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# A binding investigation of fluorescence\probes and cyclodextrin derivatives

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# A BINDING INVESTIGATION OF FLUORESCENCE PROBES AND CYCLODEXTRIN DERIVATIVES

By

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## ABSTRACT

Cyclodextrins (CDs) are cyclical carbohydrates composed of repeating glucopyranose units. The three most common CDs, α–, β–, and γ–, contain 6, 7 and 8 glucopyranose units, respectively, and have hydrophilic exteriors, which makes them water soluble. CD polymers (CDPs) have been synthesized, containing glyceryl links between CD units. These polymers are more water soluble than their monomer counterparts. CDs and CDPs, because of their hydrophobic interior cavities, form inclusion complexes with various molecules, including aromatics. This latter property is one we have investigated using fluorophores, including naphthalenes and pyrene.
We have shown, through absorption spectral and fluorescence lifetime data, that naphthalene forms a different type of complex with α–CDP than α–CD. We suggest this complex is 1:1, as the α–CD/naphthalene complex has been reported to be 2:1. We have also studied the binding of pyrene with β–CD derivatives. These derivatives have different substitution patterns, with substitution on the smaller rim of the β–CD cavity, the larger rim of the cavity, and on both rims of the cavity. We have shown, through steady-state fluorescence and fluorescence lifetime measurements, that the derivative with substitution on the smaller rim behaves similarly to β–CD. This suggests that a 2:1 complex is still forming, as reported in the literature. On the other hand, derivatives with at least some substitution on the larger rim behave more like the β–CDP. We suggest that larger rim substitution has two effects: A) the groups extend the hydrophobic cavity and B) a 1:1 complex is likely, as the groups seem to sterically hinder a 2:1 complex from forming.

## ACKNOWLEDGMENTS

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## Introduction

Cyclodextrins (CDs) are cyclical carbohydrates composed of repeating glucopyranose units. (Glucopyranose is the cyclic form of glucose.) The three most common CDs are α-, β-, and γ- cyclodextrins, with 6, 7, and 8 glucopyranose units, respectively. All have hydrophobic cavities and hydrophilic exteriors. The hydrophilic exterior makes these molecules soluble in water. The hydrophobic interior allows CDs to form inclusion complexes with various guests, including certain aromatic molecules. Table I shows the dimensions of α-, β-, and γ- cyclodextrins.

One goal of cyclodextrin research is to increase the water solubility of these molecules. This is important because β-CD, for example, is the most versatile (due, in part, to its size) of the three commercially available CDs, but also the least soluble. One way this has been achieved is by the synthesis of polymers containing α-, β- and γ- cyclodextrins. Polymers composed of cyclodextrin molecules linked by glyceryl units are now commercially available. The general formula of the polymers is:

$$[CD\text{-}(CH_2\text{-}CHOH\text{-}CH_2\text{-}O)_nX]_p$$

where CD is α-, β- or γ-; X is H or CD; p is > 1 but < 6-8 and n is > 1 but < 18 (α-CD), < 21 (β-CD) and < 24 (γ-CD). The percents of cyclodextrin present in each of the polymers are 54 (α-CD), 55 (β-CD) and 57 (γ-CD).[1]

**Table I: Dimensions of α-, β-, and γ- CDs[2,3]**

| | Height (Å) | Internal Cavity Diameter (Å) | External Cavity Diameter (Å) |
|---|---|---|---|
| α-CD | 7.8 | 5.3 - 5.7 | 14.6 |
| β-CD | 7.8 | 6.5 - 7.8 | 15.4 |
| γ-CD | 7.8 | 8.3 - 9.5 | 17.5 |

Another focus of cyclodextrin research is to understand and characterize the binding of different molecules with cyclodextrins. Xu and coworkers and Warner and coworkers have suggested that pyrene and β-CD form a 2:1 complex, where two β-CD molecules envelope one pyrene molecule in a clam-shell structure.[4,5] The driving force of this structure is to envelope the pyrene and protect it in a hydrophobic environment from the water. As a result of the close contact between the two cyclodextrin rims, hydrogen bonds may be forming between the two CDs, thereby enhancing the complex stability.

Jodie Iannacone, for her senior research at Union College, studied the binding of pyrene to β-CD polymer (β-CDP) and compared it with the binding of pyrene to the β-CD monomer.[6] Iannacone's study, using pyrene fluorescence vibronic intensity ratios and fluorescence lifetime data, shows that there is a difference in the way pyrene binds to the monomer and to the polymer. These data suggest that there may be some interaction between pyrene and the glyceryl linker units and that a 1:1 complex is more likely for pyrene with β-CDP. In any case, clam-shell binding for pyrene to β-CDP is highly unlikely.

Based on fluorescence lifetime and absorption spectral data, Kohler and coworkers have suggested that α-CD forms a 2:1 complex with naphthalene, similar to that of the pyrene/β-CD complex.[7] Kohler's primary evidence for the formation of the 2:1 complex is the sharpening of the absorption spectrum of naphthalene observed when α-CD is present. A non-polar environment surrounding naphthalene can cause this sharpening. This is consistent with the 2:1 complex, as the environment created for the naphthalene by the two cyclodextrins is non-polar (compared to the polar water solvent). Based on

Kohler's work with naphthalene, we have investigated and compared the binding of naphthalene with α-CD and α-CDP. These systems are also similar to the β-CD/pyrene and β-CDP/pyrene systems. In each case it is suggested that the monomer forms a 2:1 complex with the fluorophore.[4,5,6,7]

We have also compared the binding of pyrene with β-CD and β-CD derivatives, which were given to us by Dr. Josef Pitha. These derivatives have been synthesized with various substituents on the larger and/or smaller rim of each cyclodextrin. These substituted cyclodextrins are more soluble than the β-CD monomer.

Pyrene was chosen as the fluorophore in these cases, partly based on the previous work with the β-CD/pyrene system, and in part because of its unique ability to indicate the hydrophobicity of the environment it is experiencing. The fluorescence spectrum of pyrene has five vibronic bands. By calculating the ratio of the first (I, at 372 nm) to the third (III, at 384 nm) band (I/III ratio), one can determine the relative hydrophobicity of pyrene's environment. A low I/III ratio indicates a more hydrophobic environment.

Another important feature of pyrene is its fluorescence lifetime. The length of its lifetime will increase when pyrene is complexed by CDs, because it is protected from oxygen quenching.[4]

Steady-state fluorescence measurements, as well as fluorescence lifetime measurements, were used to study the interactions between pyrene and these substituted β-CD derivatives.

We also studied the binding of two naphthalene derivatives with the β-CD derivative having substitution solely on the smaller rim (6-0Mβ-CD). These naphthalene based probes (2-AN and 2,6-MANS) are known to form complexes with β-CD.[8] Based on this previous work, we chose these to further compare their binding with β-CD to their binding with 6-0Mβ-CD.

From these studies we have gained insight as to the effects of substitution on the larger and smaller rim of cyclodextrins on their ability to form inclusion complexes.

## Experimental

**Apparatus.** UV absorption measurements were performed on a Hewlett Packard 8452A Diode Array Spectrophotometer and a Hitachi U-3410 Spectrophotometer. Spectra were taken over a range of 250 - 350 nm for naphthalene, 290 - 400 nm for pyrene, and 300 - 400 nm for 2 - acetylnaphthalene (2-AN) and 2 - (N - methylanilino) naphthalene - 6 - sulfonic acid (2,6 - MANS).

Fluorescence measurements were taken on a Perkin Elmer LS-5B Luminescence Spectrometer. Solutions containing naphthalene were excited at 300 nm and emission spectra were acquired from 310 - 400 nm with excitation and emission slits (bandwidths) set at 3 nm. Solutions containing pyrene were excited at 320 nm and emission spectra were acquired from 350 - 500 nm with excitation and emission slits set at 3 nm. Solutions containing 2-AN were excited at 340 nm and emission intensities were acquired at 437 nm with excitation and emission slits set at 10 nm. Solutions containing 2,6-MANS were excited at 350 nm and emission spectra were acquired from 375 - 675 nm with excitation and emission slits set at 10 nm. Corrected emission spectra were obtained for pyrene and 2,6-MANS.

Fluorescence lifetime measurements were performed on a Photon Technology International (PTI) LS-100 Luminescence Spectrometer. A nitrogen lamp was used with an excitation wavelength of 297 nm and an emission wavelength of 330 nm for naphthalene solutions. An excitation wavelength of 337 nm and an emission wavelength of 384 nm were used for

pyrene solutions. The fitting procedure for the lifetime decay curve allows for 1-4 exponentials, meaning that there can be between one and four emitting species in solution. The decay curve is generated by an iterative procedure based on the Marquardt algorithm. In order to generate the curve fit, one is required to enter "guesses" for two values for each possible emitting species. These two values are A, a weighing factor, and $\tau$, the actual lifetime (ns) of the species. We found that, depending on the values we chose to begin the curve fit calculations, we would obtain different values for the lifetimes and relative fraction of the total fluorescence of each component in solution ($F_i$), defined mathematically as:

$$F_i = A_i\tau_i/\Sigma A_i\tau_i$$

In order to combat the inconsistencies we were observing, we chose to begin each curve fit with a different set of starting conditions (different values for $A_1$, $A_2$, $\tau_1$, and $\tau_2$) and take an average of the final values we obtained. (The $\chi^2$ values, which are indications of the quality of the curve fit, for the averaged lifetimes are reported as a range rather than an average. A $\chi^2$ value of approximately 1.00 indicates an ideal fit.) Another parameter used to determine whether or not the curve fit was suitable was the Durbin-Watson (DW) parameter. A satisfactory fit is indicated by a DW of >1.7 for a single exponential, >1.75 for a double exponential, and > 1.8 for a triple exponential. See Table II for an illustration of the dependence of the final values of $A_1$, $A_2$, $\tau_1$, and $\tau_2$ on the initial guesses. The values reported for naphthalene in the presence $\alpha$-CD and $\alpha$-CDP are averages from different curve fit starting conditions using two different solutions of the same CD concentration.

**Table II:** The dependence of final $\tau$ values on initial $A_1$, $A_2$, and $\tau$ values (For Naphthalene in 0.060M $\alpha$-CD)

| Initial Conditions | | | | Final Conditions | | | | |
|---|---|---|---|---|---|---|---|---|
| $A_1$ | $A_2$ | $\tau_1$ (ns) | $\tau_2$ (ns) | $A_1$ | $A_2$ | $\tau_1$ (ns) | $\tau_2$ (ns) | $F_1$ |
| 0.5 | 0.5 | 100 | 35 | 0.714 | 0.286 | 112 | 44 | 0.865 |
| 0.5 | 0.5 | 85 | 20 | 0.714 | 0.286 | 108 | 37 | 0.880 |
| 0.5 | 0.5 | 100 | 40 | 0.750 | 0.250 | 102 | 25 | 0.925 |
| 0.5 | 0.5 | 90 | 30 | 0.607 | 0.393 | 99 | 21 | 0.880 |
| 0.5 | 0.5 | 95 | 30 | 0.718 | 0.282 | 111 | 42 | 0.871 |

The average values reported for this solution are:

$\tau_1 = 106 \pm 6$ ns $\quad A_1 = 0.70_1 \pm 0.05_4$

$\tau_2 = 34 \pm 10$ ns $\quad F_1 = 0.88_4 \pm 0.02_4$

**Reagents.** Naphthalene (99%+) was used as received from Aldrich. The α-CD was a gift from Wacker Chemicals and used as received. The α-CDP was used as received from Cyclolabs R&D Laboratory Ltd. of Budapest, Hungary. The β-CD was a gift from the American Maize-Products Company. Pyrene (99+%) and 2,2,3,3,3 - pentafluoro - 1 - propanol (PFP) were used as received from Aldrich. The 2-AN was recrystallized three times from ethanol and water. The 2,6-MANS was used as received from Aldrich. The 6-0Mβ-CD, P101, P125, Randomly Methylated β-CD, heptakis (2,6-O-dimethyl)-β-CD, and dihydroxypropyl β-CD were used as received from J. Pitha of Baltimore, MD. These six derivatives are described in a letter sent to us by Dr. Pitha. Table III gives sample composition information.

6-0-Maltosyl-β-CD (6-0Mβ-CD) This is a Japanese preparation with two glucopyranosyl residues attached to β-CD by an α-glycosidic bond. Substitution is on the smaller rim of the β-CD.[9]

Dihydroxypropyl-β-CD (DHPβ-CD) A Japanese preparation with an average degree of substitution of 4.68 per molecule. This mixture contains just species with one cyclodextrin moiety and no intramolecular crosslinks. Substitution is on the larger and the smaller rim of the β-CD.[9]

Heptakis(2,6 - 0 - dimethyl) - β-CD (Hepsβ-CD) A commercial preparation which contains 70% of the title compound. The rest is closely undermethylated and overmethylated title compound. Substitution is on the larger and the smaller rim of the β-CD.[9]

Randomly Methylated β-CD (Random) A preparation from Wacker, with an average substitution of 12.32 methyls per molecule. Substitution is on the larger and the smaller rim. See Table III for sample composition information.[7]

P101 Prepared by Josef Pitha; a product of the reaction of β-CD with 1,2 - dichloroethane. Nearly all of the species present have just one cyclodextrin moiety and carry either 2 - hydroxyethyl substituents or --$CH_2$-$CH_2$-- units crosslinking the 2° and 3° hydroxyls. Substitution is on the larger rim of the β-CD. See Table III for sample composition information.[9]

P125 Prepared by Josef Pitha; a product of the reaction of β-CD with epichlorohydrin. Nearly all of the species present have just one cyclodextrin moiety per molecule. Substituents are either 2,3 - dihydroxypropyl or --$CH_2$-$CH(CH_2OH)$-- units crosslinking the 2° and 3° hydroxyls. Substitution is on the larger rim of the β-CD. See Table III for sample composition information.[9]

All solutions were prepared with Millipore deionized, doubly distilled water.

**Methods for Sample Preparation.** The naphthalene stock solutions were prepared by placing a small amount of naphthalene in a volume of water and stirring overnight. The stock solutions were filtered using a 30 cc plastic syringe and a 25 mm, 0.20 micron syringe filter, and diluted to have an absorbance of ~0.65 at 276 nm. The estimated concentration of these naphthalene stock solutions is $2.0 \times 10^{-4}$ M. (This was calculated by using a value for the molar absorptivity in ethanol; 5012 $L \cdot mol^{-1} cm^{-1}$)[10]

**Table III**: Molar percentages of glucopyranosal residues from alditol acetate analyses (based on results of analyses performed at Arrhenius Laboratory of Stockholm University)[9]

| | Random | P101 | P125 |
|---|---|---|---|
| Unsubstituted | 6.7 | 80.0 | 60.0 |
| Cyclic Substitution | | | |
| 2° and 3°† | | 13.5 | 19.9 |
| Substitution on 2-0* | 14.7 | 3.1 | 4.6 |
| Substitution on 3-0* | 4.7 | 1.6 | 10.9 |
| Substitution on 6-0** | 14.5 | 0.6 | 1.9 |
| Disubstitution on 2, 3-0 | 7.0 | | |
| Disubstitution on 3, 6-0 | 9.5 | | |
| Disubstitution on 2, 6-0 | 27.8 | | |
| Trisubstition on 2, 3, 6-0 | 15.4 | | |

† substitution crosslinks adjacent hydroxyl groups
* located on the larger rim of the cyclodextrin cavity
** located on the smaller rim of the cyclodextrin cavity

Stock solutions of 2-AN were prepared by placing a small amount of 2-AN in a volume of water and stirring overnight. 2-AN solutions were diluted to have an absorbance of < 0.05 at 340 nm. Stock solutions of 2,6-MANS were prepared in a 0.1M phosphate buffer (pH 6-7). 2,6-MANS solutions were diluted to have an absorbance of 0.02 - 0.03 at 350 nm. Stock solutions of pyrene were prepared by placing a small amount of pyrene in a volume of water and stirring overnight. Pyrene solutions were prepared to have an absorbance of < 0.05 at 320 nm. The solutions containing β-CD derivatives/pyrene used for I/III ratio measurements (steady-state fluorescence) were prepared by adding ~ 0.0150 g of the various derivatives to the pyrene stock and measuring the ratio after each addition. The final solution (with a limiting value for the I/III ratio) was then used to take the lifetime measurement. The I/III ratio experiments were performed in duplicate, and in some cases, triplicate.

For the solutions containing 2,2,3,3,3 - pentafluoro - 1 - propanol [PFP], the concentration of cyclodextrin was the concentration reached at the limiting value of the I/III ratio of pyrene, as mentioned above. To these solutions, a volume of PFP was added to make the volume of PFP in solution 1% that of the total volume.

## Results

### I. Naphthalene/α-CD and Naphthalene/α-CDP

To investigate and compare the naphthalene/α-CD system to the naphthalene/α-CDP system, three methods were employed: UV absorption measurements, steady-state fluorescence measurements and fluorescence lifetime measurements.

Since naphthalene is relatively non-polar, a preliminary experiment was performed to determine if there would be any significant loss of naphthalene to the walls of the glass vials or the walls of the glass cuvettes used in the experiments. Absorption of naphthalene onto the glass would affect a comparison of absolute fluorescence intensities of solutions, as concentrations of naphthalene in solution would not be constant.

A simple experiment was performed, whereby a naphthalene solution was prepared in water and fluorescence measurements were taken every 20 minutes over a period of two hours. These measurements were taken against a polymethylmethacrylate standard, whose intensity was set to be 100. Absorption spectra were also taken of the solution; the absorbance values reported were at 276 nm.

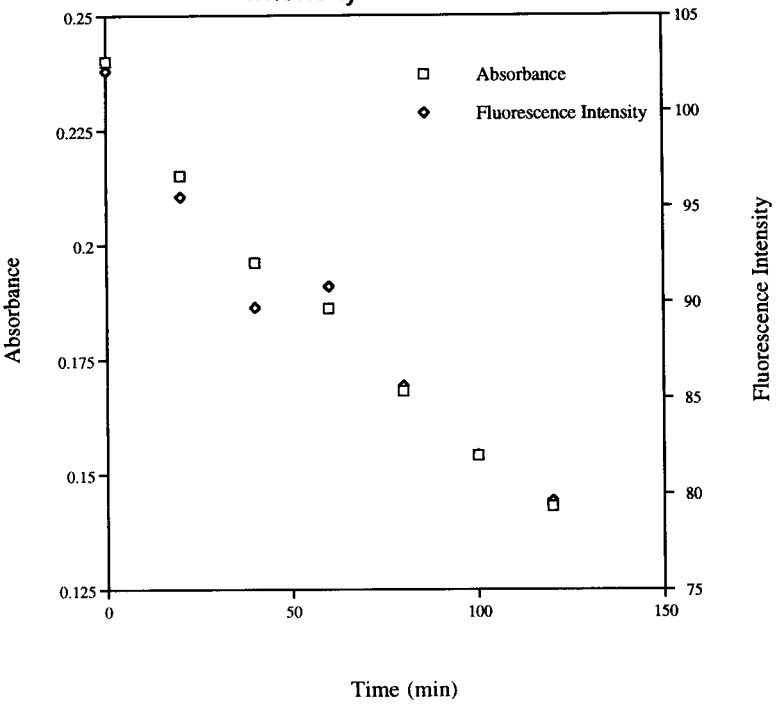
Figure 1 shows a plot of fluorescence intensity vs. time and absorbance vs. time for the naphthalene solution. Over the two hour period, the solution's fluorescence intensity drops from ~ 102 to ~ 80 and the absorbance drops from 0.24 to 0.14. These data indicate that there is a significant loss of naphthalene to the glass walls. From this experiment we conclude that we will not be able to make absolute intensity comparisons between the two CD systems.

**Figure 1:** Absorbance and Fluorescence Intensity of Naphthalene vs. Time

Red Square: Absorbance
Blue Diamond: Fluorescence Intensity

# Naphthalene

## Absorbance and Fluorescence Intensity vs. Time



We can, however, obtain reliable information from changes in the shape of absorption spectra and shifts in peak maxima. Moreover, the fluorescence lifetime measurements will not be affected by the loss of naphthalene to the walls.

*UV Absorption Measurements.* Original absorption measurements for this experiment were taken on a Hewlett Packard 8452A Diode Array. We were able to observe some difference in the spectra of free naphthalene and naphthalene in the presence of both α-CD and α-CDP but were not satisfied with the resolution. The data reported in this paper were collected on a Hitachi U-3410 Spectrophotometer at the General Electric Research and Development Center, Schenectady, NY. This scanning instrument gave us sufficient resolution to elucidate the differences between the three spectra.

The UV absorption spectrum of free naphthalene in water (Figure 2) shows a maximum at 276 nm, as well as two prominent rounded peaks at 266 nm and 284 nm. There is also a small peak at 310 nm.

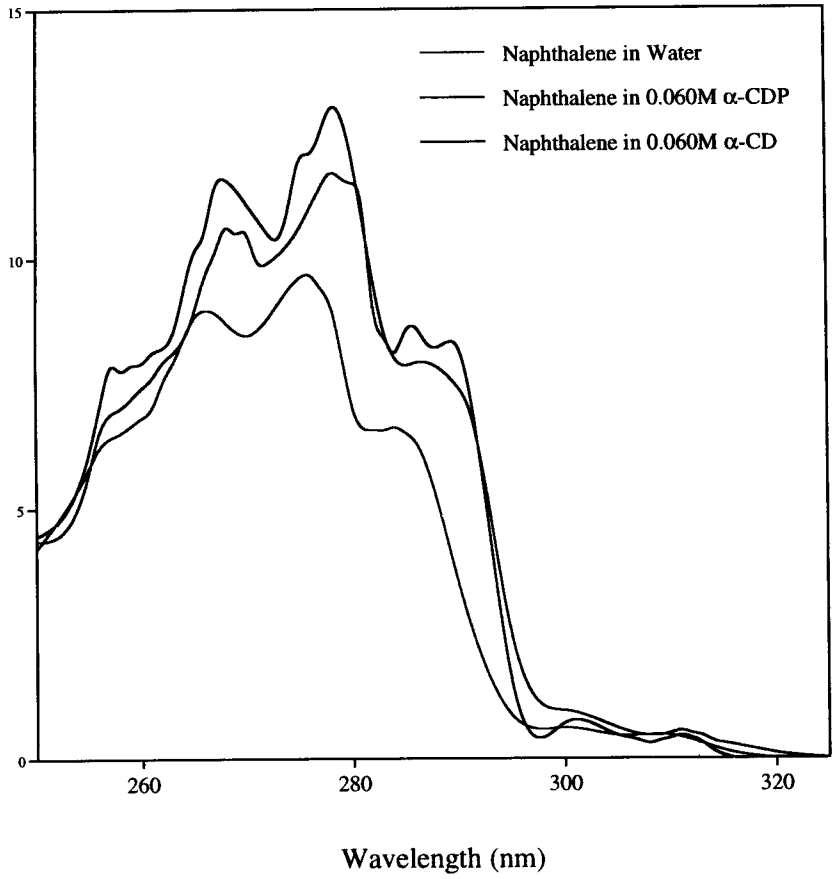
Upon addition of α-CD to the naphthalene in water solution (0.060M α-CD), there are visible changes in the spectrum, as shown by the sharpening of the peaks in Figure 2. The maximum has shifted from 276 nm to 280 nm and the peak at 284 nm now shows two distinct local maxima. There is now a more prominent peak at 310 nm compared to that of the free naphthalene. The maximum absorbance has also been increased by the addition of the α-CD monomer from ~ 0.48 to ~0.65.

The absorption spectrum of naphthalene in 0.060M α-CDP is also shown in Figure 2. While there is still a shift of the maximum to 280 nm, as in the α-CD spectrum, there is not as great a sharpening of the peaks. The region between 282 and 292 nm is not as well defined, and the peak at 268 nm

**Figure 2:** Absorbance Spectra of Naphthalene in water, Naphthalene in 0.060M α-CD, and Naphthalene in 0.060M α-CDP

Red Line: Naphthalene in Water
Blue Line: Naphthalene in 0.060M α-CD
Green Line: Naphthalene in 0.060M α-CDP

Absorbance Spectra of Naphthalene
Wavelength (nm) vs. Absorbance



is not as sharp as in the presence of the CD monomer. However, as in the presence of the CD monomer, the peak at 310 nm is more prominent than it is for free naphthalene.

*Fluorescence Lifetime Measurements.* The lifetime value of free naphthalene in water has been reported to be 35.9 ns by Warner et al.[11] We obtained a value of 35±1 ns, with a $\chi^2$ range of 1.28 - 1.34. Our value is in good agreement with the literature value within our standard deviation.

The fluorescence lifetime of naphthalene in the presence of 0.060M α-CD shows two fluorescing components. Two components were also seen in the fluorescence lifetime of naphthalene in the presence of 0.060M α-CDP. These results are shown in Table IV. The longer lived component in each case is likely the lifetime of naphthalene bound to α-CD or α-CDP. We also took lifetime measurements of the same naphthalene and α-CDP solution twice in a 24 hour period, to determine if there was any time dependence to the lifetime values we obtained. Our results for $\tau_1$ were 75 and 73 ns, while $\tau_2$ values were 7 ns for both runs. This shows that there is not a time dependence in the lifetime measurements, as the values for the lifetimes have not significantly changed in 24 hours.

## II. Naphthalenes and Pyrene Binding to Substituted β-CDs

### A. 2 - Acetylnaphthalene (2 -AN)

Steady-state fluorescence was also used to study the binding of 6-0-Mβ-CD with 2-acetylnaphthalene (2-AN). 2-AN is known to form a 1:1 inclusion complex with β-CD. When 2-AN binds inside the hydrophobic cavity of a CD, its fluorescence is quenched. Quenching is an observed

**Table IV**: Naphthalene Fluorescence Lifetime Results

| Solution: | $\tau_1$ (ns) | $\tau_2$ (ns) | $F_1$ |
|---|---|---|---|
| 0.060M α-CD | 106 ± 6 | 34 ± 10 | 0.88 ± 0.22 |
| 0.060M α-CDP | 73 ± 4 | 8 ± 2 | 0.76 ± 0.21 |

**Figure 3**: Stern Volmer Plot, [6-0Mβ-CD] vs. Fo/F

Stern Volmer Plot
[6-0Mβ-CD] vs. Fo/F

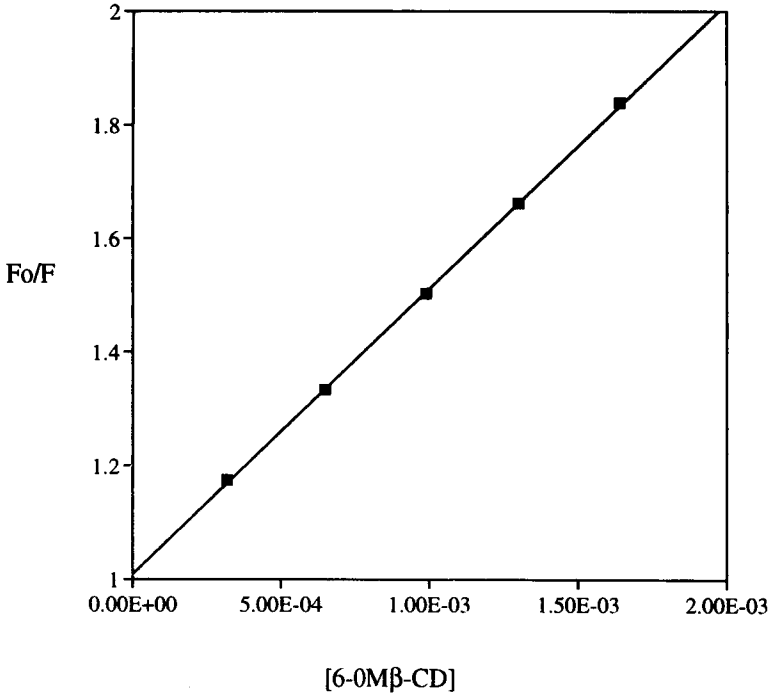


**Table V**: Stern Volmer Plot Results (2-AN in the presence of 6-0Mβ-CD)

| | Slope (K) | Intercept | $r^2$ |
|---|---|---|---|
| Run 1 | 503 | 1.009 | 1.000 |
| Run 2 | 516 | 0.947 | 0.999 |

The average slope value (K) reported for these two runs is: 510 ± 7

decrease in the intensity of the fluorescence signal that can occur dynamically or statically. Dynamic quenching occurs from diffusion of quencher to fluorophore while the fluorophore is in its excited state. Static quenching occurs because of a ground state complex between the fluorophore and the quencher.[12] If quenching is known to be static and the complex formed is 1:1, the intensity data should conform to the modified Stern-Volmer equation.[8]

(1) $$F^0/F = 1 + K[CD]$$

Werner and Warner showed the slope value (K) from equation 1 for 2-AN quenched by β-CD to be 536 ± 6.[8] A similar experiment was performed in duplicate with 6-0Mβ-CD and 2-AN. The first run is plotted according to equation 1 in Figure 3. Table V shows the slope, intercept and $r^2$ values obtained for two separate runs. The average value for the slope of equation 1 obtained for these measurements was 510 ± 7.

**B. 2 - (N - methylanilino) naphthalene - 6 - sulfonic acid (2,6 - MANS)**

Another naphthalene derivative, 2,6 - MANS, was used in a comparative binding study with β-CD and 6-0Mβ-CD. Figure 4 shows the corrected fluorescence spectrum of 2,6-MANS in the presence of both β-CD and 6-0Mβ-CD, where the concentration of both β-CD and 6-0Mβ-CD is 0.0020M. The shape of each curve is very similar and the maxima for each curve is about 503 nm.

**Figure 4**: Fluorescence spectra of 2,6 - MANS in the presence of 6-0Mβ-CD and β-CD

Blue Line: 2,6-MANS in β-CD
Red Line: 2,6-MANS in 6-0Mβ-CD

Fluorescence Spectra of 2,6 - MANS
in the presence of 6-0Mβ-CD and β-CD

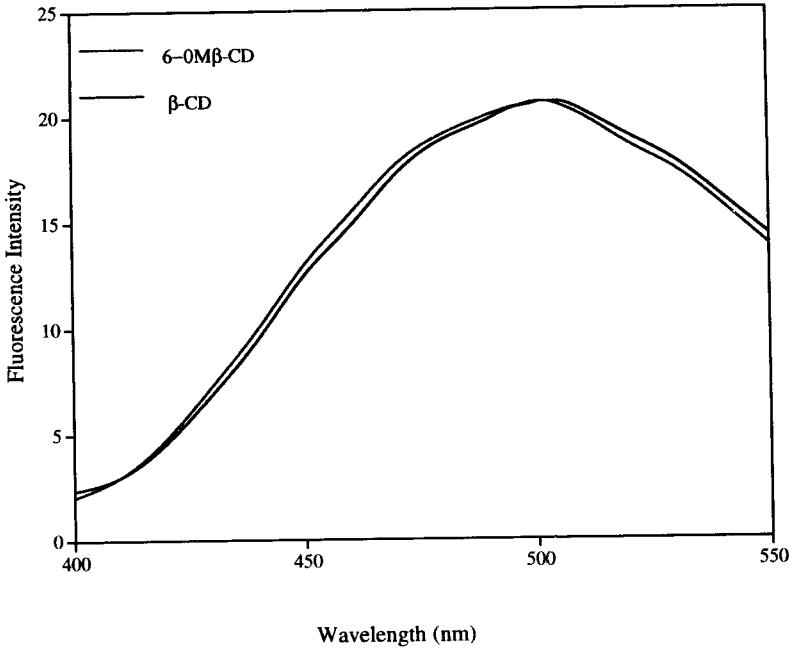
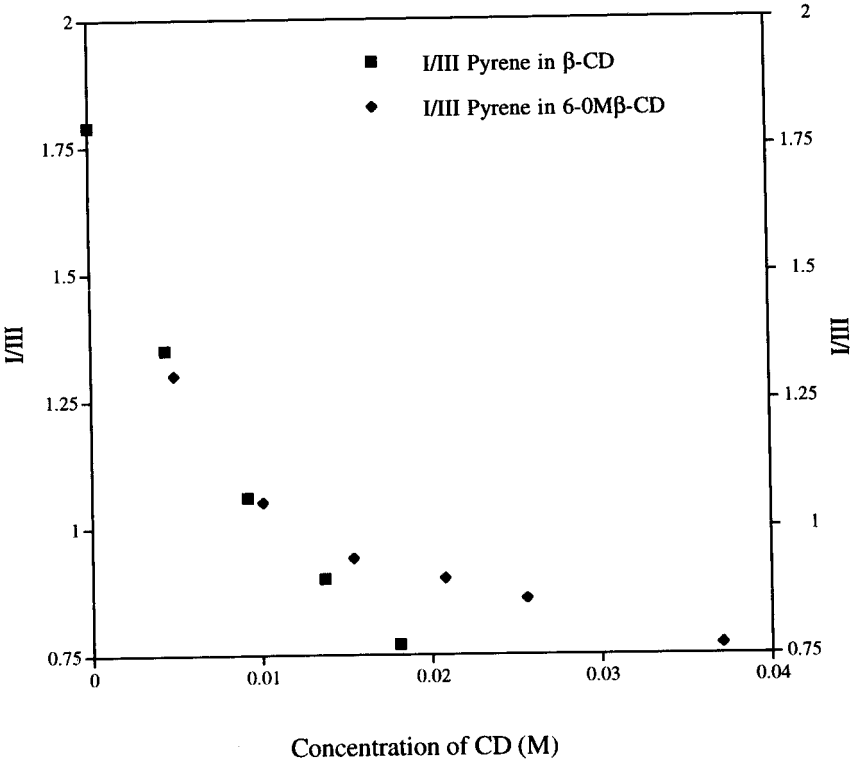


**Figure 5**: Pyrene I/III ratio vs. concentration of CD (M)

Blue Square: Pyrene in β-CD
Red Diamond: Pyrene in 6-0Mβ-CD

I/III Ratio vs. Concentration (M)
(Pyrene in β-CD and 6-0Mβ-CD)



**IV. Pyrene**

The binding of pyrene with the six β-CD derivatives sent to us by Dr. Pitha was studied using steady-state fluorescence and fluorescence lifetime measurements. The limiting I/III ratio of pyrene in the presence of β-CD has been reported to be 0.62.[5] Figure 5 shows the effect of increased cyclodextrin concentration on pyrene's I/III ratio. The limiting value for pyrene in the presence of β-CD occurs at 0.77. The difference between the literature value and our value is probably due to a difference in resolution of the instruments used during the experiment. Notice that the I/III ratio for pyrene in the presence of 6-0Mβ-CD also levels off at approximately 0.77. This is the limiting value of pyrene's I/III ratio obtained with 6-0Mβ-CD.

Based on previous work by Warner et al., pyrene I/III ratio measurements were also made for pyrene in the presence of 0.0256M 6-0Mβ–CD (the limiting concentration) and PFP.[13] PFP was added so that its volume was 1% of the total volume of the solution. (In this case, 25 μL of PFP was added to a 2.5 mL solution of pyrene/6-0Mβ–CD.) It has been suggested that this alcohol "caps" the smaller end of the CD which protects the pyrene (in the 2:1 complex with β-CD) further from the water solvent and makes its binding environment more hydrophobic.[13] Warner et al. find the I/III ratio of pyrene in the presence of β–CD and PFP to be 0.38.[13] We found the I/III ratio of pyrene in the presence of 6-0Mβ–CD and PFP to be 0.44.

Table VI shows the results of the pyrene I/III ratio experiments with each of the β-CD derivatives studied.

Once we determined the concentration at which the value of the I/III ratio ceased to change, we called this the limiting value and used this

**Table VI**: Pyrene I/III Ratio results for β-CD derivatives

| Cyclodextrin Derivative | I/III Ratio ($I_{372}/III_{384}$) |
|---|---|
| β-CD | 0.77 |
| 6-OMβ-CD | 0.77 |
| β-CD* | 0.38[13] |
| 6-OMβ-CD* | 0.44 |
| β-CDP | 1.55[14] |
| P101 | 1.32 |
| P125 | 1.55 |
| Hepsβ-CD | 1.42 |
| DHPβ-CD | 1.47 |
| Random | 1.41 |

* solutions with 1% PFP

**Table VII**: Fluorescence lifetime results for pyrene in the presense of β-CD derivatives

| **Cyclodextrin Derivative** | $\tau_1$ (ns) | $F_1$ | $\tau_2$ (ns) | $F_2$ |
|---|---|---|---|---|
| none (free pyrene) | 130[4] | 1 | ----- | ----- |
| β-CD | 300[4] | 0.57 | 130 | 0.43 |
| 60Mβ-CD | 324 ± 0 | 1 | ----- | ----- |
| β-CD* | 425 | 1 | ----- | ----- |
| 60Mβ-CD* | 418 ± 0 | 0.98 | 16 | 0.02 |
| β-CDP | 251[14] | 0.97 | 92 | 0.03 |
| P101 | 248 ± 8 | 0.63 ± 0.10 | 139 ± 4 | 0.37 ± 0.10 |
| P125 | 206 ± 1 | 1 | ----- | ----- |
| DHPβ-CD | 260 ± 2 | 0.57 ± 0.01 | 192 ± 1 | 0.43 ± 0.01 |
| Hepsβ-CD | 245 ± 13 | 0.65 ± 0.08 | 150 ± 9 | 0.35 ± 0.08 |
| Random | 206 ± 4 | 1 | ----- | ----- |

* solutions containing PFP

concentration of CD derivative to take the fluorescence lifetime measurements.

Fluorescence lifetime measurements were made for pyrene in the presence of each derivative at the limiting concentration of a given derivative. Table VII shows the lifetime values for each solution. In each case the lifetimes and $F_i$ values for each emitting species are reported. $F_i$ is the fraction of the fluorescence signal which is due to that particular emitting species.

Lifetime measurements for pyrene in the presence of the limiting concentrations of β-CD and 6-0Mβ-CD were also made on solutions containing 1% PFP. The proposed binding effect of this alcohol causes the lifetime to lengthen, as observed by Warner et al. for pyrene in the presence of β-CD, most likely because the PFP protects the pyrene further from oxygen quenching.[13]

## Discussion

### I. Naphthalene/α-CD and Naphthalene/α-CDP

We observe a more distinct sharpening in the naphthalene absorption spectrum in the presence of α-CD, than in the presence of α-CDP (see Figure 2). Kohler and coworkers have previously reported this observation for naphthalene/α-CD and attribute the sharpening of the peaks in the absorption spectrum of naphthalene in the presence of α-CD to the inclusion of naphthalene in a non-polar environment which is protected from the polar solvent, water.[7] The proposed 2:1 complex, with two α-CDs enveloping

one naphthalene molecule, provides naphthalene with such a protected, non-polar environment . The clam-shell conformation of the α-CDs may also allow the larger rims of these CDs to hydrogen bond with each other. We also observe this sharpening with the monomer but less so with the polymer, therefore we conclude that the polymer is not forming the same kind of complex with naphthalene as the monomer does.

We see that there is a marked difference between the naphthalene lifetime values in the presence of the monomer and the polymer. The fact that we see two lifetimes for each system indicates that there is a complex formed in each case, but the difference in length of the major component lifetime values suggests that the naphthalene is binding in a distinct manner with each CD. A longer lifetime indicates that naphthalene is protected more from oxygen quenching, supporting the formation of a 2:1 complex between α-CD and naphthalene. On the other hand, the shorter lifetime observed in the presence of α-CDP indicates that naphthalene's fluorescence is being partially quenched by oxygen because it is more exposed.

Based on the absorption data for α-CDP and naphthalene as well as the fluorescence lifetime data, we suggest that naphthalene and α-CDP are forming a 1:1 complex. This is supported by the less structured absorption spectrum (compared to naphthalene in the presence of α-CD), the shorter lifetime values (compared to naphthalene in the presence of α-CD) and structural differences between α-CD and α-CDP. While two α-CDs are structurally able to form the 2:1 complex, the steric hindrance created by the glyceryl linkers of the polymer chains likely prevents two CDs in a polymer chain from forming the proposed clam-shell structure.

## II. Fluorophore Binding to Substituted β-CDs

### A. Pyrene/β-CD and Pyrene/6-0Mβ-CD

The β-CD derivative 6-0Mβ-CD is the only derivative we studied with substitution solely on the smaller rim of the β-CD cavity. This substitution should not have a significant effect on the binding of this derivative with pyrene, if the pyrene enters the CD through the larger rim. Moreover, it is the only β-CD derivative that might be expected to readily form the 2:1 clam-shell structure.

This theory is clearly supported by the measured I/III ratios of pyrene in the presence of each of these cyclodextrins. (See Table VI) The I/III ratio in each case was found to be 0.77, indicating that pyrene is experiencing an equally hydrophobic environment in each case.

The I/III ratio of pyrene was also measured with solutions containing each of the cyclodextrins and PFP. Warner et al. suggest that PFP "caps" the smaller rim of the cyclodextrin, protecting the pyrene further from the solvent, water, based on the low I/III ratio for pyrene in the presence of β-CD and PFP (0.38).[11] The I/III value we obtained for pyrene in the presence of 6-0Mβ-CD and PFP of 0.44 is also very low, though not as low as the value measured in the presence of β-CD. This suggests that pyrene is experiencing a more hydrophobic environment than without the alcohol present, but it is not as hydrophobic as when it binds with β-CD. One explanation for the difference in I/III ratios is a difference in resolution between the instruments used in each experiment. Our fluorometer may not be able to sufficiently resolve the I and III bands. Another possibility is that, since the PFP caps the smaller end of the CD cavity where 6-0Mβ-CD substitution occurs, the

maltosyl groups may be interfering with PFP's ability to tightly cap this rim. This leaves pyrene more exposed to water, thus increasing the I/III ratio.

Table VII shows lifetime values for pyrene in the presence of β-CD and 6-0Mβ-CD, as well as the lifetime for free pyrene.

First, the table shows that the lifetime of pyrene is lengthened when it is bound to a CD derivative. This is due to the fact that pyrene, when bound inside any of the CDs, is protected from oxygen quenching. The longer lifetime reported for pyrene in the presence of β-CD ($\tau_1$) is due to pyrene in the complex formed with β-CD. The second, shorter lived species, is likely free pyrene in solution. Its lifetime value, 130 ns, agrees with the reported value of 130 ns for free pyrene. Pyrene's lifetime in the presence of 6-0Mβ-CD (324 ns), is close to that of pyrene in β-CD (300 ns). It is likely that we only see one emitting species for this solution because the concentration of 6-0Mβ-CD is considerably higher than the concentration of β-CD. The higher concentration of CD allows all of the pyrene to be complexed, whereas in the case of β-CD, there may not be enough cycloaextrin present to bind all of the pyrene. These similar lifetime values support the I/III ratio data, indicating that β-CD/pyrene and 6-0Mβ-CD/pyrene form similar complexes.

Lifetime measurements were also taken on the solutions used in the I/III ratio experiments to which PFP was added. For pyrene in the presence of β-CD, the lifetime has been greatly lengthened to 425 ns. This is consistent with the idea that PFP caps the smaller rim; this type of structure would further protect pyrene from oxygen quenching and lengthen its lifetime. The lifetime of pyrene in the presence of 6-0Mβ-CD has also been lengthened by addition of PFP. The longer and more dominant species at 418 ns is from the pyrene/CD complex. The shorter lived species, at 16 ns, makes an insignificant contribution to the overall fluorescence signal and is

therefore a highly inaccurate value. The two lifetime values for the complexes are very close, further supporting our assertion that β-CD and 6-0Mβ-CD form the same type of complex with pyrene.

As mentioned previously, the proposed binding structure for β-CD and pyrene is a 2:1 complex, where two β-CD molecules envelop one pyrene molecule in a clam-shell structure. Since all of our data indicate that pyrene and 6-0Mβ-CD are forming the same type of structure as pyrene and β-CD, we suggest that this complex is also 2:1. The maltosyl groups on the 6-0Mβ-CD, present only on the smaller rim of the CD cavity, do not interfere with binding and do not prevent this 2:1 complex from forming. Our data can also be considered confirmatory for the pyrene and β-CD complex structure.

### B. Naphthalenes/β-CD and Naphthalenes/6-0Mβ-CD

Further proof that β-CD and 6-0Mβ-CD form similar complexes with fluorophores is shown by our studies of binding with 2-AN and 2,6 - MANS. The 2-AN quenching studies show that the slope (K) obtained using equation 1 for 2-AN in the presence of β-CD is very similar to that of 2-AN in the presence of 6-0Mβ-CD (Table V). The value reported by Werner and Warner for β-CD is 536 ± 6,[8] while our value for 6-0Mβ-CD is 510 ± 7.

The fluorescence spectra of 2,6 - MANS in the presence of β-CD and 6-0Mβ-CD (Figure 4) are almost identical, with maxima in each case at approximately 503 nm. This also shows that β-CD and 6-0Mβ-CD form similar complexes with naphthalene based derivatives.

**B. Pyrene/β-CDP and Pyrene/β-CD derivatives**

The remaining β-CD derivatives all have at least some substitution on the larger rim of the β-CD cavity. (Some also have substitution on the smaller rim.) The I/III ratio of pyrene measured in the presence of these β-CD derivatives (Table III) are similar to the I/III ratio of pyrene in the presence of the commercially available β-CDP. Pyrene, in the presence of some of the derivatives, including P101, Hepsβ-CD, DHPβ-CD and Randomly methylated, has a lower I/III ratio than with the β-CDP. We suggest this is due to the substituents on the larger rim of the cyclodextrin extending the hydrophobic cavity. This would increase the hydrophobicity of pyrene's binding environment, thus decreasing its I/III ratio.

The I/III values for pyrene in the presence of these derivatives are higher than those measured for pyrene in the presence of β-CD and 6-0Mβ-CD, indicating that pyrene is in a much less hydrophobic environment in the latter case. This is consistent with the suggested 1:1 complex for pyrene and β-CDP. A 1:1 complex would leave pyrene exposed to water, increasing its I/III ratio in comparison with the ratio observed for the 2:1 complex with β-CD and 6-0Mβ-CD.

The fluorescence lifetime results (Table VII) also support the idea of similar complex formation between pyrene and β-CDP and pyrene and these five derivatives. In comparison to the lifetime of free pyrene (130 ns) we see again that in the presence of any one of the cyclodextrins, pyrene's lifetime is lengthened. This lifetime ranges from 206 to 260 ns for the various larger rim

substituted derivatives. In these cases, the second component ($\tau_2$), when present, is likely to be free pyrene or pyrene bound to another binding site.

The lifetime values are longer than that of free pyrene, but shorter than those in the presence of β-CD and 6-0Mβ-CD. These values indicate that pyrene is not as protected from oxygen quenching and is in a more open environment when the β-C. has substitution on the larger rim. This supports the formation of a 1:1 complex for pyrene and β-CDP as well as a 1:1 complex for pyrene and the five derivatives with substitution on the larger rim.

We can make general conclusions about the effects of cyclodextrin substitution on binding with pyrene based on these data. First, substitution solely on the smaller rim of the cyclodextrin cavity does not prohibit the 2:1 complex observed in unsubstituted β-CD/pyrene from forming. This was illustrated by the similar results for β-CD and 6-0Mβ-CD binding.

Second, substitution on the larger rim of the cyclodextrin cavity has two effects:

A) The substituents extend the hydrophobic cavity, as shown by the lower I/III ratios compared to commercial β-CDP.

B) A 1:1 complex is more likely than a 2:1 complex. This is shown by the higher I/III ratios, and the shorter lifetime values. This makes sense structurally as well because groups on the larger rim would prevent two CDs to have close contact between these rims. The groups may sterically hinder the rims from hydrogen bonding and forming a clam-shell complex.

We also observe similar binding complexes for cyclodextrin monomers and cyclodextrin polymers. We have confirmed the proposed 2:1

complex for both α-CD/naphthalene and β-CD/pyrene, as well as suggesting a 1:1 complex for α-CDP/naphthalene similar to the proposed 1:1 complex for β-CDP/pyrene. These data suggest that cyclodextrin monomers have the ability to fully envelop some fluorophores entering into their cavity by forming a 2:1 complex. Our data also suggest that cyclodextrin polymers are not able to achieve the close contact between cyclodextrins in the chain necessary for the formation of a 2:1 complex.

## APPENDIX

Based on work by Warner et al., we have used our pyrene I/III ratio data to quantitatively determine the stoichiometry and binding constant of the complexes formed between pyrene and β-CD and pyrene and 6-0Mβ-CD. Warner et al. use the following equations to determine whether the complex formed is a 1:1 complex (eq. (2)) or a 2:1 complex (eq. (3)).[5]

$$1/(R_o - R) = 1/(K_1(R_o - R_1)[CD]_0) + 1/(R_o - R_1) \quad (2)$$

$$1/(R_o - R) = 1/(K_1(R_o - R_2)[CD]_0^2) + 1/(R_o - R_2) \quad (3)$$

Where the parameters $R_o$ and $R_1$/ $R_2$ denote the I/III ratios for pyrene in water and in the complex, respectively, and R is the measured ratio at a given CD concentration. In the case of a 1:1 complex, equation (2) is appropriate and a plot of $1/(R_o - R)$ vs. $1/[CD]_0$ should give a straight line. In the case of a 2:1 complex, equation (3) is used and a plot of $1/(R_o - R)$ vs. $1/[CD]_0^2$ should yield a straight line.[5]

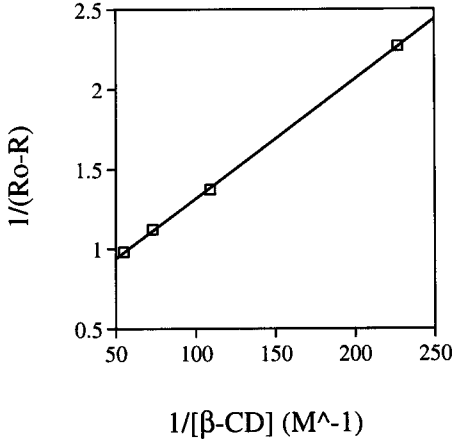
Warner et al. find that the β-CD/pyrene complex is a 2:1 complex, as their data are fit to a straight line by equation (3).[5] Figure 6 shows our double reciprocal plots for pyrene in the presence of β-CD. We obtained the same results for pyrene in the presence of 6-0Mβ-CD (Figure 7). In each case,

although all our prior data indicate that both these CDs form 2:1 complexes with pyrene, we find that our data are fit by a straight line using equation (2). This would indicate that a 1:1 complex is forming between the cyclodextrins and pyrene.

A possible explanation for these results is that our fluorometer can not sufficiently resolve the I/III ratio differences at different CD concentrations. If this is the case, these I/III measurements should be repeated on a different fluorometer with better resolving power.

**Figure 6**: Double Reciprocal Plots (pyrene in the presence of β-CD)

Top: $1/(Ro-R)$ vs. $1/[\beta\text{-CD}]$ (eq. 2)
Bottom: $1/(Ro-R)$ vs. $1/[\beta\text{-CD}]^2$ (eq. 3)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1
0.5
1/(Ro-R)
50
100
150
200
250
1/[β-CD] (M^-1)

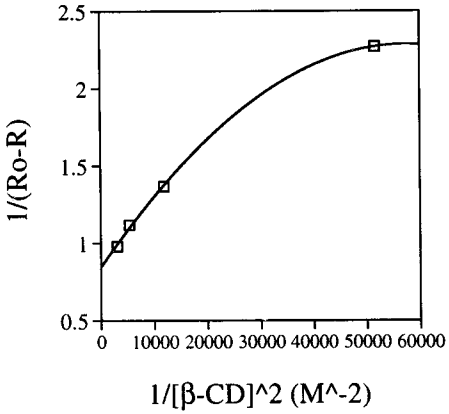
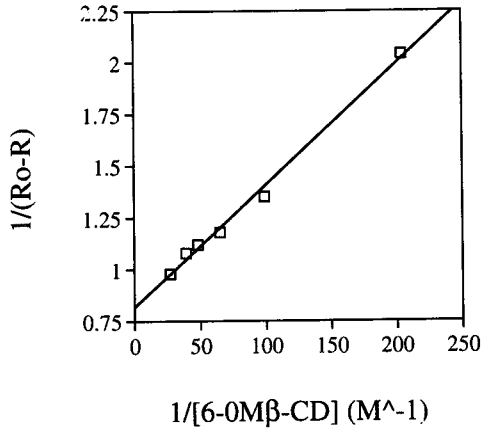
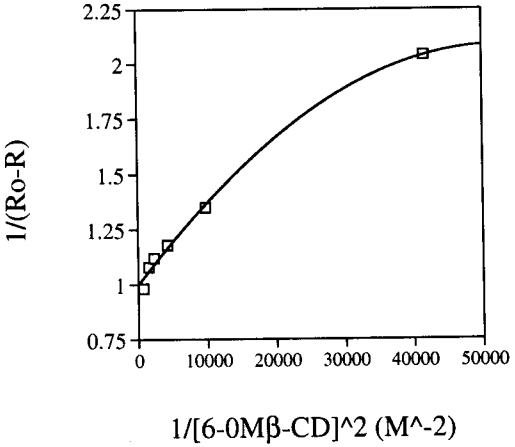

2.5
2
1.5
1
0.5
1/(Ro-R)
0
10000
20000
30000
40000
50000
60000
1/[β-CD]^2 (M^-2)

**Figure 7**: Double Reciprocal Plots (pyrene in the presence of 6-0Mβ-CD)

Top: $1/(Ro-R)$ vs. $1/[6\text{-}0M\beta\text{-}CD]$ (eq. 2)
Bottom: $1/(Ro-R)$ vs. $1/[6\text{-}0M\beta\text{-}CD]^2$ (eq. 3)


2.25
2
1.75
1.5
1.25
1
0.75
1/(Ro-R)
0
50
100
150
200
250
1/[6-0Mβ-CD] (M^-1)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2
1.75
1.5
1.25
1
0.75
1/(Ro-R)
0
10000
20000
30000
40000
50000
1/[6-0Mβ-CD]^2 (M^-2)

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