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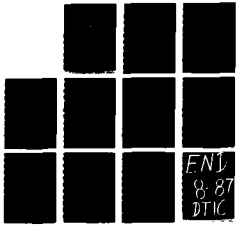

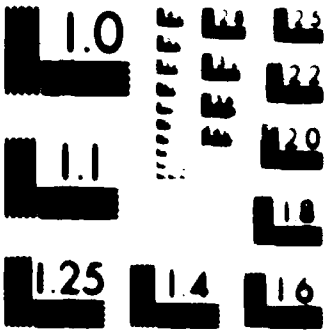
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INCLUSION COMPLEXES OF DIISOPROPYL FLUOROPHOSPHATE WITH CYCLODEXTRINS

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INTRODUCTION:

The catalytic hydrolysis of Diisopropylfluorophosphate (DFP) and other phosphorus esters have been of great interest to the Navy. The discovery of the enzyme DFPase shows great promise for the hydrolysis of DFP, but a major problem with enzymes is their poor stability and limited available quantities. By investigating the use of enzyme mimics for the hydrolysis of DFP the stability and quantity problems can be avoided. One of the best known molecules used in biomimetic studies is cyclodextrin (CD). In this research we studied the inclusion complex of DFP with alpha, beta, and gamma cylcodextrin using Nuclear Magnetic Resonance (NMR) techniques.

Cyclodextrins are cyclic oligomers of glucose. The most common ones are the hexamer(α),heptamer(β), and the octamer (δ) which have a molecular weight around 1,000 (Figure 1). Cyclodextrins have a conical doughnut shape with hydroxyl groups on the outer surface while the cavity is hydrophobic, similar to what would be expected for a miniature enzyme. The solubility of cyclodextrin in water is good, making the inclusion of various apolar molecules possible (1).

In solution a 1:1 molar ratio guest-host complex is usually formed. This interaction enables enhanced reaction rates by placing reactants in close proximity to each other. Functional groups can be attached directly to the cyclodextrin molecule making it catalytically active, but first an inclusion complex must be formed with the guest molecule.

The first research of DFP and cyclodextrins was completed by C. Van Hooidonk and J. Breebaart-Hansen (2). They investigated the kinetics and thermodynamics of the reaction of DFP with α-CD in aqueous alkaline media. By calorimeteric measurments they determined that an inclusion complex was formed. A recent article (3) on the interaction of phosphorus containing aromatic compounds with α-CD indicate that the addition of the phosphate ester group makes the molecule to bulky too form an inclusion complex. This work addresses the question of whether DFP forms an inclusion complex with α, β, or γ-CD.

The characterization of the guest-host inclusion complex can give valuable information as to what type of cyclodextrin derivative should be synthesized to enable greater success in obtaining the catalytic activity desired. The use of ^{1}H-NMR to study the inclusion of aromatic hydrocarbons by cyclodextrins has been previously studied (4). Peaks for the H-3 and H-5 atoms of α-cyclodextrin, which are directed toward the interior of the cyclodextrin cavity (Figure 2), showed significant chemical shifts when

substituted benzoic acids were added to cyclodextrin solutions in D_2O. Atoms which reside on the exterior of the cavity, H-1, H-2, H-4, had only marginal shifts. The large upfield shift for the H-3 and H-5 atoms is due to the anisotropic shielding effect of the aromatic rings of the benzoic acids included in the cyclodextrin cavity.

The change in chemical shift of the internal protons due to the inclusion of a guest molecule can help determine the guest-host molar ratio complex, the binding constant (K) and the molecular disposition of guest compounds. We have studied the DFP/CD complex by ^{1}H-NMR and ^{31}P-NMR. The ^{31}P-NMR was helpful in confirming the ^{1}H-NMR data and also in clarifying the molecular disposition of DFP in the cyclodextrin cavity.

EXPERIMENTAL:

The following materials were used: α-CD, β-CD, γ-CD, DFP (Sigma Chemical Co., St. Louis, Mo.). All cyclodextrins were recrystalized twice from water.

For the proton NMR studies all cylcodextrins were dried in a vaccum oven at 60 ^{o}C for 24 hrs. For the binding studies all solutions were made with 99.9% D_2O to give a final cyclodextrin concentration of 4.4 mM, and varying amount of DFP were added. No internal ^{1}H NMR reference was added since the possibility of reference binding to the

cyclodextrin could not be excluded. An external DDS (in D_2O) sample was used.

The spectra were recorded on a Varian 200-MHz spectrometer. In aqueous solution the resonances from only the nonexchanging hydrogens attached to carbons are detected. The assignment of the CD spectra were determined from previous work (4),(5).

The chemical shift value of the i^{th} hydrogen of CD is referenced to the chemical shift of the hydrgen on C_1, H-1. The external H-1 hydrogen was chosen as a reference since DFP inclusion would have a minimal effect on the H-1 chemical shift. Any changes in the $\Delta\delta_i$ for inner-surface hydrogens is due to the added guest and may be related to the nature of the adduct and not to pH or solvent effects. Figure 3, 4 and 5 show the $\Delta\delta$ of alpha, beta and gamma-CD respectively over the molar ratio of DFP/CD. Table 1 shows the change in $\Delta\delta$ over the complete range of molar ratios of DFP/CD, (R). The range of R was only 2.0 for α-CD since only a 1:1 inclusion complex was feasible. The range was extended to 5.0 and 10.0 for β and γ-CD respectively since a 2:1 complex was a possibility for these two cyclodextrins.

For the ^{31}P-NMR experiments a 5 mL 2.27mM DFP (in D_2O) sample was used with the addition of varying amounts of solid CD. The ^{31}P-NMR spectra were recorded on the same instrument as the ^{1}H-NMR data. An external reference of

phosporic acid was used. A graph of δ_P vs. R of the three cyclodextrins is shown in Figure 6 (δ_P = the average of the DFP doublet).

RESULTS AND DISCUSSION:

^{1}H-NMR STUDIES: The use of ^{1}H-NMR for the detection of the inclusion of aromatic compounds by cyclodextrins has been demomstrated by other researchers (4), (6). They have shown that if inclusion takes place, the screening environment produced by the ring current should be sensed by hydrogens on the inner suface (H-3 and H-5) of the cyclodextrins. With the same reasoning we expected measurable screening of the internal protons even with the inclusion of a non-aromatic molecule such as DFP. The amount of shielding the internal protons would experience by the inclusion of DFP was expected to be less than the inclusion of an aromatic since there is no ring current effect. We also would expect that the outer protons would not be effected by the inclusion of DFP, as was found with the inclusion of aromatic substances.

α-Cyclodextrin binding studies with DFP are shown as a plot of the values of $\Delta\delta$ vs. R in Figure 3. There was no change in $\Delta\delta$ by the addition of DFP, indicating that an inclusion complex is not formed. All $\Delta\delta$ are constant to within 0.010 ppm over the complete range of R. It may be

that α-CD is too small for the bulky isopropyl groups of DFP to fit into the cavity making it impossible for inclusion.

Figure 4 shows the β-cyclodextrin ^{1}H-NMR binding studies which proved to be more interesting than the α-CD data. Like the α-CD the $\Delta\delta_2$ and $\Delta\delta_4$ remain almost constant within 0.010 ppm for the complete range of R. However, a large change in shielding is experienced by both the H-3 and H-5 atoms. The $\Delta\delta_3$ initially at R=0 is 1.104 ppm and increases to 1.143 ppm. $\Delta\delta_5$ is initially at 1.232 ppm and also increases to an upper limit of 1.288 ppm. The H-6 hydrogens which are located on the smaller end (primary hydroxyl side) of cyclodextrin and directed inwards in the gauche gauche conformation (4) are also effected, $\Delta\delta_6$ increased slightly with a total change of .016 ppm.

The ^{1}H-NMR data of γ-cyclodextrin binding to DFP is diplayed in Figure 5. $\Delta\delta_2$, $\Delta\delta_4$, and $\Delta\delta_6$ are all constant within 0.011 ppm. Both $\Delta\delta_3$ and $\Delta\delta_5$ increase with the addition of DFP upto 1:1 molar ratio. The total increase for the range of R=10 for $\Delta\delta_3$ was 0.033 ppm and $\Delta\delta_5$ was 0.049 ppm which is similar to β-CD. There appears to be no break at 1:1 for the complex as seen with β-CD. This may indicate loose binding of DFP with γ-CD compared to tighter binding with β-CD.

We can assume that the $\Delta\delta_i$ for R=0 are the values for the water-included adduct or the empty cyclodextrin and that at large R represent the DFP-CD complex (4). With this assumption and that a 1:1 complex is formed, the $\Delta\delta$ data can

be used to estimate the binding constant, K for the DFP-CD adduct. Table 2 shows the $\Delta\delta$ values of complexed and uncomplexed β-CD and γ-CD for H-3 and H-5 atoms and also the calculated binding constants associated with these atoms. For β-CD K is 1.4×10^3 M^{-1}, and 1.25×10^3 M^{-1} for H-5 and H-3 respectively. The K values for γ-CD are 69.5 M^{-1} and 75.97 M^{-1} for H-5 and H-3. The calculated K by H-5 or H-3 agree nicely for both beta and gamma-CD. The H-5 proton in both cyclodextrins experiences a greater interaction with DFP than H-3. This may be due to a tighter fit of the isopropyl groups of DFP into the smaller part of cyclodextrin. Neither in β-or γ-CD does the $\Delta\delta$ of H-6 move significantly indicating that the DFP molecule does not enter from the smaller end of the cavity. The change in ^{1}H-chemical shift data gives evidence that DFP may enter from the larger opening and fit more snuggly into the smaller part of CD. From the the ^{1}H-NMR the molecular disposition of DFP has not yet been determined but it is expected that the isopropyl groups are in the cavity and the phosphorus is outside of the larger end of both β and γ-cyclodextrin.

^{31}P-NMR STUDIES: The ^{31}P-NMR studies look at the guest instead of the host molecule, the reverse of the ^{1}H-NMR experiments. These experiments were done by keeping the DFP concentration constant and adding solid CD to the NMR tubes. To interpolate the data a molar ratio of CD to DFP (R) had

to be used. Figure 6 shows the change in chemical shift of the phosphorus in DFP (δp) over a range of R from 0.0 to 4.0 for all three cyclodextrins. This data indicates a 1:1 complex for both β and γ-CD. In both cases we see a break approxomately at 1:1 and then a steep increase in chemical shift as more CD is added. The phosporus is not hidden in the cavity and is effected by the CD solution effects to a large extent at molar ratios above 1.5:1. A theoretical chemical shift maximum associated with only complexation must be used to determine the binding contstant.

The binding constants K, for β and γ-CD were calculated using the uncomplexed p, -9.885 ppm which is the value for DFp with α-CD below 1:1 molar ratio. This value was chosen because it takes into account any change in chemical shift due to the addition of a sugar that does not form an inclusion conplex with DFP. The complexed p that was used for the determination a K was extrapolated to be -10.00 ppm for β-CD and -9.975 ppm for γ-CD. Assuming a 1:1 complex K was determined to be K β-cd = 1,837, and K γ-cd = 169. These K values are not as accurate as the values calculated by the H-5, H-3 protons, but are relatively close.

CONCLUSION:

Understanding the orientaion and type of binding of a guest molecule to cyclodextrins can give insight into the engineering of functionalized cyclodextrins. These results indicate that α-CD is not a good candidate for

derivatization since it appears not to make an inclusion complex with DFP. The ^{1}H-NMR studies seem to be much more accuarate in determining if and inclusion complex has been formed then observing a change in thermodynamic parameters (2). The ^{1}H-NMR studies are not affected by any change in solvent or pH while enthalpy and entropy calculations can be.

Both β and γ-CD are able to make a 1:1 inclusion complex with DFP. The molecular disposition of DFP in the cavity of both β and γ-CD apears to be the same. The isopropyl groups are in the cavity and the phosphorus is outside of the secondary hydroxyl end of cyclodextrin, this orientation is displayed in Figure 7.

From this data it appears that functional groups for the hydrolysis of DFP should be attached to the 2^{o} hydroxyl end of β and γ-CD. This would enable the reacting groups tobe in close proximity to the P-F bond. At this moment it is difficult to predict which CD, beta or gamma will have the best catalytic rate once derivatized. Even though β-CD has much tighter association with DFP then γ-CD, this does not automatically mean it will have better activity. Previous work (1) has shown that the catalytic activity of cyclodextrins can be independent of associaton constants. Hopefully the work presented in this paper will not only help guide in the construction of enzyme mimics for the hydrolysis of DFP but also in the catalysis of other phosphorus esters as well.

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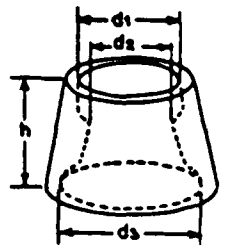
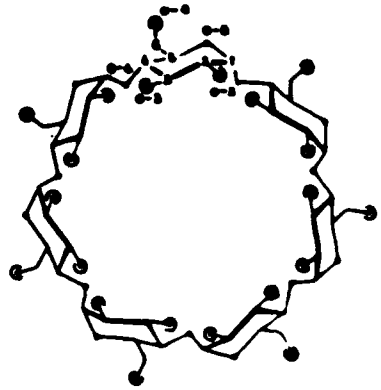




Cyclo-dextrin	d_1 (Å)	d_2 (Å)	d_3 (Å)	h(Å)
α	5.6	4.2	8.8	7.8
β	6.8	5.6	10.8	7.8
γ	8.0	6.8	12.0	7.8

Figure 1: Shape and structure of the cyclodextrin cavity.

Figure 2: The approximate location of the non-exchangeable protons of cyclodextrin are shown in this figure.

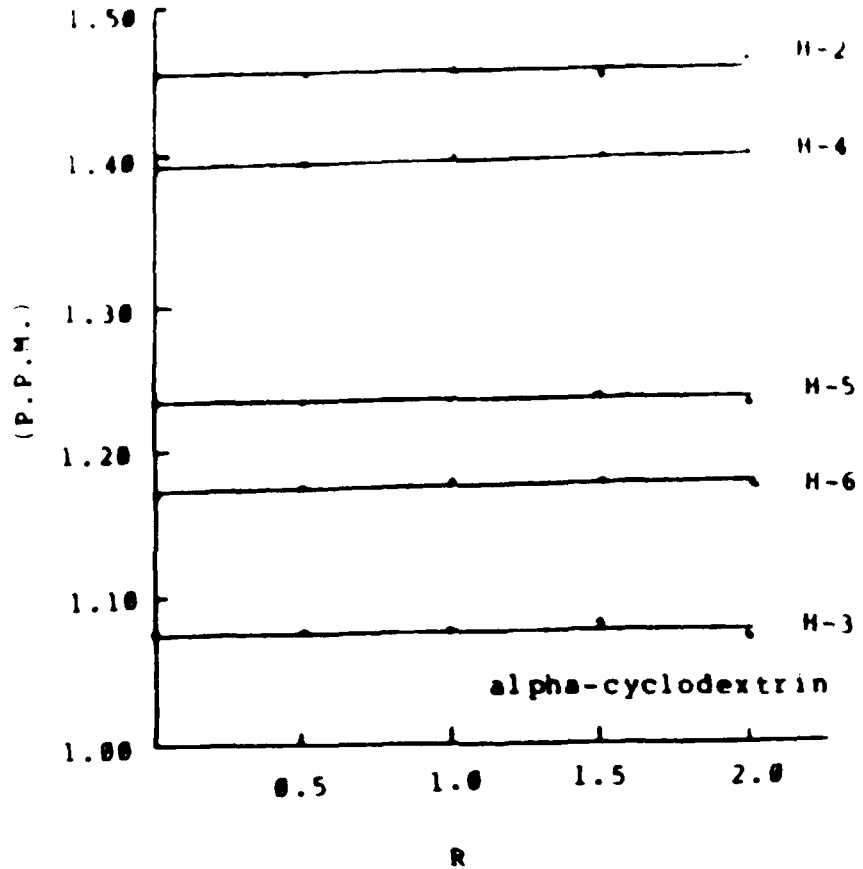


Figure 3: ^{1}H-NMR results of alpha-cyclodextrin with DPP displayed as a graph of p.p.m. vs. R (R equals the molar ratio of DPP to CD).

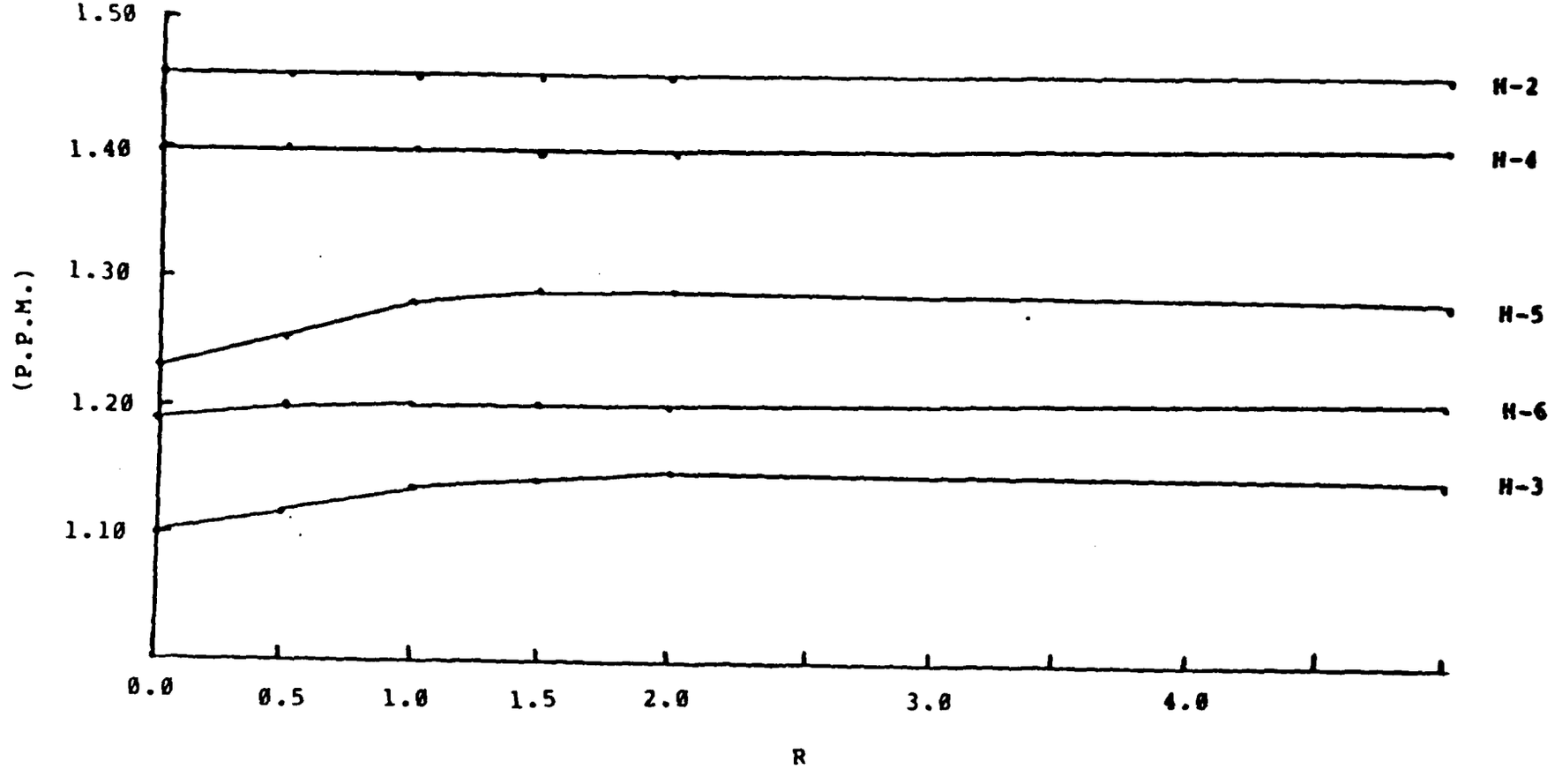


Figure 4: ^{1}H-NMR results of beta-cyclodextrin with DFP displayed as a graph of p.p.m. vs. R (R equals the molar ratio of DFP to CD).

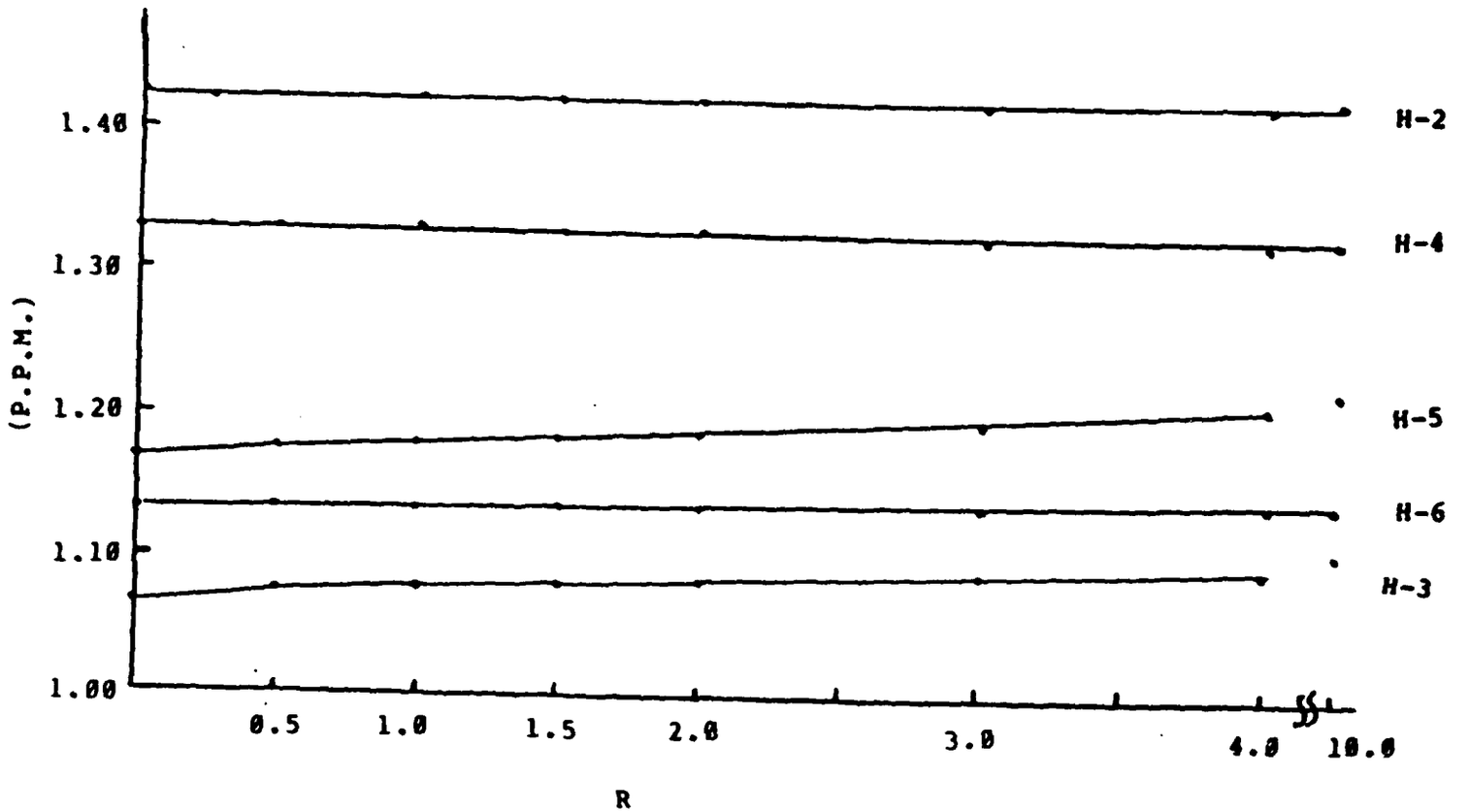


Figure 5: ^{1}H-NMR results of gamma-cyclodextrin with DFP displayed as a graph of p.p.m. vs. R (R equals the molar ratio of DFP to CD).

Figure 6: ^{31}P-NMR results of alpha, beta and gamma cyclodextrin with DFP displayed as a graph of p.p.m. vs. R (R is equal to the molar ratio of CD to DFP).

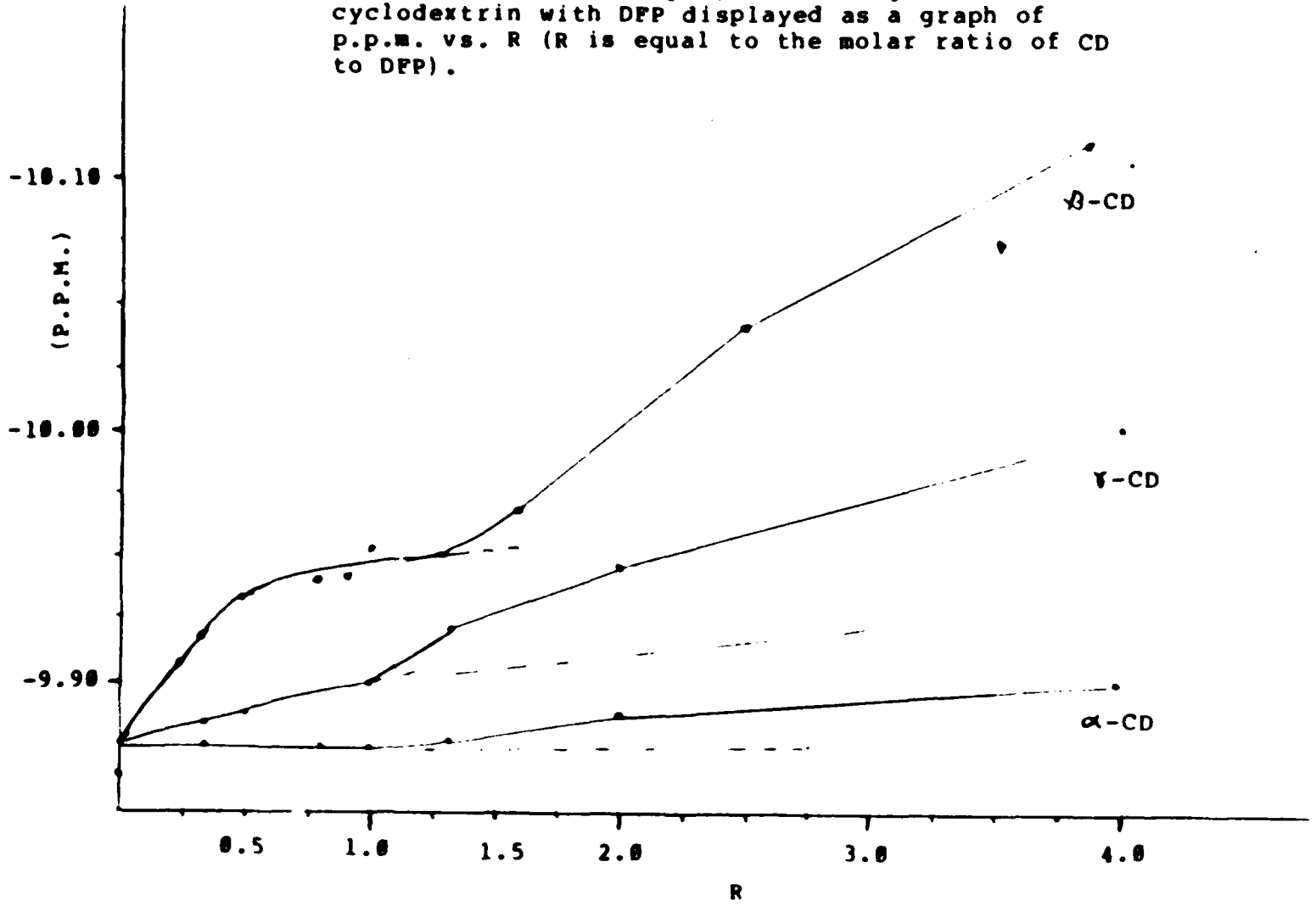


Table 1: The change in ^{1}H-NMR $\Delta\delta_i$ (ppm.) for α, β, and γ-cyclodextrins over R.

R	H-2	H-3	H-4	H-5	H-6
α-CD	0.006	0.010	0.009	0.003	0.010
β-CD	0.008	0.039	0.009	0.056	0.016
γ-CD	0.011	0.033	0.011	0.049	0.004

The R is the range of DFP/CD molar ratio that the change in $\Delta\delta_i$ was measured. $\Delta\delta_i$ is the chemical shift of the i^{th} proton in reference to H-1 proton.

R for α-CD from 0 to 2.0.
R for β-CD from 0 to 4.0.
R for γ-CD from 0 to 10.0.

TABLE 2: Complexed and uncomplexed delta chemical shifts for H-3 and H-5 of β-CD and γ-CD. Binding constants for β-CD and γ-CD.

	β-cyclodextrin		γ-cyclodextrin	
	H-3	H-5	H-3	H-5
$\Delta\delta u$	1.184	1.232	1.174	1.271
$\Delta\delta c$	1.143	1.288	1.208	1.328
K (M^{-1})	1,258	1,488	69.5	75.97

$\Delta\delta u$ = uncomplexed CD. $\Delta\delta c$ = complexed DPP/CD (values taken from Figure 2 and 3). K was determined by the and chemical shift equation: $\Delta\delta_r = \Delta\delta_u N_u + \Delta\delta_c N_c$.
$\Delta\delta_r$ = $\Delta\delta$ at a specific R (DPP/CD molar ratio).
N_c = mole fraction of complexed CD.
N_u = mole fraction of uncomplexed CD.

SYNTHESIS OF FUNCTIONALIZED CYCLODEXTRINS

All of the synthetic work was done with beta cyclodextrin (CD) becuase of the low cost of beta-CD and the many literature reports on beta-CD derivatization. Also it was believed that beta-CD was the most likely CD to be able to catalitically hydrolyze Diisopropylfluorophosphate (DFP).

One synthetic approach involved the functionalization of the primary hydroxyl or smaller end of -CD while the other experiments involved the specific derivatization of the larger end (secondary hydroxyl) of the molecule. It is believed that attaching functional groups to the larger end might be more useful since ^{1}H-NMR indicates that the P-F bond is exposed at this end.

The first part of this section will go into experimental detail on the synthesis of each compound and then show the progress on the isolation of these derivatives.

Regiospecific A,C-Disulfonate Capping of Beta-Cylodextrin

Reference followed:

Tabushi, I., Kuroda, Y., Yokota, K., and Yuan, L. C., J. A. C. S., 103, pp. 711-712, 1981.

The procedure described in the above reference was used for the benzophenone-3,3'-disulfonyl chloride (B) capped

beta-CD. 2 g of beta-CD dissolved in 50 mL of distilled pyridine was treated with 0.68 g of B (1) at 60°C and stirred for 3 hrs.

The work up used with the best results was as follows: The sample was brought to dryness by rotoevaporation. The reaction mixture was dissolved in 15-20 mL of $EtOH/H_2O$ (3/1 by vol.). This solution was then slowly added to 200 mL CH_3CN/H_2O (5/1 by vol.) with constant stirring at rm. temperature. The precipitate was filtered off and the filtrate was evaporated to 20 mL. The precipitate was mostly beta-CD and some polymeric material. The filtrate contained the capped CD as determined by TLC. The concentrated filtrate was then added to 200 mL of acetone. The precipitate was recoverd and found to contain crude product by TLC. The TLC mixt used was CH_3CN/H_2O (5/1) with silica plates. The pure capped material was obtained by preparative TLC and characterized by ^1H-NMR to be the desired product.

1. The benzophenone-3,3'-disulfonyl chloride used was purified by recrystallization of material obtained by the procedure in J. of Polymer Science; Part A-1, 6, pp. 2022, 1968.

Selective Sulfonation of a Secondary Hydroxyl Group of Beta-Cyclodextrin

The procedure used was from: Ueno, A., and Breslow, R., Tet. Lett., 23, pp. 3451, 1982.

The information given in this article is minimal for the synthsis of the secondary toylated beta-CD.

I was able to make the 3-nitrophenyl tosylate by the following procedure. R. S. Tipson, J. Org. Chem., 9, pp. 238, 1944.

p-Toluenesulfonyl chloride (1.1 moles per mole of alcohol) was added to the phenol (10 g) in dry pyridine (100 mL), cooled to -5° in an ice-salt bath. The mixture was shaken in an ice-bath until the sulfonyl chloride was dissolved, and then at 0° for a further 2h. It was then treated with portion (1, 1, 1, 2, and 5 mL) of 5° water at intervals of 5 min, followed by a further 100 mL of water. The toluenesulfonate then crystallized from solution and was filtered off.

Several attempts were made to reproduce this Breslow paper but were unsuccessful getting the CD and the 3-nitrophenyl tosylate dissolved in the alkaline solution.

Preparation of 3A,3C and 3A,3D Beta-cyclodextrin disulfonates

The attachment of the beta-naphthalenesulfonyl group to the 2° end of beta-CD was accomplished by following the

procedure in the article by Fujita, K. Tahara, T. Imoto, T. and Koga, T., in J.A.C.S., 108, pp. 2030, 1986.

Changes made in the workup of the reaction mixture were as follows. After filtration of the excess beta-naphthalenesulfonylchloride, the filtrate was rotoevaporated to a small volume then added to acetone (200 mL). The precipitate contained crude product as determined by TLC. The TLC mixture used was n-propanol: ethylacetate: water, 7:7:5 by vol.

Nicotinoyl Functionalized Beta-Cyclodextrin

Recent catalyitic studies of DFP with nicotinyl groups gave these two cyclodextrin derivatives special interest. The experimental procedure used was from Goren, Z. Dan, P. and Willner,I., Chemistry Letters, pp. 845-848, 1984.

The 3-nitrophenylnicotinate and the isonicotinate ester were made by the same procedure as 3-nitrophenyl tosylate described above. Changes from the literature procedure in the beta-CD nicotinate synthesis are as follows. For the nicotinoyl group the reaction had to reflux for atleast 24 h. The isonicotinoyl reaction was left for 48-62 h. The reaction mixtures were rotoevaporated to a syrup, and then dissolved in 10-15 mL of water. This solution was then added to 20-40 mL of TLC solvent to precipitate out most of the unreacted beta-CD. The TLC mix was n-butanol: ethanol: water, 5:4:3 (by vol.). The nicotin yl-beta-CD was purified

by semi-prep HPLC and characterized to be at least 70% pure by ^{1}H-NMR.

Purification of Crude Reaction Products

Many of the derivatized CD compounds have similar properties to beta-CD, thus making them difficult to separate. The most common technique used is column chromatography. I tried silica, and sephdex G-25 columns with no success. The purification procedure that showed the best results was the use of a C_{18} HPLC column. Samples that were separated by analytical HPLC were the benzophene capped-CD and the two nicotinoyl derivatives. The semi-preparative column was only used on the nicotinoyl-CD derivatives. The conditions used are given below.

Column: C_{18} analytical

Flow rate: 1 mL/min.

Detection: Ab 214 nm

Injection: 20 uL (6 mg/mL)

Program: 5% methanol in water for 0.5 min. At .5 min. change to 25% methanol for 20 min. wash column at 80% methanol, then re-equilibrate at 5% mathanol.

Semi- Preparative HPLC

Column: C_{18}

Flow rate: 4 mL/min.

Detction: Ab 214 nm

Injection: 1.0-1.5 mL (100-150 mg)

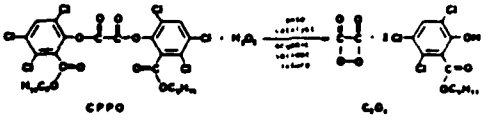
Program: 5% methanol for 3 min. 25% methanol for 65 min. Then wash with 80% methanol and re-equilibrate with 5% methanol.

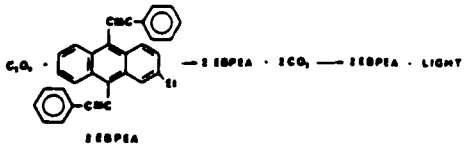
The size of fraction taken depended on the size of the peak. The fractions were rotoevaporated to dryness and then ran on a TLC to determine their purity. Also ^{1}H-NMR were ran on the samples having the R_f of the nicotinoyl and isonicotinoyl derivatized beta-CD.

EFFECTS OF CYCLODEXTRIN ON THE CHEMILUMINESCENCE OF "CHEMILITE" DYES

The Naval Weapons Center has been involved in the research and development of chemilite sticks for many years. The chemilite sticks have been useful for rescue and navigation. One problem the light sticks have is that the chemiluminescence (CL) reaction is inhibited under antartic and artic conditions. Several additives have been tried to enhance CL output at freezing temperatures but nothing seemed to work. Recent literature reports on CL and fluorescence enhancement in the presence of cyclodextrins (CD) have initiated our investigation of the interaction bettween CD and the chemilite reaction (1), (2).

The CL reaction is illustrated by the following equations:





The oxidation of bis(2,4,5-trichloro-6-carbopentoxyphenyl)oxalate (CPPO) by hydrogen peroxide (H_2O_2) to give dioxetanedione (C_2O_4) is catalyzed by weakly basic salts in organic solvents and the reaction is undoubtedly more complex than is shown here. The C_2O_4 intermediate, an unstable, energy-rich species, transfers chemical energy to the fluorescor, which is excited to its first electronic

singlet state. The excited molecule then fluoresces with the emission of its characteristic color. The three dyes studied were 2-ethyl-9,10-bis(phenyethynyl)-anthracene (2EBPEA) (green), rubrene (orange) and 9,10-diphenyl-anthracene (blue). The solutions were made up following Dr. H. Richter's procedure (3).

All three dyes where studied with all three cyclodextrins, alpha, beta and gamma. In general the alpha -CD did not enhance or prolong CL. Both beta and gamma had some effect on all the dyes. The results from visual estimation are shown below on a scale from 0 (control) to 4 (longest prolonged CL).

DYES	GREEN	ORANGE	BLUE
control	0	0	0
<u>beta-CD</u>			
1mg/ml	1	1	1
10mg/ml	3	3	4
<u>gamma-CD</u>			
1mg/ml	2	2	3
10mg/ml	4	4	4

Similar results were found when the % transmittance was checked using a UV VIS spectrophotometer with CL from the sample as the only light source. The observation made was that CD is not very soluble in the oxalate solvent, Dibutylphthalate (DBP). The CD precipitant on the bottom of the test tube appeared to have the most amount of CL. When the test tube was mixed so that the CD solid was suspended, the % transmittance would go up. This suggested that the CD was trapping the CL intermediate, thus prolonging the CL reaction.

The concentrations of dyes and CPPO used in these experiments were much lower than what is used in the chemilite sticks. The use of CD is limited by solubility, and a possible future project would be to derivatize CD molecules to make them more soluble in DBP. If the derivatized CD are still able to enhance and prolong CL at applicable concetrations, this system maybe of great use to the Navy.

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