tryptophan within the CD cavity as this phenomenon is expected to occur in the picosecond time-domain and cannot be measured with our time-resolved single photon counter (time resolution of ca. 1 ns).

3.4. Tryptophan and β-CD complexation

The observed chiral discrimination can only be due to the different interactions of the chiral host, ß-CD, with D- and L-tryptophan, since pyrene is an achiral molecule. The efficiency of tryptophan complexation with ß-CD and the conformation of the inclusion complex are two important parameters that could explain the chiral discrimination observed. NMR was used to obtain a qualitative picture of the Trp/ß-CD complex. In addition, a competitive binding experiment was used to estimate the binding constants, since the results in the literature are contradictory (7.9 M^{-1} and 214 M^{-1}).[120-122]

3.4.1. NMR study

The proton NMR spectra of D- and L-tryptophan (1.0×10^{-2} M) in D_2O and in the presence of 1.0×10^{-2} M ß-CD were recorded. The literature assignment[127] is shown in **Figure 3.14**.

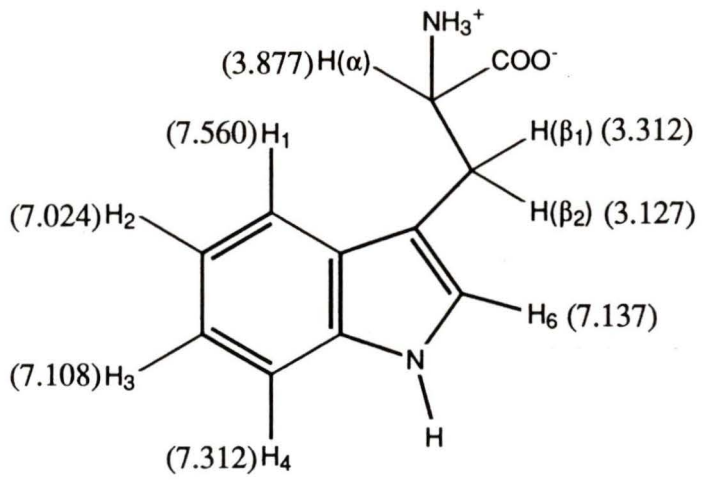


Figure 3.14: Proton NMR chemical shifts of free tryptophan in D_2O as assigned in reference 127.

Differences in the chemical shifts for tryptophan in the presence and absence of ß-CD and those for ß-CD in the presence and absence of tryptophan were observed. (**Figure 3.15a** and **3.15b**).

The following observations of the proton NMR of tryptophan in the presence of ß-CD are relevant: A). The change of chemical shift for Trp/β-CD is smaller than for Trp/α-CD[127] suggesting that tryptophan binds more strongly with α-CD than with ß-CD. B). The tryptophan protons that have biggest changes for their chemical shifts are: H_6, H_3 and H_2, and H(β). C). The different change of the proton chemical shifts between D- and L-tryptophan ß-CD complexes is small (**Table 3.3**).

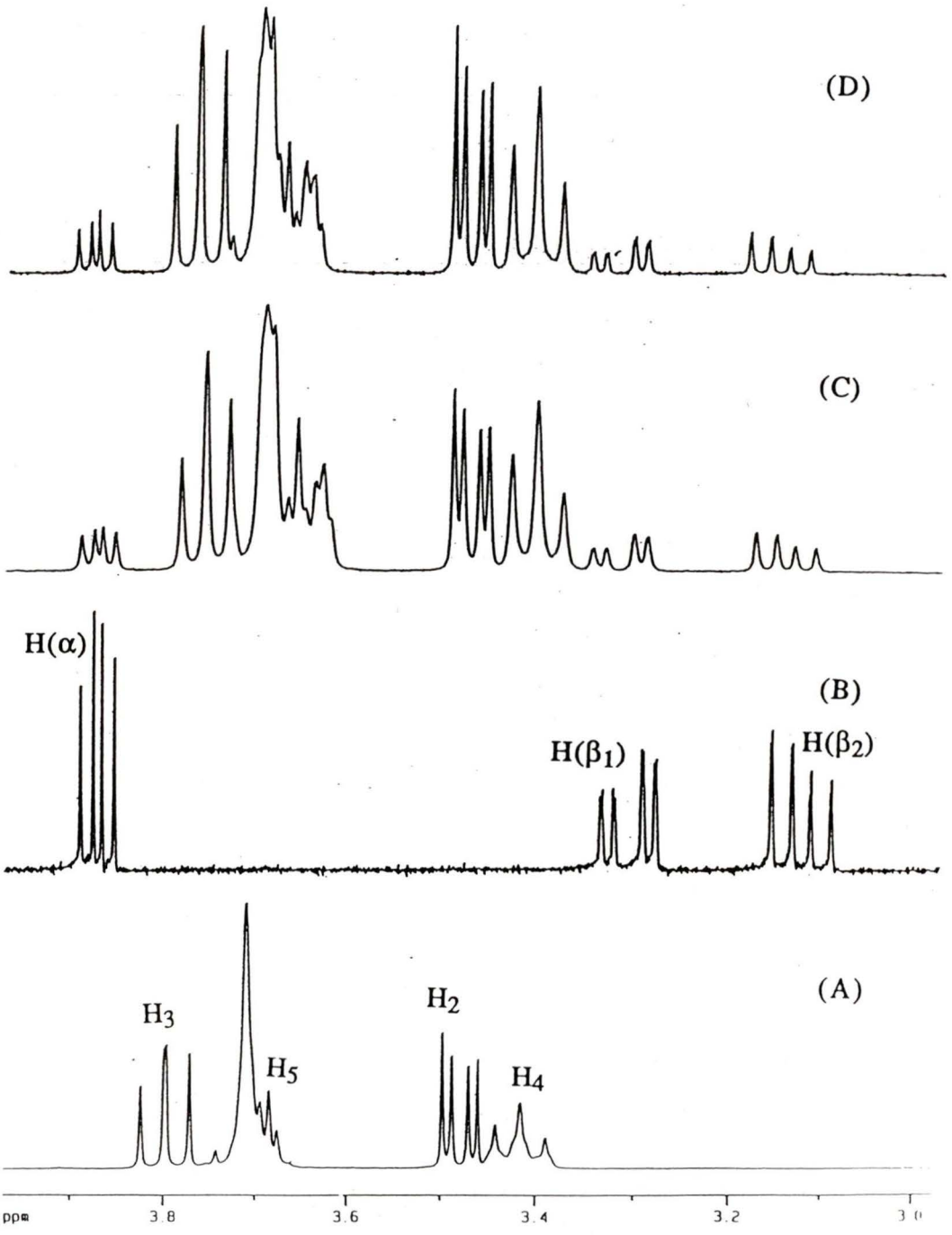


Figure 3.15a: Proton NMR spectra of tryptophan, ß-CD and their complex (aliphatic region). (A). ß-CD only. (B). tryptophan only. (C). L-tryptophan (1.0×10^{-2} M)/ ß-CD(1.0×10^{-2} M). (D). D-tryptophan (1.0×10^{-2} M)/ß-CD (1.0×10^{-2} M).

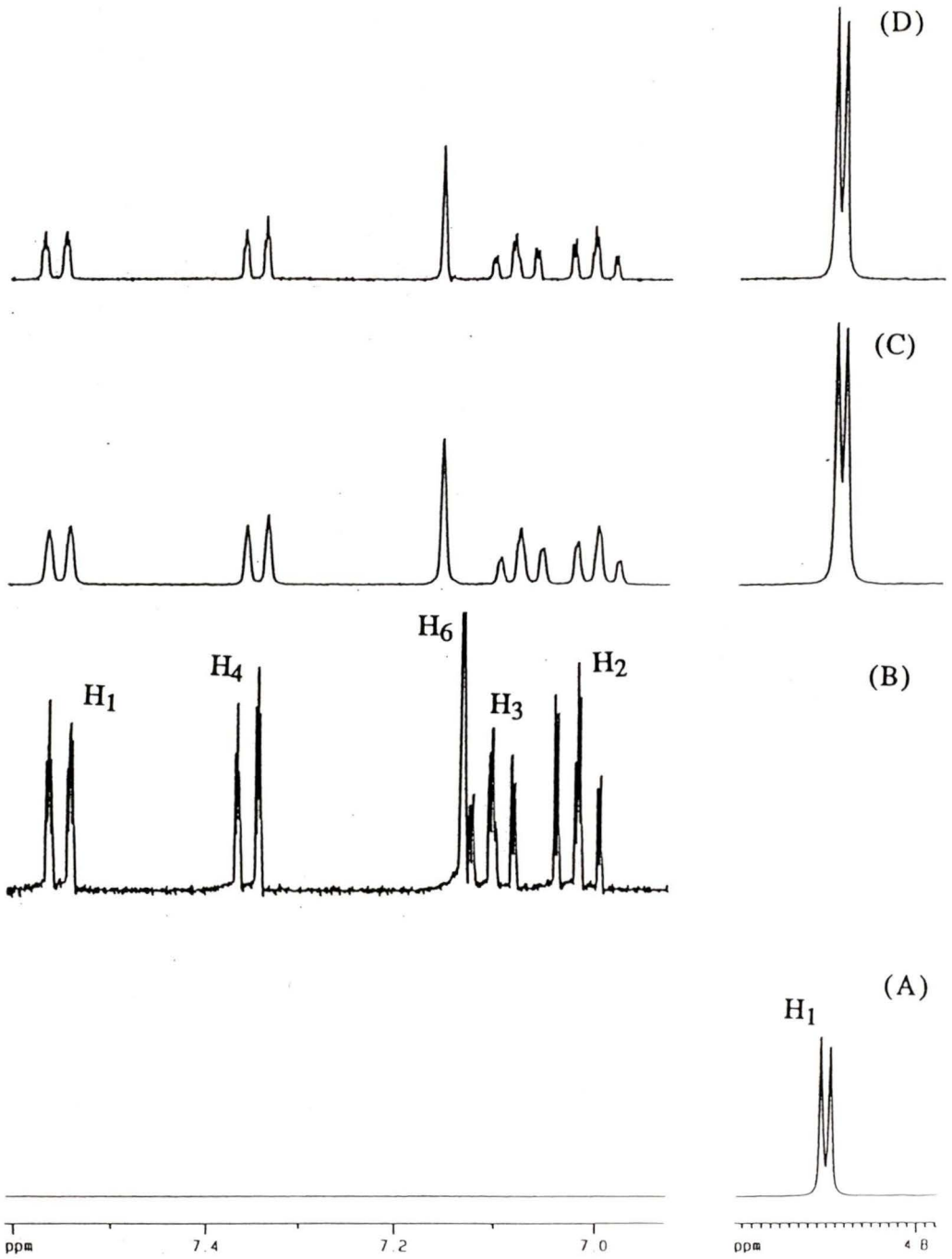


Figure 3.15b: Proton NMR spectra of tryptophan, ß-CD and their complex (aromatic region). (A). ß-CD only. (B). tryptophan only. (C). L-tryptophan (1.0×10^{-2} M)/ ß-CD(1.0×10^{-2} M). (D). D-tryptophan (1.0×10^{-2} M)/ß-CD (1.0×10^{-2} M).

Table 3.3: Difference of proton chemical shifts $\Delta(\Delta\delta)$ of tryptophan in the absence and presence of ß-CD.[a]

Proton	$\delta(F)-\delta(D)$ $(\Delta\delta(D))$	$\delta(F)-\delta(L)$ $(\Delta\delta(L))$	$\Delta(\Delta\delta)$ $(\Delta\delta(D)-\Delta\delta(L))$
H_1	-(1.85 ± 0.02)	-(0.58 ± 0.01)	-(1.27 ±0.02)
$H(\beta_1)$	-(4.1 ± 0.1)	-3.6	-(0.4±0.1)
$H(\beta_2)$	-9.92	-(6.9±0.1)	-(3.0±0.1)

[a]: Chemical shifts are expressed in Hertz. $\delta(F)$, $\delta(D)$ and $\delta(L)$ are the chemical shifts for free tryptophan and D- and L-tryptophan in the presence of ß-CD respectively. The concentrations of tryptophan and ß-CD were 1.0×10^{-2} M. All data are the averages of two determinations.

Complexation of tryptophan also affected the chemical shifts of the ß-CD protons. The H_3 proton on the ß-CD ring has the biggest change in chemical shift. Unfortunately, no NOE or NOSEY signals were observed for the complex. This probably reflects the weak complexation of tryptophan to ß-CD and the local mobility of tryptophan within the cavity.[53]

3.4.2. Determination of association constants of tryptophan with ß-CD

The Benesi-Hildebrand approach could not be used to study the complex formation between tryptophan and ß-CD as no measurable changes were observed in the circular dichroism, UV-Vis or fluorescence spectra of tryptophan in the presence of ß-CD.

Competitive binding is an alternative method to measure these small association constants.[23,121] An environment (polarity) sensitive probe whose equilibrium constant with ß-CD can be determined accurately is employed. If

tryptophan competitively binds to CD, it will displace the probe from the cavity. Consequently, the fluorescence intensity of the probe will change. 1-Anilino-8-naphthalensulfonate (ANS) is such a probe.[130,134,135] It can form a twisted intramolecular charge transfer (TICT) species in the excited state. The TICT has a fluorescence quantum yield that is very dependent on the solvent polarity.[136,137] The following equilibria were involved in the competitive binding experiment:

$$\text{Trp} + \text{ß-CD} \overset{K_1}{\rightleftharpoons} \text{Trp-CD} \qquad (3.1)$$

$$\text{ANS} + \text{ß-CD} \overset{K_2}{\rightleftharpoons} \text{ANS-CD} \qquad (3.2)$$

When [Trp] » [CD] » [ANS] and at constant concentrations of CD and ANS, the changes of the ANS fluorescence intensity with changes of tryptophan concentrations follows:

$$\Delta I = \frac{K_1 \Delta\Phi [ANS]_t [CD]_t}{1 + K_2 [Trp]_t + K_1 [CD]_t} \qquad (3.3)$$

where ΔI is the ANS fluorescence intensity change at a particular tryptophan concentration; $\Delta\Phi$ is the difference in the fluorescence quantum yield between bound and free ANS. $[ANS]_t$, $[CD]_t$ are total concentrations of ANS and ß-CD, respectively.

The Benesi-Hildebrand approach was employed to determine K_2 ((77 ± 4) M^{-1} at 20°C pH 6.7 phosphate buffer 0.1 M) (**Figure 3.16**). This value agrees

reasonably well with previous literature determinations, 85 M^{-1}, (25 °C)[138] and 58 M^{-1} (sodium salt, 25 °C pH 8.9 borate buffer).[139]

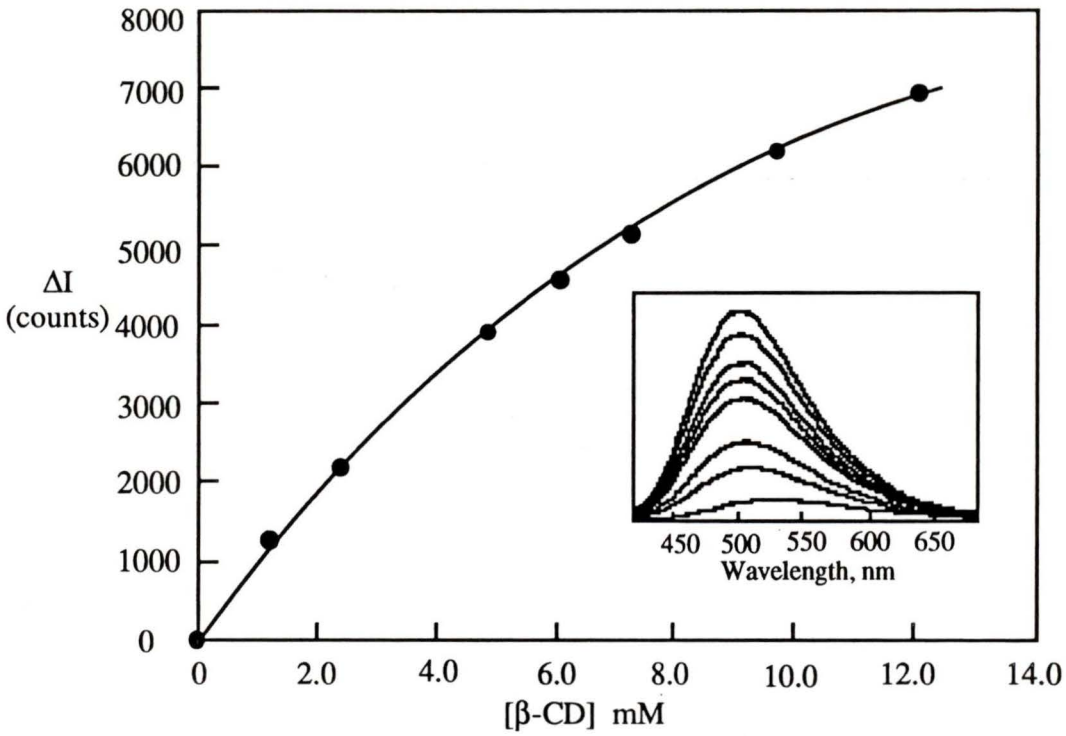


Figure 3.16: Changes of fluorescence intensity of ANS in the presence of ß-CD. Intensities were integrated between 470 nm and 570 nm. The inset shows the ANS fluorescence spectra in the presence of β-CD. The β-CD concentration increases from the bottom (0 M) to the top (1.2×10^{-2} M).

The association constant between L-tryptophan and ß-CD was determined by using Eq. (3.3). The K_1 value obtained ((10 ± 1) M^{-1} at 20 °C pH 6.7) agrees with one of the values reported previously.[121] The impurity emission of tryptophan prevented the acquisition of precise data for D-tryptophan.

3.5. Discussion

The mode of pyrene and tryptophan inclusion inside the CD cavity and the mechanism for interaction are important to understand the chiral discrimination observed for the quenching process. CPK models[111] and molecular modeling[112] of CD pyrene complexes showed that pyrene does not completely fit within the β-CD cavity. The sizes of pyrene and ß-CD are shown in **Figure 3.17**. It is for this reason that at high CD concentrations a complex with a 1:2 pyrene:ß-CD stoichiometry is formed.

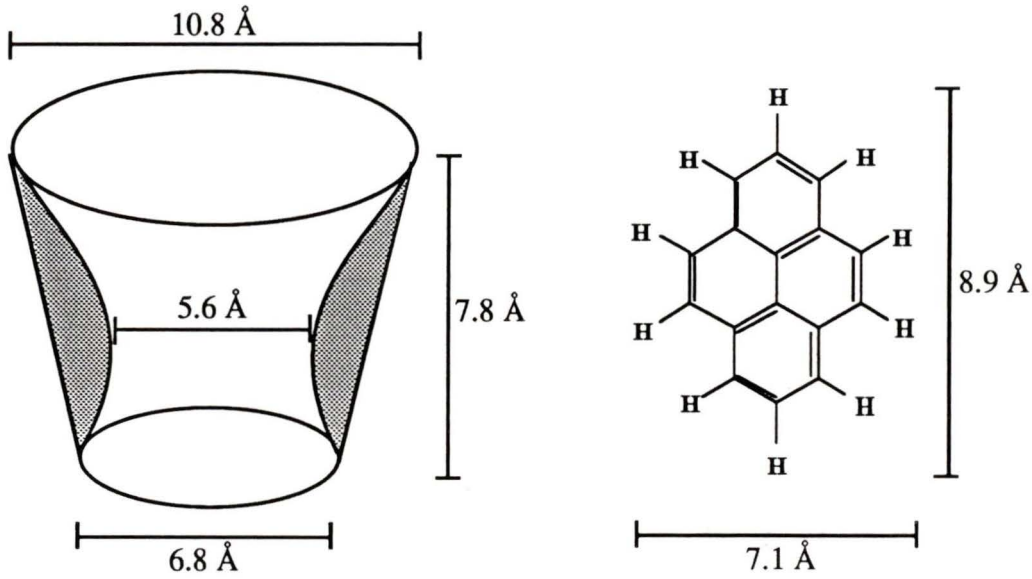


Figure 3.17: Estimated sizes of ß-CD[58] and pyrene.

One possibility for the quenching of complexed pyrene by tryptophan is a complex in which the tryptophan is included inside the CD cavity and pyrene is capping one of the entrances. This mode of interaction was suggested for the static quenching of pyrene/β-CD by amines.[118] This possibility can be excluded, since the low R(I/III) ratios for the complexes in the presence of alcohols or alkyl

sulfates as well as the protection from oxygen quenching indicate that pyrene was included within the CD cavity and the third component was protecting the excited state from interaction with molecules residing in the homogeneous solvent. Shielding effects by alcohols were previously observed for the quenching of triplet 1-bromonaphthalene by oxygen when included in glucosyl-β-CD[140] and the iodide quenching of 1-cyanonaphthalene in β-CD[141].

For static quenching to occur, the indole moiety of tryptophan must be located in the vicinity of pyrene within the same CD complex. Due to the dimensions of pyrene and tryptophan, the complex probably involves two CDs. Indeed, studies on the enhancement of the association constant between pyrene and β-CD by amino acids suggests a 1:2 stoichiometry for pyrene and CD at high CD concentrations (**Chapter 4**). The important point to emphasize is that regardless of the stoichiometry of the complex the chiral discrimination observed is due to the different complexation pattern of D- and L-tryptophan with β-CD.

Our NMR study showed weaker complexation of tryptophan with ß-CD than with α-CD[127]. This previous study suggested that the inclusion mode of both enantiomers is similar (indole moiety partially included inside the cavity and hydrogen bonding to hydroxyl groups) but there is a greater number of hydrogen bonds formed with the R-enantiomer (D-tryptophan). This is reflected in the stronger binding of D-tryptophan when compared to its enantiomer. In addition, the orientation of the indole moiety within the CD cavity is slightly different for the D- and L- enantiomers. Therefore, D- and L-tryptophan bind to CD not only with different strength but also in a different location within the cavity.

One possibility for the chiral discrimination observed in our quenching experiments is that the binding constants for the enantiomers are different. The

value for the L-tryptophan/ß-CD association constant that we determined ($(10 \pm 1)\ M^{-1}$) is close to the value determined at pH 8.9 and 12 °C.[122] Chiral discrimination was suggested in this latter study. The ratio between the association constants for D- and L-tryptophan was reported to be 1.1. Thus, differential binding could account for a ratio of 1.1 between the K_{sv} values for D- and L-tryptophan. This value is much lower than the discrimination observed. We do not believe that achiral molecules, such as alcohols and alkyl sulfates can affect the differential binding of the tryptophan enantiomers to β-CD to such an extent as to lead to quenching efficiency ratios ($K_{sv}(D)/K_{sv}(L)$) higher than 2. Thus, more subtle differences in the relative positioning of tryptophan with respect to pyrene may account for the discrimination observed.

The quenching mechanism of singlet pyrene by tryptophan has not been established, but charge transfer interactions have been proposed.[100] The indole moiety of tryptophan is responsible for the quenching as the quenching behavior of tryptophan is very similar to that of indole[100,101] and other amino acids do not quench singlet pyrene (**Chapter 4**). A charge transfer mechanism is also supported by the observation of exciplex emission for the interaction of pyrene derivatives with 1,2-dimethylindole.[102] For static quenching to occur through a charge transfer mechanism, the pyrene and indole moieties have to be in close proximity. In all cases, we observe quenching by both enantiomers indicating that the indole group is included into the CD cavity. One of the possible models for this static quenching is shown on **Figure 3.18**. Chiral discrimination could be due to the different relative location of the tryptophan indole moiety with respect to the pyrene π system. Evidence for different location of the indole ring within CD cavities has been previously described.[127]

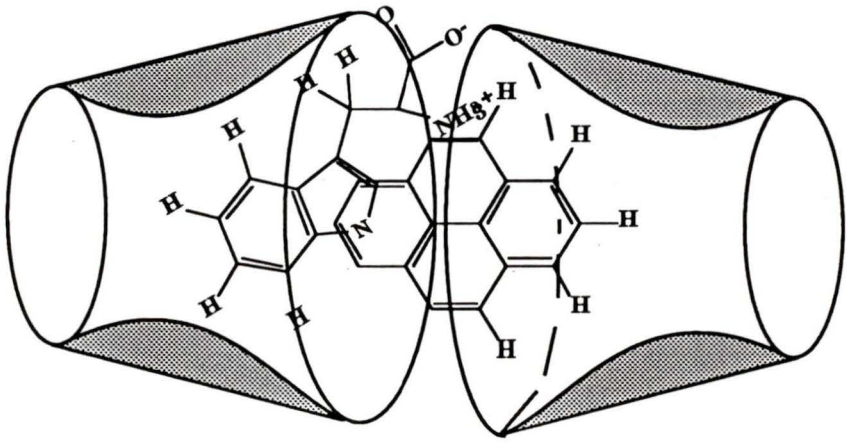


Figure 3.18: Proposed model for tryptophan quenching pyrene bound to ß-CD.

For all of the alcohols or alkyl sulfates except for C_6-SO_4 the quenching efficiency by D-tryptophan is the same within the experimental error (**Table 3.2**). The major contribution for the different ratios (**Table 3.2**) of the Stern-Volmer constants (K_{SV}(D-Trp)/K_{SV} (L-Trp)) is the change of the quenching efficiency by L-tryptophan for different alcohols or alkyl sulfates. This result indicates that the positioning of L-tryptophan, which binds CD more weakly, is affected more easily by the alcohols or alkyl sulfates than the positioning of D-tryptophan.

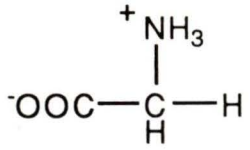
In summary, the chiral discrimination in the quenching by D- or L-tryptophan of excited singlet pyrene complexed to β-CD/alcohols or alkyl sulfates occurs through a static mechanism. This requires tryptophan to be in close proximity to pyrene. The major effect leading to chiral discrimination is probably the different relative positioning of the indole ring with respect to pyrene although differential binding efficiencies of tryptophan to CD may also play a minor role. The

structure of the added alcohols or alkyl sulfates has a marked effect on the quenching efficiency of L-tryptophan; the enantiomer that has a weaker complexation to the β-CD cavity. These results confirm that chiral environments can lead to chiral discrimination for a photophysical process where only one of the partners is chiral.

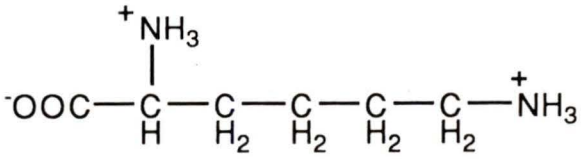
4. Ternary complex formation between pyrene/ß-cyclodextrin and amino acids

During the course of the study on tryptophan quenching of excited singlet pyrene complexed to ß-CD we noticed a change in the pyrene UV-Vis absorption spectra when the amino acid was added to the solution. This change, an increase in the absorption of the complexed pyrene, suggested the formation of a ternary complex for pyrene, ß-CD and tryptophan. To establish the generality of this phenomenon, we studied the effect of nine amino acids (**Figure 4.1**) on the pyrene ß-CD complexation.

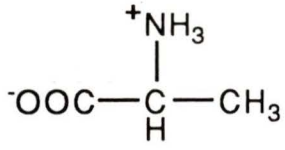
The amino acids employed can be divided into three groups. The first category contains glycine, alanine, valine, leucine and phenylalanine which have nonpolar side chains. The second category contains tryptophan and serine which have polar but uncharged side chains. The third category contains histidine and lysine which have polar charged side chains.



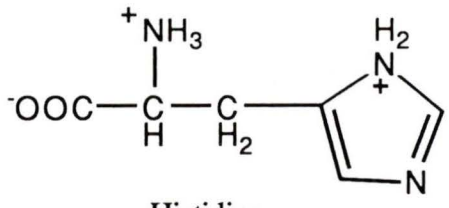
Glycine



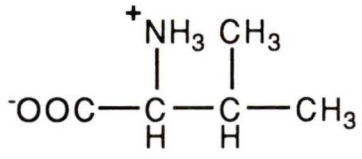
Lysine



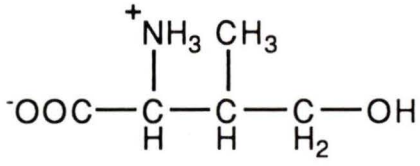
Alanine



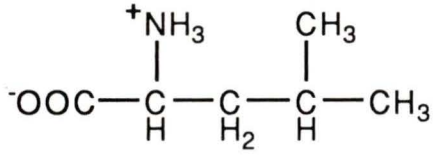
Histidine



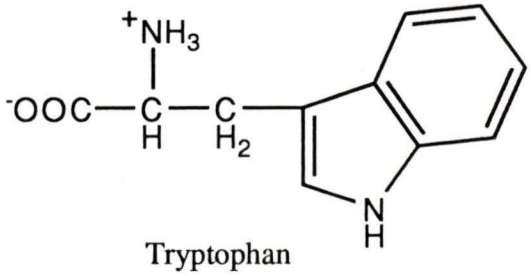
Valine



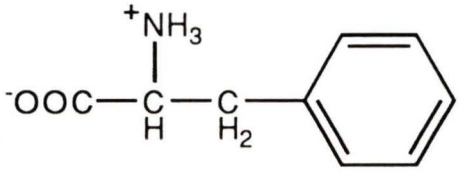
Serine



Leucine



Tryptophan



Phenylalanine

Figure 4.1: Structure of the amino acids employed in this study.

4.1. Quenching of pyrene by amino acids

The quenching of singlet pyrene by the amino acids listed in **Figure 4.1** was checked by steady-state and time-correlated fluorescence techniques. No quenching was detected at the concentrations measured (higher than the concentrations employed in the complexation study) for glycine, alanine, valine, leucine, serine, lysine and histidine. The quenching rate constant for phenylalanine was smaller than 2.5×10^6 $M^{-1}s^{-1}$. The quenching by tryptophan was reported in **Chapter 3**.

4.2. Spectroscopic evidence for ternary complex formation

Fluorescence spectroscopy. Several pyrene fluorescence spectra in the presence of 1.0×10^{-2} M ß-CD and 1.0×10^{-2} M amino acids are shown in **Figure 4.2**. Most amino acids tested (glycine, alanine, valine, serine, lysine and histidine) do not change the R(I/III) ratio for pyrene/ß-CD measured in the absence of amino acids (**Table 4.1**). However, for leucine and phenylalanine a marked decrease of the R(I/III) ratio was observed. These R(I/III) values are similar to those observed for pyrene/ß-CD in the presence of alcohols or alkyl sulfates (**Table 3.1**). The decrease in the R(I/III) value suggests the formation of ternary complexes.

Table 4.1: Effect of amino acids on the complex formation between pyrene and ß-CD.[a]

Amino Acid	R(I/III)	Spectroscopic Change		
		Fluorescence emission spectra	SPC decay trace	UV-Vis absorption spectra
none	0.99	—	—	—
glycine	0.96	no	no	no
alanine	0.97	no	no	no
valine	0.95	no	no	no
leucine	0.60	yes	yes	yes
phenylalanine	0.76	yes	yes	yes
serine	0.98	no	no	no
tryptophan	b	b	b	yes
lysine	0.97	no	no	no
histidine	0.96	no	no	no

[a] The concentrations of pyrene, ß-CD and amino acids were 5×10^{-7}, 1.0×10^{-2} and 1.0×10^{-2} M, respectively.
[b] These parameters can not be measured since tryptophan quenches the pyrene fluorescence.

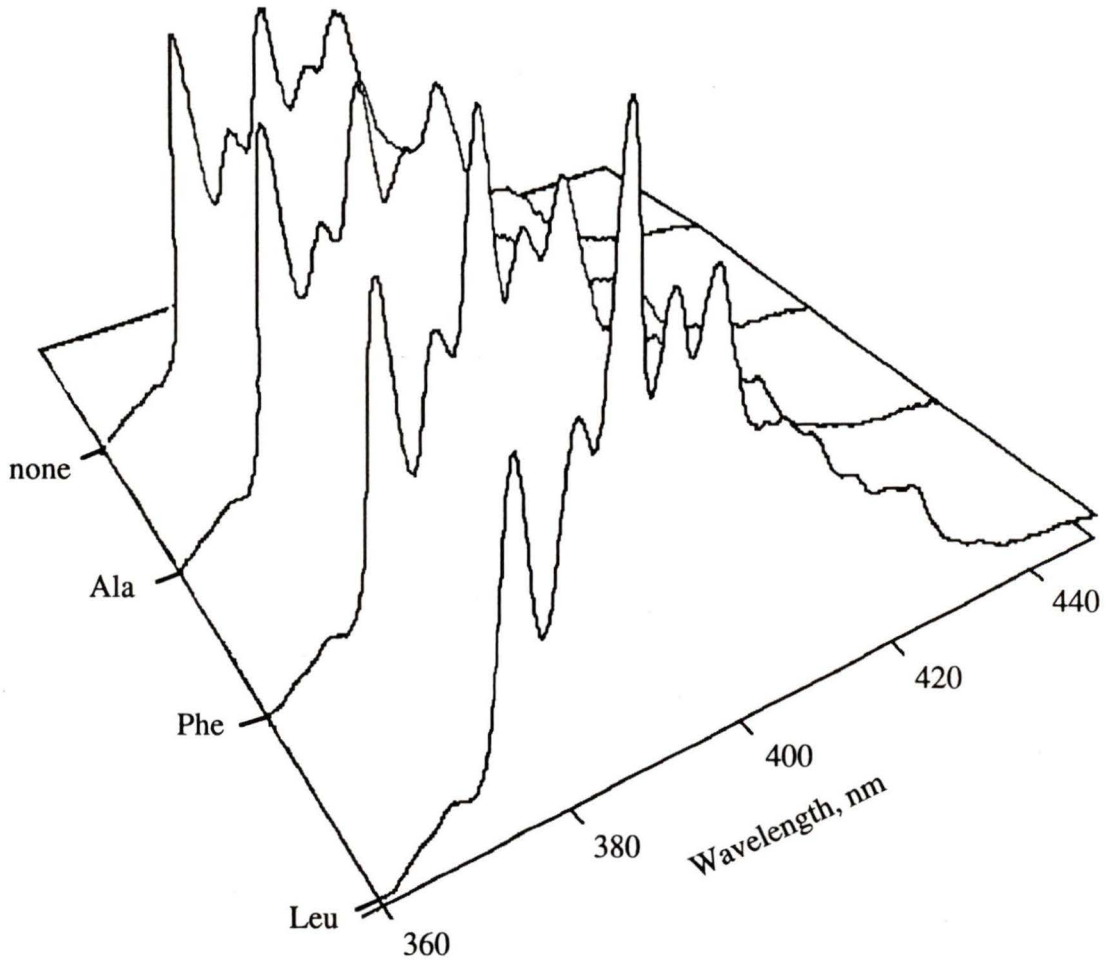


Figure 4.2: Fluorescence of pyrene (5×10^{-7} M) in the presence of β-CD (1.0×10^{-2} M) and amino acids (1.0×10^{-2} M).

Time-correlated SPC. The lifetime of pyrene complexed to ß-CD increases only in the presence of leucine and phenylalanine. These were the same amino acids for which a decrease of the R(I/III) ratio of pyrene was observed. Tryptophan quenches pyrene and ternary complex formation can not be tested by this methodology. In the presence of all other amino acids the fluorescence decay was similar to that observed just in the presence of β-CD.

UV-Vis spectroscopy. Free pyrene and pyrene complexed to β-CD had absorption maxima at 334 nm and 339 nm, respectively. In the presence of increasing concentrations of leucine, phenylalanine and tryptophan the absorption at 339 nm increased at the expense of the absorption at 334 nm (**Figure 4.3** and **Table 4.1**) indicating the formation of a ternary complex. None of the other amino acids led to any change of the pyrene absorption spectrum.

Absorption is an extremely valuable technique to study the ternary complex formation with amino acids such as tryptophan which quench *excited* singlet pyrene. Indeed, absorption was the only technique by which we could unequivocally establish the ternary complex formation in the presence of tryptophan.

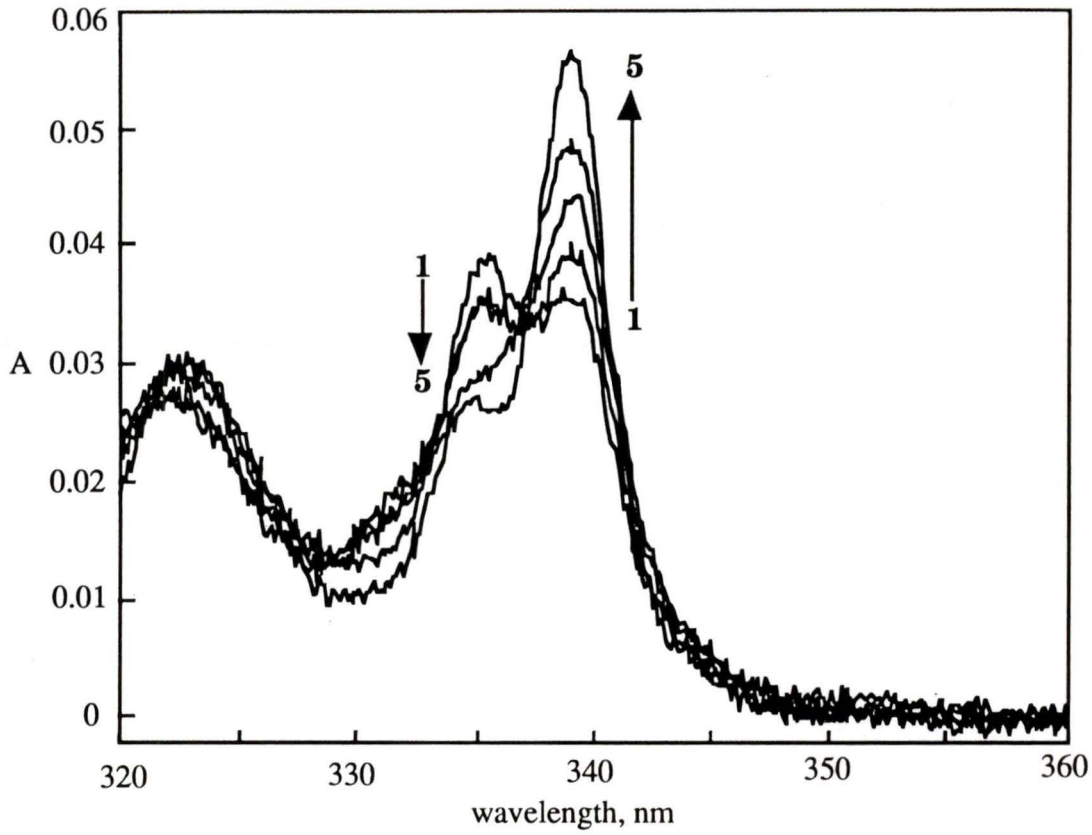


Figure 4.3: Absorbance of pyrene (5×10^{-7} M) in presence of ß-CD (1.3×10^{-2} M) and tryptophan (**1**: 0; **2**: 1.4×10^{-3}; **3**: 2.8×10^{-3}; **4**: 1.1×10^{-2} and **5**: 2.2×10^{-2} M).

In summary, leucine, phenylalanine and tryptophan form ternary complexes with pyrene and CD and enhance the binding of pyrene to ß-CD. To further characterize the ternary complex formation we chose leucine for detailed studies.

4.3. Complexation between leucine and ß-CD

The association between leucine and ß-CD was studied in order to establish how much of this complex was formed in the absence of pyrene. The competitive binding method was employed by using ANS as a probe molecule.

The experiments were performed at pH 6.7 and the concentrations for ANS and ß-CD were 1.4×10^{-5} and 1.1×10^{-3} M, respectively. The concentrations of leucine were varied between 8×10^{-2} M and 8.0×10^{-1} M. Only a very small decrease of fluorescence emission intensity was observed with the addition of leucine (**Figure 4.4**). The change of the integrated ANS fluorescence intensity (integration between 470 nm and 570 nm) with leucine concentration was fitted to Eq. (3.3) (**Figure 4.4**). The association constant for D- or L-leucine with ß-CD was estimated to be (1.5 ± 0.3) M^{-1}.

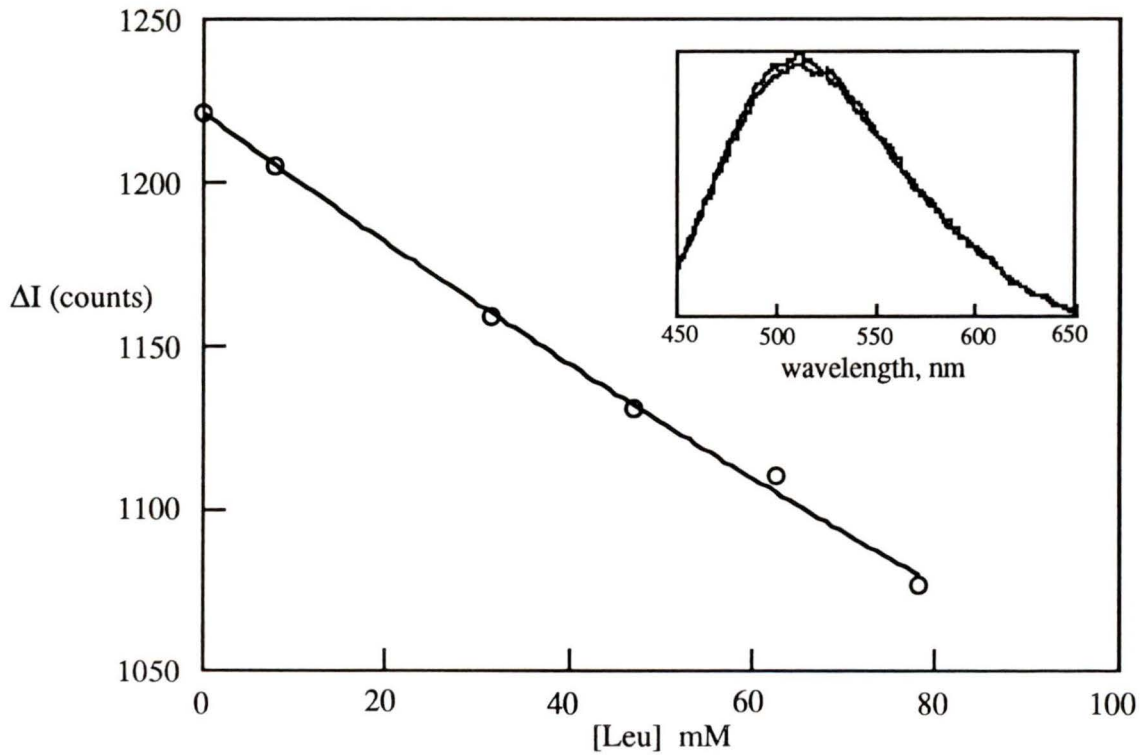


Figure 4.4: Changes of the fluorescence intensity of ANS (1.4×10^{-5} M) in the presence of ß-CD (1.1×10^{-3} M) at different D-leucine concentrations. Inset shows the ANS fluorescence spectra at leucine concentrations of 0 (top) and 7.8×10^{-1} M (bottom).

For the competition method to be applicable to estimate the equilibrium constant between leucine and β-CD we had to ensure that ANS and leucine did not form a ternary complex with the CD. As for pyrene, the photophysics of ANS is very dependent on the solvent polarity. The absorption maximum of ANS shifts to longer wavelengths when the solvent polarity increases.[130] The maximum of the ANS fluorescence spectrum shifts from 528 nm in water to approximately 500 nm in the presence of ß-CD ($\geq 1.0 \times 10^{-2}$ M). However, upon addition of leucine no further shift of the ANS spectrum was observed, suggesting that no ternary complex between ANS, leucine and β-CD is formed.

4.4. Determination of the pyrene/ß-CD stoichiometry in ternary complexes with amino acids

Previous reports for ternary complex formation suggested either a pyrene:ß-CD stoichiometry of 1:1[104] or 1:2.[114] To unravel which binding stoichiometries were important in the case of ternary complex formation with amino acids we studied the effect of temperature on the pyrene fluorescence spectra and lifetimes. The temperature was kept below 60 °C as CDs become unstable at higher temperatures.[41]

The effect of temperature on the fluorescence spectra of pyrene at low (1.3×10^{-3} M) and high (1.1×10^{-2} M) ß-CD concentrations in the presence of leucine (1×10^{-2} M) are shown in **Figure 4.5**. The overall fluorescence intensity dropped and the R(I/III) ratios increased with increasing temperatures. At high ß-CD concentrations (1.1×10^{-2} M) the intensity of peak I is independent on temperature, whereas in the presence of 1.3×10^{-3} M ß-CD the intensity of peak I decreases slightly. It is also very important to note that at high ß-CD

concentrations an isoemissive point was observed at 378 nm (Inset of **Figure 4.5B**), whereas no clear isoemissive point was observed at low ß-CD concentrations. This observation indicated that at 1×10^{-2} M ß-CD two pyrene species related by one equilibrium were present, but in the presence of 1×10^{-3} M ß-CD more than two pyrene species existed.

To be able to interpret the changes in the R(I/III) ratios for the ß-CD systems, we studied the temperature effect on this ratio for pyrene in water and cyclohexane. The later solvent was chosen to mimic the polarity within the CD cavity. The temperature effect on the R(I/III) ratio for pyrene in both solvents and in the presence of β-CD/leucine at low and high CD concentrations is shown in **Figure 4.6**.

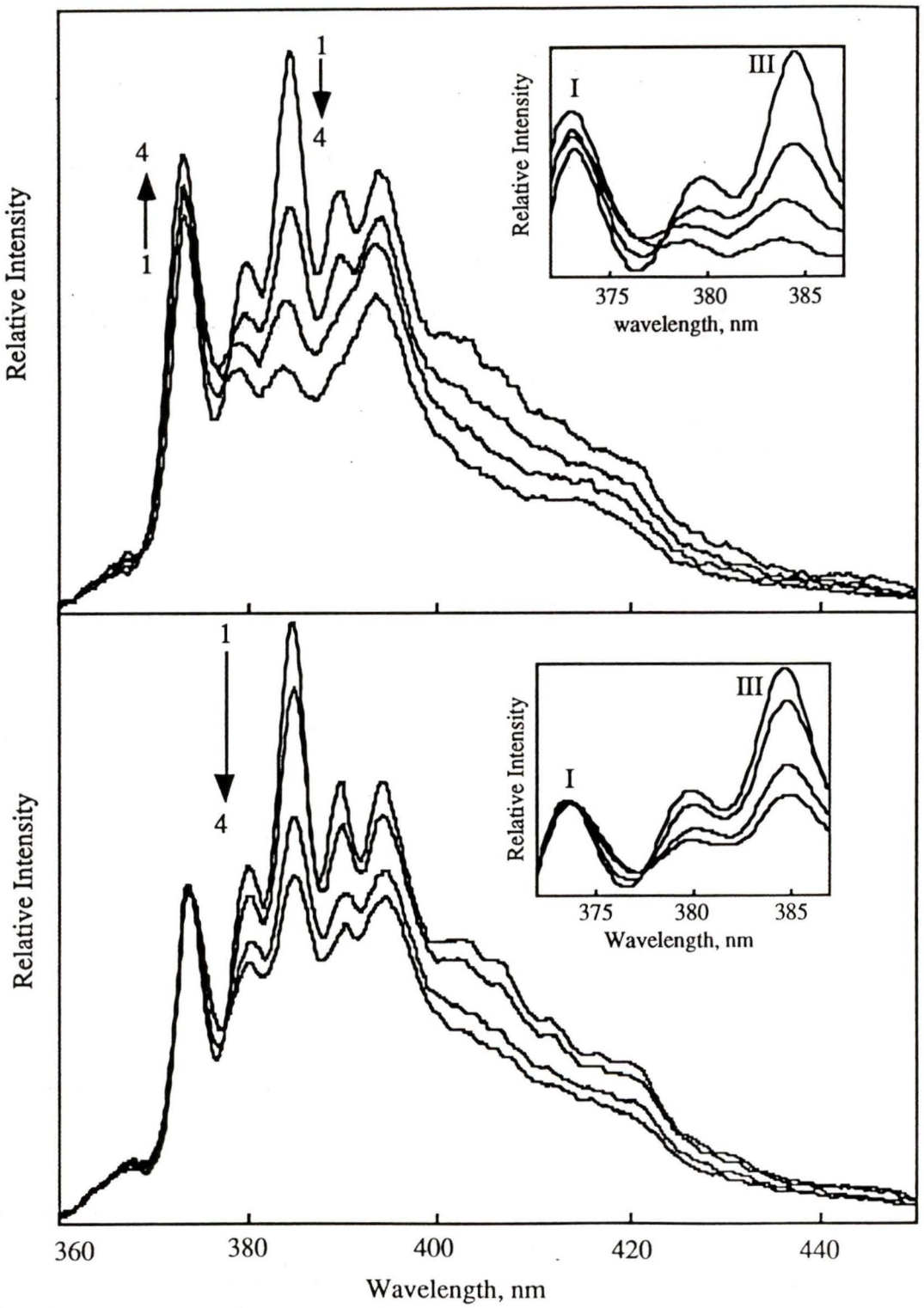


Figure 4.5: Temperature dependence on the fluorescence spectra of pyrene (5×10^{-7} M) in the presence of ß-CD (Plot A, 1.3×10^{-3} M; Plot B 1.1×10^{-2} M) and leucine (1×10^{-2} M). The temperatures are: (A) **1**: 7 °C; **2**: 20 °C; **3**: 33 °C; **4**: 51 °C. (B) **1**: 8 °C; **2**: 26 °C; **3**: 44 °C; **4**: 50 °C.

No change was observed for R(I/III) ratio in cyclohexane whereas in water the R(I/III) ratio decreased.

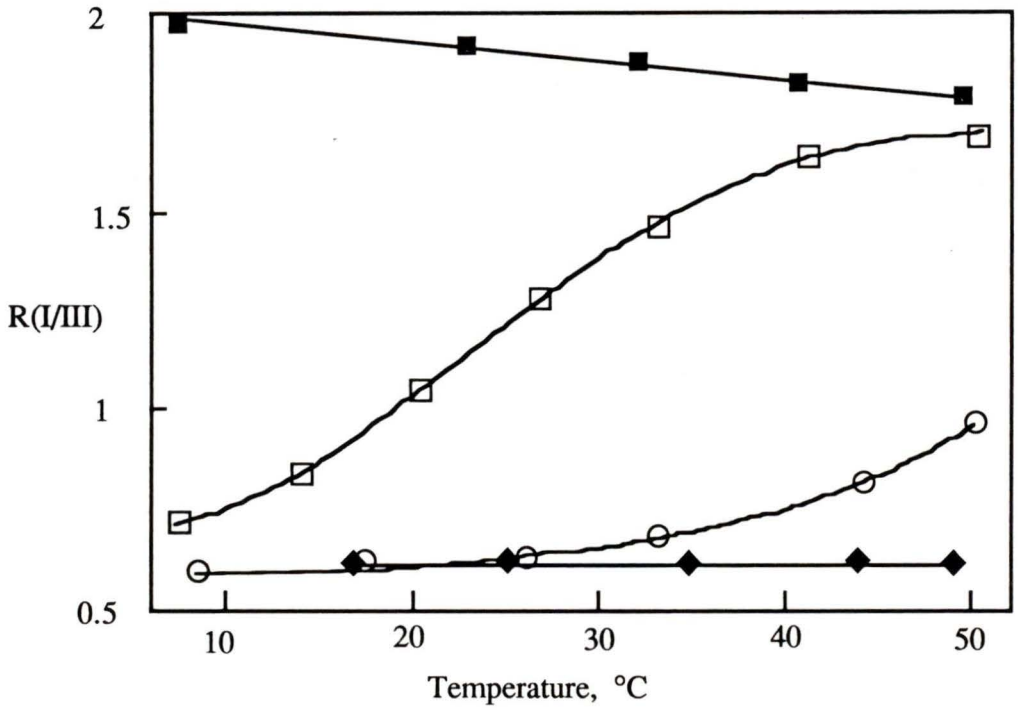


Figure 4.6: Temperature dependence of pyrene R(I/III) ratios in water (■), cyclohexane (◆), and in the presence of leucine (1×10^{-2} M) with ß-CD ((○) 1.1×10^{-2} M; (□) 1.3×10^{-3} M).

In the presence of ß-CD and leucine the R(I/III) ratio corresponds to the combined value for all pyrene species. At 1×10^{-2} M ß-CD only two pyrene species are detected (vide infra) and we assigned these to complexes with pyrene:ß-CD stoichiometries of 1:1 and 1:2. The equilibrium was displaced towards the complex with 1:1 stoichiometry with increasing temperatures. At low temperatures, the 1:2 complex predominated and the measured R(I/III) values (0.57 at 8 °C and 0.59 at 17 °C) showed that pyrene in the 1:2 complex was in a

non polar environment. At the low ß-CD concentration (1×10^{-3} M) the R(I/III) ratio increased with temperature to a value close to that for pyrene in water. Thus, at high temperatures and 1×10^{-3} M ß-CD most of the pyrene was free in solution. If pyrene would only complex with ß-CD in the presence of leucine with a 1:2 stoichiometry, an isoemissive point should also have been observed for the temperature dependence when the CD concentration was 1×10^{-3} M. No clear isoemissive point was observed at the lower β-CD concentrations and at high temperatures pyrene was predominantly in water. Thus, at least two other pyrene species should be present and we assigned these to pyrene complexes with 1:1 and 1:2 pyrene:β-CD stoichiometries. This assignment is supported by the SPC data presented below.

Previous work[112] established that for the pyrene/ß-CD binary system, only two distinguishable lifetimes could be observed although free pyrene, pyrene/ß-CD with 1:1 and 1:2 stoichiometries were present. The similar lifetimes of free pyrene and pyrene/ß-CD 1:1 complex made it impossible to distinguish these two species spectroscopically.

We noticed that in the presence of leucine the fluorescence decay data at 1×10^{-3} M ß-CD were fitted best by the sum of three exponentials. At higher β-CD concentrations (1×10^{-2} M) the data were satisfactorily fitted to a double exponential (**Figure 4.7**) and no improvement on the quality of the fit was observed when the data were fitted to a triple exponential (**Table 4.2**). Indeed, for the sum of three exponentials two of the lifetimes had the same value indicating that the decay corresponds to a double exponential.

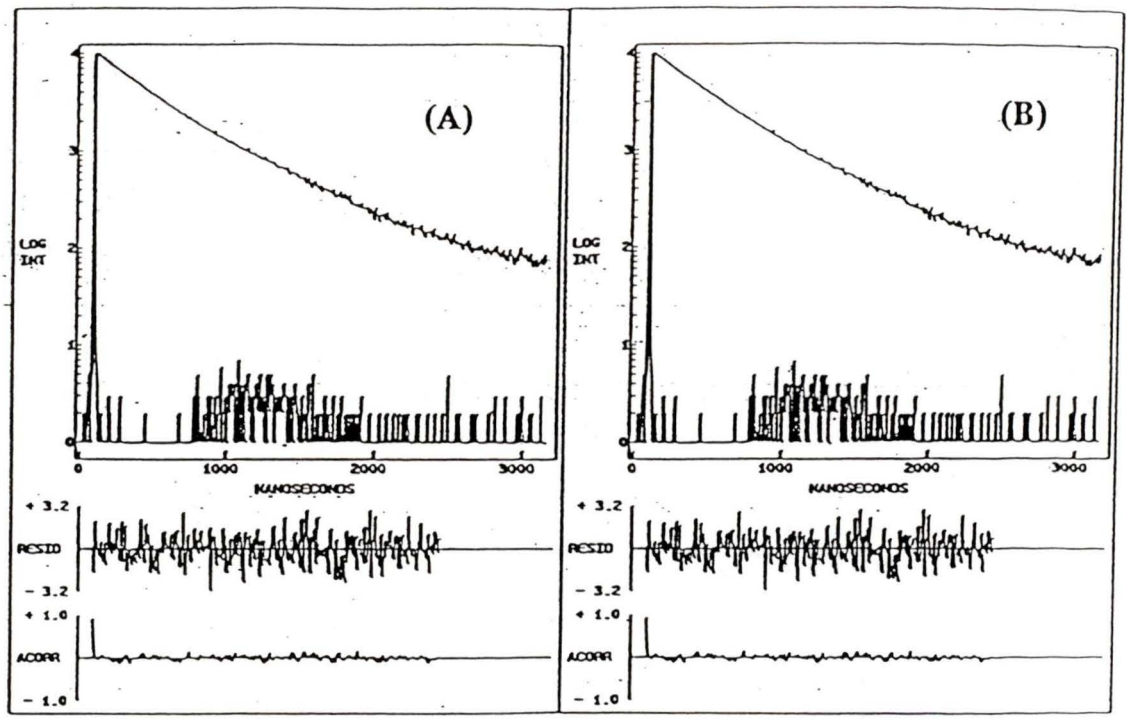


Figure 4.7: SPC decay trace and fit for pyrene (5×10^{-7} M) fluorescence in the presence of ß-CD (1.0×10^{-2} M) and leucine (1.0×10^{-2} M). The decay trace was fitted to the sum of two (A) and three (B) exponentials.

The two lifetimes determined at 1×10^{-2} M ß-CD were 191 and 449 ns. The longer lifetime was similar to the lifetime (460 ns) obtained with 0.14 M leucine when the pyrene fluorescence decay was mono exponential. The short lifetime was much longer than that for pyrene in water (127 ns). Thus, none of the two species correspond to free pyrene. We assigned the lifetimes with 190 and 450 ns to pyrene fluorescence when complexed to β-CD with 1:1 and 1:2 stoichiometries, respectively.

The pyrene fluorescence decay in the presence of 1×10^{-3} M β-CD and 1×10^{-2} M leucine was fitted to the sum of three exponentials and the recovered lifetimes were (116 ± 23) ns, (170 ± 26) ns and (457 ± 33) ns. The errors for these lifetimes were estimated from fits to three different experiments and by

employing different initial guesses for each experiment. The relatively large errors suggest that the data set does not contain enough information for a more precise determination of the lifetimes when all 6 parameters (3 pre-exponential factors and 3 lifetimes) for a fit to the sum of three exponentials are floated. Nevertheless, the lifetimes recovered are close to the lifetime for pyrene in water and the two lifetimes obtained at higher β-CD concentrations.

Table 4.2: Data analysis of the fluorescence decays for pyrene/ß-CD/leucine ternary complexes.[a]

Parameter	Number of exponentials		
	single	double	triple
τ_1 (ns)	413	449	449
τ_2 (ns)	—	—	448
τ_3 (ns)	—	191	191
A_1	1	0.82	0.68
A_2	—	—	0.14
A_3	—	0.18	0.18
χ^2	1.617	1.059	1.064
DW parameter	0.787	1.912	1.912
Z value	-5.154	-1.345	-1.345
Residue	bad	good	good

[a] The pyrene, ß-CD and leucine concentrations were 5×10^{-7}, 1.0×10^{-2} and 1.0×10^{-2} M, respectively. Excitation and emission wavelengths were set to 337 nm and 384 nm. T = 20 °C. The fitting range was from channel 17 to channel 400 with a total channel number of 512.

SPC data were also collected at 48 °C. In the presence of 1×10^{-3} M ß-CD the decay was single exponential and the lifetime was the same as for pyrene in water. Thus, at 48 °C no pyrene is complexed to β-CD as already suggested by the high R(I/III) values (**Figure 4.6**). At high ß-CD concentrations the fluorescence decay was fitted satisfactorily to the sum of three exponentials suggesting that pyrene in water and both complexes with β-CD were present.

Table 4.3: Temperature dependence of pyrene lifetimes in different systems.[a]

Temp (°C)	Pyrene only	Pyrene in presence of leucine		
		[CD] = 0 mM	[CD] = 1 mM	[CD] = 10 mM
20	128 (1)	127 (1)	116 ± 23 (0.44±0.18)[c]	
			170 ± 26 (0.39±0.16)[c]	191 (0.18)
			457 ± 33 (0.17±0.04)[c]	449 (0.82)
48	112 (1)	b	118 (1)	108 (0.24)
				194 (0.43)
				392 (0.33)

[a] Lifetimes in nanosecond. Excitation and emission wavelengths were set to 337 nm and 384 nm, respectively. The number in parenthesis are pre-exponential factors of the fits. Pyrene and leucine concentrations were 5×10^{-7}, and 1.0×10^{-2} M, respectively.
[b] Not measured.
[c] Three determinations.

The pyrene fluorescence decay in the presence of 1.0×10^{-2} M ß-CD and 1.0×10^{-2} M D- and L-leucine was determined to investigate if any chiral discrimination occurred for the formation of ternary complexes. No chiral discrimination was observed (**Table 4.4**).

Table 4.4: Comparison of pyrene fluorescence decay parameters in the presence of ß-CD and D- or L-leucine.[a]

Leucine	τ_1 (ns)	A_1	τ_2 (ns)	A_2
D-	191	0.18_2	449	0.81_8
L-	196	0.18_5	447	0.81_5

[a] Excitation and emission wavelengths were set to 337 nm and 384 nm, respectively. τ and A are the fluorescence lifetimes and pre-exponential factors recovered from the fit to the experimental data. [Pyrene]=5×10^{-7} M.

4.5. Determination of conditional association constants for ternary complex formation

To evaluate the efficiency of ternary complex formation in the presence of amino acids we determined conditional equilibrium constants for pyrene/ß-CD in the presence of 1×10^{-2} M leucine. The equilibrium constants for 1:1 (K_1) and 1:2 (K_2) stoichiometries could be determined from single photon counting data as all three species had different lifetimes. Thus, the sequential binding constants can be calculated from:

$$K_1 = \frac{A_{PyCD}}{A_{Py}} \frac{\varepsilon_{Py}}{\varepsilon_{PyCD}} \frac{k_{Py}}{k_{PyCD}} \frac{1}{[CD]_t} \qquad (4.1)$$

$$K_2 = \frac{A_{CDPyCD}}{A_{PyCD}} \frac{\varepsilon_{PyCD}}{\varepsilon_{CDPyCD}} \frac{k_{PyCD}}{k_{CDPyCD}} \frac{1}{[CD]_t} \qquad (4.2)$$

where A, k and ε are pre-exponential factors, radiative rate constants and molar absorption coefficients, respectively. The subscript of Py, PyCD and CDPyCD represent free pyrene, pyrene/ß-CD complexes with 1:1 and 1:2 stoichiometries, respectively.

The samples were excited at 337 nm, the isosbestic point. Thus, the ratio for the molar absorption coefficients was equal to unity. We measured the integrated fluorescence intensity of free pyrene in water and pyrene in the presence of leucine (9.5×10^{-2} M) and ß-CD (1.3×10^{-2} M) for deaerated samples. For the latter sample all the pyrene was complexed to β-CD either with a 1:1 or a 1:2 stoichiometry. Within 10%, the same emission quantum yield was observed. It is important to measure the quantum yields in deaerated samples since pyrene free in the aqueous solution is efficiently quenched by oxygen whereas no quenching occurs for complexed pyrene. The fluorescence quantum yield and radiative lifetimes are related by Eq. (1.5). As the emission quantum yields were equal, the ratio of radiative rate constants was equal to the inverse ratio of the lifetimes in deaerated solutions. The lifetime values employed were 198, 190 and 450 ns for free pyrene and complexed pyrene with 1:1 and 1:2 stoichiometries in deareated solutions, respectively.

As mentioned above, a precise determination of the lifetimes and pre-exponential factors was not possible when all parameters were floated for a fit to the sum of three exponentials. To obtain precise values for the pre-exponential factors we fixed the lifetimes for free pyrene and pyrene/ß-CD with 1:1 and 1:2 stoichiometries at 127, 190 and 450 ns, respectively. The value for pyrene in water was determined independently, since leucine does not quench the pyrene fluorescence. The lifetime values for complexed pyrene with 1:1 and 1:2

stoichiometries were those determined at high β-CD concentrations when no free pyrene was present.

SPC data were obtained for samples with pyrene, ß-CD and leucine concentrations of 5×10^{-7} M, 1.0×10^{-3} M and 1.0×10^{-2} M, respectively. The conditional association constants calculated from Eq. (4.1) and (4.2) were $(3.8 \pm 1.2) \times 10^{2}$ M^{-1} and $(1.8 \pm 0.2) \times 10^{3}$ M^{-1}, respectively. This gave an overall association constant ($K_{12} = K_1K_2$) of $(6.8 \pm 2.3) \times 10^{5}$ M^{-2}.

In the case of phenylalanine, the lifetime of pyrene with ß-CD with a 1:1 stoichiometry was similar to that for free pyrene. Therefore, we could not employ SPC to determine the conditional association constants. SPC can also not be employed in the case of tryptophan due to the quenching of pyrene fluorescence by this amino acid. For this reason we were not able to determine the individual equilibrium constants for the sequential association process. However, in order to compare our data with those in the literature when alcohols were employed as ternary complexation agents, we determined overall conditional equilibrium constants (K_{12}) by assuming that pyrene is mostly associated to β-CD with a 1:2 stoichiometry. This was the same assumption employed for the work with alcohols.[114] The enhancement of the pyrene absorption at 339 nm or emission intensity at 385 nm with increasing concentrations of β-CD at constant amino acid concentrations was measured. The values for K_{12} were determined from the fit of the experimental data to Eq. (1.19) for n=2. The values for K_{12} determined by different techniques are shown in **Table 4.5**. The values are considered to be the same within the experimental errors.

Table 4.5: Conditional equilibrium constants (K_{12}) of pyrene/ß-CD in the presence and absence of amino acids (1.0×10^{-2} M).

Determination method	K_{12} (10^5 M^{-2})[a]			
	none	leucine	phenylalanine	tryptophan
UV-Vis absorbance	0.64 ± 0.03 (1)	4.9 ± 2.0 (3)	3.2 ± 0.2 (1)	11 ± 1 (1)
Fluorescence emission	0.47 ± 0.10 (2)	4.3 ± 0.9 (2)	3.8 ± 0.3 (1)	b
Fluorescence lifetime	b	6.8 ± 2.3 (3)	b	b

[a] Number in parentheses indicate the number of independent experiments performed.
[b] Method not applicable.

Warner et al. [114] determined overall conditional equilibrium constants for the formation of ternary complexes with 1:2 pyrene:β-CD stoichiometry for several alcohols. The alcohol concentration employed was 1% v/v, which corresponds to approximately 0.1 M. To compare their data with ours we calculated conditional equilibrium constants at alcohol concentrations of 1×10^{-2} M (**Table 4.6**). For most alcohols a larger enhancement of the pyrene/ß-CD complexation was observed for the ternary complex than in the case of amino acids.

Table 4.6: Estimated conditional association constants (K_{12}) of pyrene/ß-CD in the presence of alcohols (1×10^{-2} M).[a]

Alcohol	K_{12} (10^5 M^{-2})
ethanol	0.086
benzyl alcohol	2.5
1-pentanol	8.1
1-propanol	9.3
phenol	13
cyclohexanol	14
1-butanol	15
2-propanol	17
tert-butanol	51
cyclopentanol	97

[a] Original data were determined for alcohol concentrations of 1% v/v.[114] Molecular weights and densities were obtained from reference 142.

4.6. Discussion

Ternary complexation involving CDs have been described for several cases.[104,105,108,114,118,143,144] For some guest molecules, such as pyrene the addition of ternary complexation agents enhances the equilibrium constant of the guest with CDs.[104,105,114] For other molecules such as naphthalene, azulene and acridine the complexation with a ternary complexation agent decreases the association constant of the guest molecule with CD.[105,145,146]

Ternary complexation agents that were shown to enhance the association of pyrene with β-CD include alcohols, alkyl sulfates, alkyl ammonium chlorides

and amines. The main feature of these molecules is that they have a hydrophobic moiety and contain atoms that can be hydrogen bonded to the hydroxyl groups of CDs. Previous studies also determined that the enhancement effect was dependent on the structure of the alcohols[104,114] or alkyl sulfates.[105] In the case of alcohols the authors[114] suggested that the stability of the ternary complex is directly related to the geometry and volume of the alcohol molecule. Higher stability is achieved when a bigger space of the void volume within the CD cavity that is unoccupied by pyrene is filled by the alcohol. In the case of alkyl sulfates as ternary complexation agents the stability of the complex decreases with an increase of the alkyl chain length. This observation was again explained in terms of the "space-filling" model. Longer chains are too big to accommodate the alkyl sulfate and pyrene within the same cavity.

The binding of amino acids to CDs has been described in several publications.[120-122] However, the use of amino acids as ternary complexation agents had not been previously reported. Some amino acids have the structural properties necessary for ternary complex formation, i.e. hydrophobic moieties and hydrogen bonding atoms. Our experiments established that there is a strong correlation between the hydrophobicity of the amino acid side chain and their ability to form ternary complexes. Amino acids with charged side chains do not form ternary complexes. This is the case for histidine which has a side chain with approximately the same size as phenylalanine. No enhancement of the pyrene complexation to β-CD was observed even for amino acids with long and charged side chains such as lysine, which could thread through the β-CD cavity. This suggests that the amino acid binds to the entrance opposite to that occupied by pyrene.

The amino acids that form ternary complexes have either polar side chains (category II) or nonpolar hydrophobic side chains (category I). But even within these categories there is a strict requirement as to the structure of the amino acid side chain. Amino acids with small side chains, such as glycine, alanine and valine do not form ternary complexes. However, it is surprising that valine and leucine have such a different behavior. The structural difference between these amino acids is the additional methylene group in leucine. This observation shows that a strict stereochemical requirement exists for ternary complex formation in the presence of amino acids. The general structure for amino acids to be able to act as ternary complexation agents is shown in **Figure 4.8**. Amino acids will only function as ternary complexation agents when the side chain contains more than 3 carbons, the side chain is uncharged and the γ-carbon is a secondary carbon.

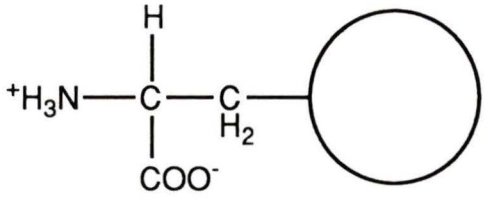


Figure 4.8: Proposed general structure of amino acid that can enhance the complexation between pyrene and ß-CD.

Amino acids form ternary complexes with conditional equilibrium constants that are smaller than those observed for the enhancement in the presence of alcohols. However, the structural requirements for amino acids to act as ternary complexation molecules are stricter than those observed for alcohols. For example, valine and alanine which have similar chain lengths as 1-propanol and 1-butanol do not enhance complex formation between pyrene and β-CD. The

higher degree of stereochemical control of the ternary complexation with amino acids could be due to the fact that these molecules can undergo hydrogen bonding at two sites, whereas only one site is available for alcohols, alkyl sulfates and alkyl ammonium chlorides. Our results show that stereochemical control of ternary complex formation does not parallel with the stability of the complexes formed. This might be of importance when trying to establish optimum conditions for the use of CDs for analytical purposes. Our results suggest that maximum discrimination due to stereochemical constrain might not necessarily correlate with higher stability constants.

In summary, we have shown that amino acids are capable of forming ternary complexes with pyrene and β-CD which lead to an increase in the equilibrium constant between pyrene and the CD. Ternary complex formation is driven by the hydrophobicity of the amino acid, but strict structural requirements were observed.

5. Conclusion

The quenching of excited singlet pyrene complexed to ß-CD by D- and L-tryptophan was studied in the absence and presence of alcohol or alkyl sulfates. Alcohols and alkyl sulfates were employed to enhance the equilibrium constant between pyrene and ß-CD. Quenching occurs through a static mechanism which requires tryptophan to be in close proximity to pyrene within ß-CD complex. The major effect leading to chiral discrimination is probably the different relative positioning of the indole ring with respect to pyrene within the CD complex, although differential binding efficiencies of tryptophan to ß-CD may play a minor role. We suggest that quenching occurs in a complex with 1:2:n stoichiometry for pyrene:ß-CD:tryptophan. These results confirm that chiral environments can lead to chiral discrimination for a photophysical process where only one of the partners is chiral.

The effect of amino acids on the complex formation between pyrene and ß-CD was studied to establish the formation of ternary complexes. Pyrene/ß-CD/amino acid ternary complexes were observed for tryptophan, leucine and phenylalanine. This is the first example in which a zwitterionic compound enhances the equilibrium constant of a host with ß-CD. Glycine, alanine, valine, serine, histidine and lysine did not alter the equilibrium constant between pyrene and ß-CD. The results indicate that ternary complex formation is critically dependent on the structure of the amino acid. In this study we also observed for the first time direct spectroscopic evidence for three different pyrene species in pyrene/ß-CD complexes.

6. References

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2. C. Bohne and H. Yang, Interaction of Excited Singlet Pyrene Complexed to ß-Cyclodextrin with Amino Acids (*oral presentation*), *The 77th CSC Conference*, Winnipeg, Manitoba, May, 1994.

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4. H. Yang, K. Stefaniak and C. Bohne, Preliminary Studies on the Dynamics of Singlet and Triplet Probe Molecules Complexed to Cyclodextrins, *The IAPS 5th Winter Conference*, Florida, January 1993.

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