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Transition State Stabilization by Potential Inhibitors

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Abstract

In the presence of potential inhibitors the cleavage of *p*-nitrophenyl acetate (pNPA) by ß-cyclodextrin (ß-CD) in aqueous base was demonstrated to differ in its kinetic behaviour when compared to the cleavage of *m*-nitrophenyl acetate (mNPA). Whereas the reaction of mNPA is inhibited in the manner expected, that of pNPA is generally not inhibited to the same extent and in some cases, acceleration is observed. Thus, various potential inhibitors (I) of the pNPA cleavage reaction actually mediate the cleavage of this substrate. Alcohols, carboxylate and sulfonate ions exhibited a strong correlation between the affinity of binding to ß-CD and rate constants for the I-mediated reaction of pNPA. Depending on the dimensions of the potential inhibitor that was used, the effect it had on the cleavage of pNPA in the presence of CDs varied from straight inhibition, to inhibition that falls short of the extent expected for competitive inhibition, to mild catalysis. Straight inhibition of pNPA cleavage was demonstrated with ß-CD in the presence of 1,6-hexanediol and iodide ions as well as the dianions of suberic and adipic acid. Likewise, with α-CD it was found that t-butanol and neo-pentanol inhibited the reaction. Most of the other potential inhibitors behaved as "molecular spectators" and five: n-propanol, iso-propanol, n-butanol, 2-butanol and t-butanol showed mild catalysis with ß-CD.

Overall, the results are consistent with cleavage of mNPA occurring with its aryl moiety inside the CD ring, whereas cleavage of para esters must occur with the phenyl ring lifted out of the CD to facilitate attack by the nucleophilic hydroxyls lining the perimeter of CD. The potential inhibitors that aid the reaction act as inert

spacers between substrate and CD, assisting in the achievement of transition state geometry for the reaction.

More pronounced rate accelerations were observed with CD mediated cleavage of *p*-nitro-phenyl hexanoate (pNPH) than with pNPA, but the catalytic effect showed saturation at high [I]. The results were interpreted in terms of two significant rate processes, cleavage of pNPH in the medium via a CD·S complex and reaction through a ternary complex (S.CD.I). The analyses of the data in the pNPH experiments afforded by application of the principles of transition state theory were useful as a probe of mechanism. A number of linear free energy relationships were plotted to demonstrate the correlations between ground state complexation and transition state binding. It was found that binding of the potential inhibitors in the proposed ternary complexes *and* in the transition state increase in proportion to their affinity for the cyclodextrin cavity. It was deduced that the structures of the transition state and of the proposed ternary complexes are similar, at least as pertains to binding of the potential inhibitor.

The principles of transition state theory that were used to explain the kinetic behaviour and mecahnism of reaction for these simple enzyme models are already being used, in the field of medicine, as a strategy for developing new pharmacophores. In the future, more advanced applications of transition state theory may guide the discovery of compounds with true "magic bullet" properties, that could be used for the amelioration of mutant enzyme function.

Acknowledgements

I would like to thank Dr. Tee for having given me a chance to further my education. I extend thanks to the faculty of Concordia for their colorful personalities and their friendliness, which made my stay in this University a pleasant one.

I wish to thank my parents, for being the best parents a person could possibly think of.

I also wish to give credit to Dr. Fred Ablenas, Brian Takasaki, Tim Gadosy and everyone else I have come into contact with for their explanations, help and friendship during the course of my studies.

Above all, I wish to thank God for giving me Faith and all of the the other good things in life and for decreeing the Laws of physics so that we may have something interesting to study!

> God said, "Let the waters under heaven come together into a single mass and let dry land appear." Genesis 1:9.
>
> "We are indeed in his hand, we ourselves and our words with all our understanding, too, and technical knowledge. It was He who gave me true Knowledge of all that is, who taught me the structure of the world and the properties of the elements." Wisdom 7: 16-17

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Introduction

Structure of Cyclodextrins

Cyclodextrins (CDs), also known as cycloamyloses, cycloglucans, or Schardinger dextrins, were first discovered in 1891, obtained as the result of the action of amylase from *Bacillus macerans* on starch.[1] The main structures are termed α-, ß-, and γ-cyclodextrins, comprising of 6, 7, and 8 glucose units, respectively, in which the D-glucopyranose units are linked α-(1-4) in a cyclic array.(Figure 1).[1] All the glucose units in CDs are in the relatively undistorted C_1 conformation. As a result, each CD is a torus-shaped molecule, resembling a shallow, bottomless bucket.[2]

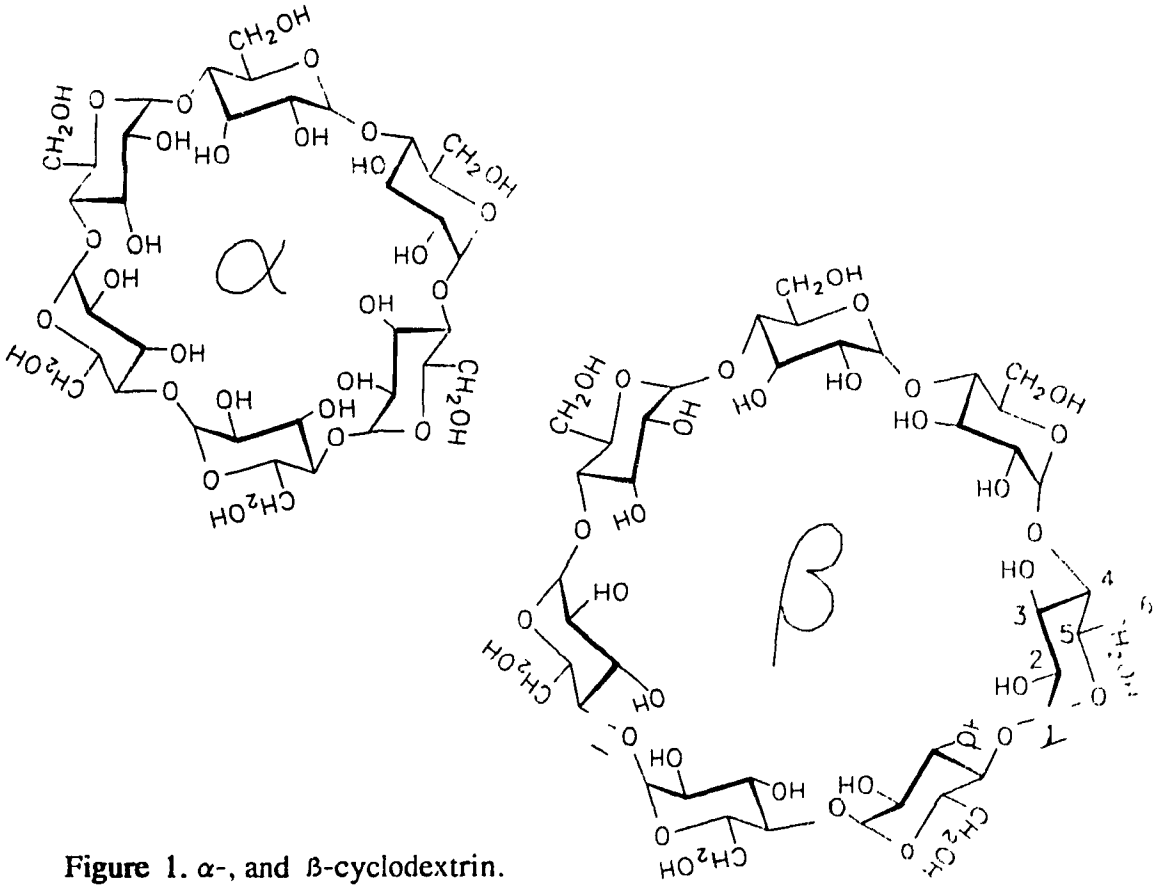


Figure 1. α-, and ß-cyclodextrin.

The hydroxyls of CD molecules are arranged on the both sides of the bucket. The side of the torus which has a wider opening is the one with the secondary hydroxyls (at C_2 and C_3, (figure 2) whereas the primary hydroxyls are rotated in such a manner that they partially block access to the cavity.[1,2]

The cyclodextrin cavity is lined by C-H bonds and glycosidic oxygen bridges.[2] This arrangement forms a relatively hydrophobic interior, surrounded by two rings of hydrophilic hydroxyls, and it is responsible for the interesting binding properties of CDs.

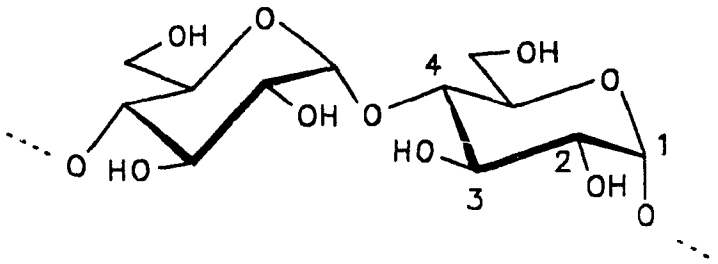


Figure 2. Schematic diagram of two glucopyranose units of a cyclodextrin.[1]

Isolation of Cyclodextrins[1,2]

Due to their different dimensions (Figure 3), cyclodextrins are selectively isolated from solution by complexation with suitably-sized organic molecules.[1,2] The formation of a "host-guest" complex has the effect of reducing the solubility of the CD in water, and the size of the CD cavity dictates which guest is most likely to interact with the cyclodextrin host. For example, α-CD is selectively precipitated from aqueous solution by cyclohexane, ß-CD by fluorobenzene, and γ-CD by tetra or pentacyclic triterpenoids or anthracene. This physical property is also of use in

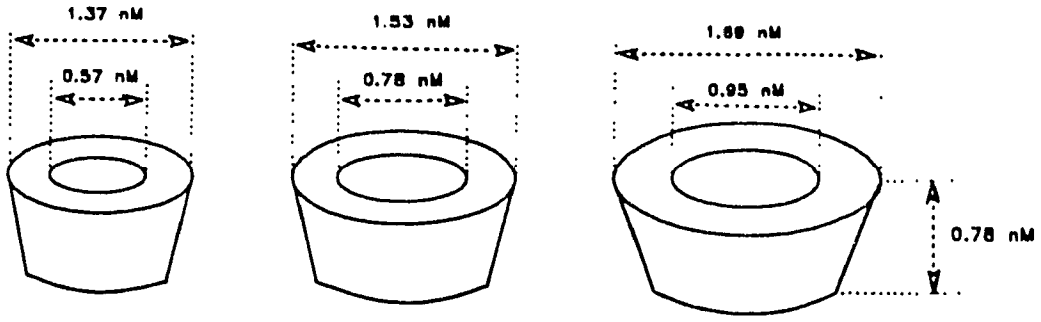


Figure 3. Dimensions of the commonly isolated CDs.

production of the CD rings, as a template to the enzyme-mediated ring closure of the oligosaccharide. Thermodynamically, the formation of ß-CD presents the smallest energy barrier. However if, during the enzyme-mediated process, a compound that is specific for one of these rings is included into the incubation mixture, a highly increased yield for the ring in question is obtained.[2] This strategy has been used to increase significantly yields of α-CD which is otherwise hard to isolate due to the low amounts produced.[5]

The ring shape of cyclodextrins is related to the possible helical structure that amylose and starch adopt in solution, thus enabling the appropriate enzyme to effect ring closure. The helicity of amylose also enables it also to form inclusion complexes and they may play a role in the formation of CDs. Apparently, in aqueous solution the amylose helix usually comprises 6 or 7 glucose units per turn, but the tightness of the helix can be influenced by introducing a variety of organic compounds that complex with it.[2] These considerations may explain the relative abundances of the CDs: ß-CD > α-CD > γ-CD. Strain and steric constraints probably prevent the

formation of rings which are smaller than α-CD.

Properties of Aqueous CD solutions

One of the most important reasons for the attention CDs receive is their ability to form inclusion complexes, which gives them the potential to be enzyme models. The inclusion complexes are formed most effectively with substrates that possess marginal solubility in water, indicating that the transfer of the substrate from aqueous solvent to apolar CD is an important driving force for complex formation.[3] CD will still form inclusion complexes, albeit not as strongly, in dimethylformamide and dimethyl sulfoxide. In more apolar organic solvents such as alcohol, ether, dioxane or pyridine no complexation is observed.[3]

It is not surprising that any given molecule would not form an inclusion complex with CD in dioxane, since spectral analysis of bound molecules reveals a similarity in the spectra of molecules dissolved in dioxane or complexed in CD. This suggests that: 1) The cavity of the CD is less polar than water; 2) the driving force for complexation with CD is counterbalanced if dioxane is the solvent. The interior of a CD has 2 rows of methine (CH) groups, one row of glycosidic oxygens and no hydroxyl groups, whereas dioxane has 4 methylenes and two ethereal oxygen atoms. Thus, it may be expected that any reaction which is encouraged by a lower dielectric medium, should proceed faster in the presence of CD provided its reactants or products, as the case may be, can be bound in the torus of a CD.[3]

The size of the CD pocket imposes fairly rigid limitations on the manner and

strength of complexation. Molecular recognition is important in determining the ease of formation of a complex with CD. Cramer and Hettler documented the effect that the placement and bulkiness of substituents has on the complexation of a typical substrate, a nitrophenol, with α-CD (Table 1). By increasing substituent size at specific positions, it becomes increasingly difficult for the guest to fit into the CD. The 3,5-dimethyl derivative does not complex at all.[2]

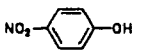
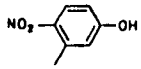
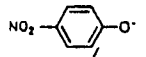
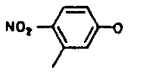
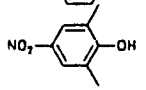
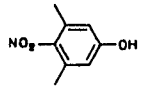
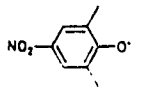
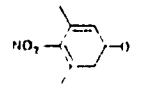
Guest Compound	Dissociation Constant K_d (mM)	Guest Compound	Dissociation Constant K_d (mM)
	53		No binding
	0.4		42
	18		No binding
	0.094		No binding

Table 1 Dissociation constants with alpha-CD at 25 deg.

Uses and Effects of CD

Practical Applications

By virtue of their tendency to form complexes, and because of their low toxicity, CDs have great potential in the food industry as preservatives and taste enhancers.[5] For example, vanillin-glucose mixtures stored in open Petri-dishes at room temperature showed practically no detectable aromatic substance after 240 days, whereas the vanillin ß-cyclodextrin complexes, even after a much longer time the loss was only about 20%. The loss measured with the latter was attributed to the incomplete complexation of analyte with CD.[5]

Besides preventing the breakdown of labile food additives, ß-cyclodextrin may be used to decrease the bitterness of milk casein hydrolysate, ginseng, and propylene glycol, to reduce the disagreeable odour of bone powder, (used as a Calcium supplement in animal fodders), yeast extract, and sodium caseinate solution. It can also be used to modify the physical properties of certain food products such as water retention, emulsion stability, egg-white beatability, retention of volatile components and improvement of soft-drink texture.[5,a]

The pharmaceutical industry also makes extensive use of compounds clathrated by CD to maximize the beneficial effects they provide while minimizing their secondary effects and other shortcomings. By coupling pharmacophores with CD, notable improvements have been made in the reduction of evaporation, hydrolysis and side-effects of drugs before they reach their target tissues.[2] Furthermore, researchers have managed to increase solubility, change liquids to powders, improve ill-tasting and smelling drugs, and reduce the hemolytic and ulcerogenic effects of drugs such as indomethacin, phenothiazines and flufenamic acid by complexation with CD. The sequestering activity of cyclodextrin causes some reactions to be inhibited, eg. hydrolysis of certain compounds, oxidation, light-induced composition, and thermal decomposition.[1,2] Chemists have taken advantage of the complexing ability of CDs to design novel syntheses and enzyme mimics, as detailed in the next three sections.

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[a] Personal experience has also revealed the unpleasant odor of organic acids is decreased if they are in aqueous solution with CD. Also, the solubility of longer-chained acids is increased by α- and ß-CD.

The effects that CDs have on chemical reactions can be loosely categorized under two designations: inhibition and catalysis. Catalysis may be further divided into covalent and non-covalent.[1] Non-covalent catalysis occurs as a result of the favourable microdielectric environment and/or the steric constraints placed on the substrate molecule by the interior of the CD ring. Covalent catalysis is primarily driven by direct involvement of the CD through formation of a covalent bond to the substrate.[1]

Inhibition of Reactions

Cyclodextrin can, in some instances, shield reactants from each other by complexing them. If the reaction is a simple intramolecular rearrangement, it is also possible for CD to lock the molecule into a conformation which is unfavourable for reaction. Also, complexation of a molecule may decrease the rate of a photoactivated reaction.

Many examples exist in literature sources citing the protective and inhibitory effects mediated by CD.[1-5] For example, the hydrolyses of various ethyl and methyl benzoates are all greatly impeded by the presence of CD.[1] Similarly, the intramolecular carboxylate ion attack in glutaric acid esters, the benzidine rearrangement of hydrazobenzene, the photochemical *cis-trans* isomerization of methyl orange, and the photodehalogenation of monohalobenzoic acids are also inhibited or prevented by CD.[1]

New and interesting applications are constantly being found for CD's inhibitor

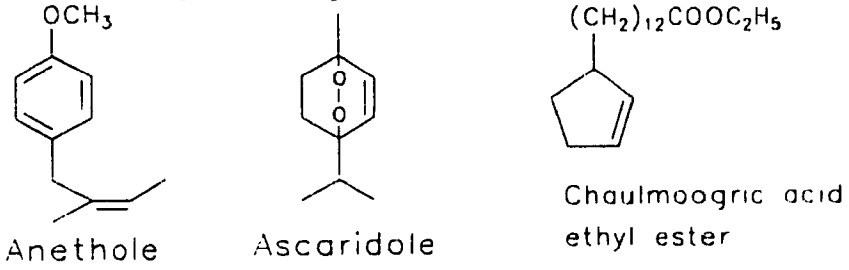
effect. So far, most of the practical applications have come about as the result of the inhibitory effects that CDs exert on chemical processes, a property of use to a number of industries. For example, researchers recently discovered that the photodecomposition (by N-dealkylation) of Coumarin-47, was retarded by a factor of 12 in the presence of ß-CD.[44] This discovery may be of significance to the food industry since coumarin is used as a flavour additive. In agriculture, several patents have been applied for by Japanese companies for procedures pertaining to the stabilization of unstable or very volatile insecticides by encapsulation.[5]

Since post-synthetic reactions of pharmaceutical products are clearly unwanted, considerable research involving CDs is devoted simply to the packaging of drugs.[5] Labile drugs must undergo careful packaging to ensure that consumers receive what is synthesized. For example, Ampicillin oligomerizes in aqueous solution to produce antigenic molecules (dimers to pentamers) which may cause dangerous allergic reactions in some patients. It was found that ß-CD prevents the oligomerization of ampicillin, at neutral pH, without adversely affecting the integrity of the ß-lactam moiety,[45] the preservation of which is crucial to the maintenance of efficacity.[24] The prevention of post-synthetic side reactions is not the only criterion for utilizing CDs in packaging. Extensive evidence has been collected indicating that CD complexation can increase the bioavailibility of certain drugs. Moreover, a significant reduction of deleterious side-effects has been observed upon administration of CD complexed drugs[5]. This factor alone may allow the usage of higher doses before complications arise, leading to a more effective treatment of

diseases with existing drugs.

One of the most important potential applications concerning inclusion complexes is the protection of guest molecules against oxidation.[5] Inclusion of a compound within the CD cavity renders physical access to atmospheric oxygen much more difficult. For example, the oxygen uptake of complexed anethole was found to be ten times less when it was complexed to ß-CD.[2] Likewise, linolic acid, ascaridole and the ethyl ester of chaulmoogric acid which serve as: essential nutrient, Anthelmintic and antileprotic respectively[54], (Figure 4) showed no oxidation after an 8-month period in tabletted form as complexes of ß-cyclodextrin.[2]

Cyclodextrins are not inhibitory to all reactions. The more interesting aspect of CD chemistry is related to the *catalytic* effects they exhibit under certain reaction conditions. The interest stimulated in this regard is due the possibility it offers to gain a deeper understanding of enzyme mechanisms. "One of the principal reasons for its use as an enzyme model is the formation of inclusion complexes between the catalyst and the substrate preceding the catalysis, which is comparable to the Michaelis-Menten complex in enzymatic reactions."[3]



$CH_3(CH_2)_4\text{-}CH{=}CH\text{-}CH_2\text{-}CH{=}CH\text{-}(CH_2)_7\text{-}COOH$

Linolic acid

Figure 4. Labile compounds which are protected by CD

Non-covalent Catalyses[2]

In catalysis, the CD cavity provides a restrictive apolar cavity which instead of inhibiting the reaction, provides a lower energy pathway than reaction in the absence of CD. Non-covalent catalysis is a result of the microsolvent effect that CD affords due to its apolar cavity or to conformational effects due to the geometrical requirements of inclusion.[1]

An example of a favourable microsolvent effect is provided by the catalytic effect of CD observed in a reaction that proceeds through a transition state that is less solvated than the initial state. Thus decarboxylations (Figure 5) of the anions of activated acids, such as α-cyano and ß-keto acids, that are extremely solvent dependent are speeded up by complexation of the substrate anion in the relatively apolar CD pocket.[1]

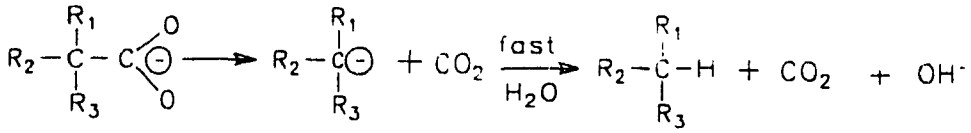


Figure 5 A Heterolytic cleavage which benefits from the presence of CD.

The microsolvent effect may also speed a reaction by shifting the keto-enol equilibrium of a substrate towards the more reactive form, such as in the oxidation of α-hydroxyketones to α-diketones (Figure 6). Cyclodextrin catalyzes this reaction by emphasizing the shift.[1] A more specific example of the conformational effect is shown by the divergent behaviour of α- and ß-CD towards the same reactant.

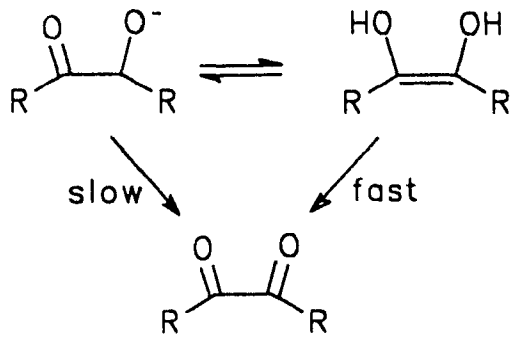


Figure 6 A keto-enol equilibrium influenced by CD.

In the intramolecular decarboxylation of benzoylacetic acid, ß-CD exhibited a considerably larger acceleration than could be expected from only the microsolvent effect, whereas α-CD caused inhibition of the reaction.[1] The discrepancy was reconciled when it was observed that the cyclic transition state of the reaction could not be accommodated by the smaller cavity of α-CD and that ß-CD, on the other hand, *can* include the cyclic ground state conformer.[1] The last experiment illustrates the importance of complementary geometry, hence the term "conformational effect."

Sometimes, the addition of CD can act in conjunction with another catalytic effect to produce a cooperative effect. In fact, the catalytic or cocatalytic effect of cyclodextrin can be quite dramatic. For instance, in the debromination of 4-bromo 4-methyl-2,5-cyclohexadienone, a large rate enhancement is observed when there is a carboxylate group attached ortho to the keto group. Another rate enhancement of similar magnitude occurs when the reaction is carried out in the presence of α-cyclodextrin.[7] (Figure. 7) Here we may say that CD is behaving as a non-covalent cocatalyst. It is interesting to note that the two rate accelerating effects i.e. intramolecular proton transfer and microsolvent effect do not interfere with each

other and are therefore multiplicative, resulting in a very large rate enhancement when both are in effect.[7]

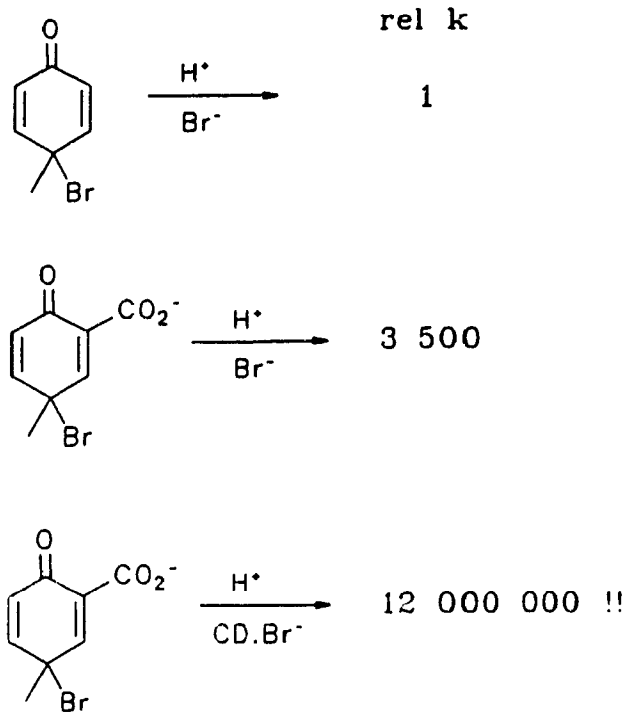


Figure 7. Cocatalysis of debromination by α-CD

Covalent Catalyses

When CD participates directly in a reaction, by forming a covalently bound intermediate, it is termed a covalent catalysis. An example is the reaction with aryl esters. The first step in such a mechanism is the formation of a complex between CD and a substrate, followed by nucleophilic attack on the ester by one of the ionized secondary hydroxyls on the periphery of the CD ring. The covalent tetrahedral intermediate then undergoes cleavage yielding an alcohol or phenol and an acylated CD. The acyl CD then undergoes hydrolysis, regenerating the CD.[1] As

with non-covalent catalysis, the geometry of the substrate is important if it is to interact with the CD. In fact, it is pivotal to covalent catalysis that electrophilic moieties on the substrate be proximal to the nucleophilic hydroxyl groups of CD. If the reactive moieties do not match, then CD may simply protect the guest, as observed earlier.

Some experiments have been conducted in an attempt to maximize the nearness of reactive moieties and the complementarity between guest and host,[11] with the premise that a better fit between substrate and host during reaction will give more effective complexation, which should give a higher probability of reaction. The proper choice of suitably-shaped substrates or substituted host can increase the rate of the process.

The attempt by researchers to find optimal substrates for cyclodextrin mediated reactions parallels the lock and key fit of the enzyme and substrate, postulated by E. Fischer.[24,46] The search for a substrate that will fit the host perfectly is akin to inserting many different keys into a lock. Many keys will fit into the socket, but not all will actuate the tumblers. Thus it is observed that some guests complex well with CD but do not undergo reaction readily because their functional groups are not close enough to the catalytic hydroxyls.[22] The lock and key model was superceded by the more refined induced-fit model of enzyme action when scientists allowed for the fact that enzymes are not totally rigid. This theory states that an enzyme may change conformation to complex a substrate more firmly during reaction. The large number of bonds that comprise an enzyme confer considerable

plasticity, making this possible.[22,23] Thus, the induced fit model of enzyme action is more accurate in its representation of enzyme behaviour. Likewise, in CD chemistry there is a parallel to induced-fit because CD molecules are also not completely rigid. On the contrary, x-ray and neutron bombardment analyses have revealed that CD *does* alter its shape upon binding a guest, becoming more circular.[5] Figure 8 is a schematized representation of the lock and key model and the induced-fit model.

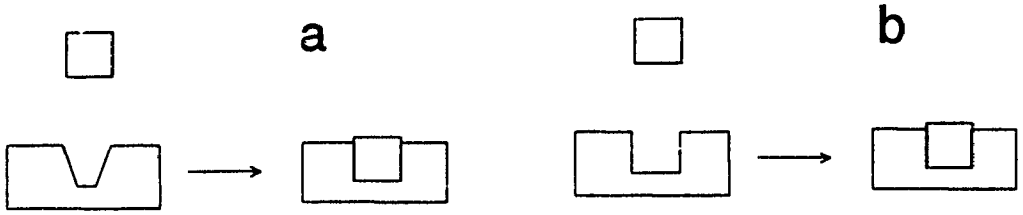


Figure 8. a) Induced Fit b) The Lock and Key fit

The driving force for complex formation between a CD host and a guest is the sum of a number of weak forces, namely: 1) The release of poorly solvated "high energy water" on the interior of the bucket; 2) ring-strain release; 3) Van der Waals interactions; 4) London dispersion forces and; 5) Hydrogen bonding between guest and host.[1,4] The addition of functional groups to CD is known to affect any one or all of the enumerated properties and can add to or diminish the overall rate of catalysis depending on conditions.

The main thrust of research into reactions catalysed by CD has utilized these principles, achieved empirically and by observing the nature of enzymes, to increase the rate and selectivity. In other words, researchers are striving to make CD

catalyzed reactions more enzyme-like.[22] Since many simple chemical processes generally do not proceed with high yield of a single desired product, free of impurities from side reactions, it is more important to obtain improvements in this regard, because from a synthetic organic chemists point of view an increase in selectivity is preferable over merely increasing the rate of reaction.[22]

An example of the selectivity obtained by using CD in a reaction is observed in the chlorination of anisole by hypochlorous acid, which normally yields *o*- and *p*-chloroanisole. When the same reaction occurs in the presence of α-CD, the geometric constraints imposed by complexation with CD prevent the formation of the *ortho* isomer, and enhance the formation of the *para* isomer (Figure 9).[22] The specificity of products thus obtained is potentially useful in applications to synthesis.

Because of the bonding that occurs between the substrate and CD host during covalent catalysis, it facilitates the construction of geometrically correct space-filling (CPK) molecular models. These models give an accurate appraisal of the structures

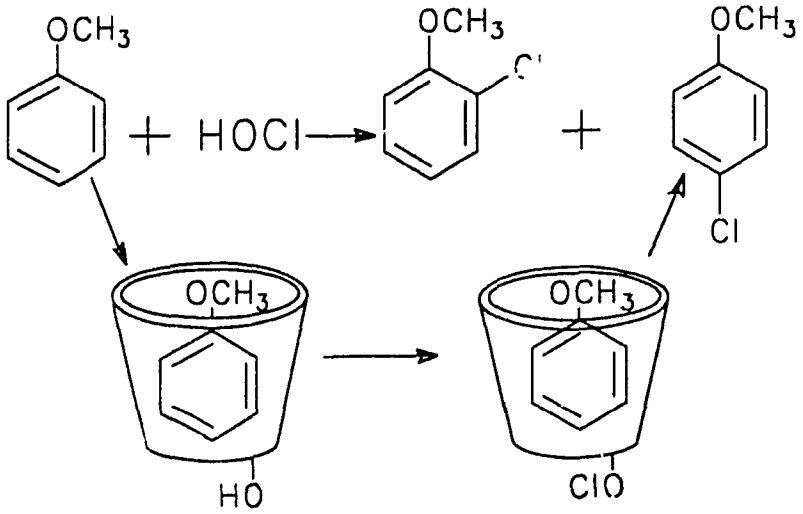
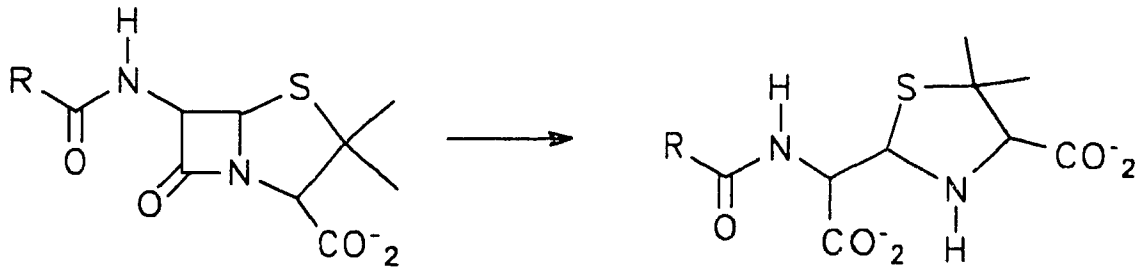


Figure 9. The cyclodextrin-directed chlorination of anisole.[22]

and the spatial relationships during reaction,[1] because not much room for movement is left over if the substrate is both complexed and bonded to the ring. For this reason, that is, the geometric constraints imposed during reaction, the task of guessing the geometry of transition states is rendered feasible, which makes CD catalysed reactions attractive for modelling. For example, it is known that ester cleavage occurs by formation of a tetrahedral adduct. Given the dimensions of the CD ring, a researcher could deduce whether a fully complexed ester of known dimensions undergoing nucleophilic attack would be strained or not. If the reaction proceeds quickly in the presence of CD, then it is safe to say that CD is providing a lower energy barrier for the rate determining step.[16,17] If it is not, then one should be able to pinpoint why.

Another example of covalent catalysis, influenced by CD, is the accelerated cleavage of the strained ß-lactam ring of penicillins (Figure 10). Experiments with these substrates provide another interesting example of the positional effects and the pH dependence of catalysis.[1] Note first of all that CD *protects* Ampicillin[45] from oligomerization and does not appreciably promote its cleavage at neutral pH. At alkaline pH, however, the hydroxyl groups ionize and become nucleophilic.[5] For various side chains (R) on the the penicillin the acceleration hardly varies (21-89) for a range of K_s (3.85-75 mM), implying that transition state binding parallels that of the substrate. Apparently, the "flexible molecular structure"[1] of the penicillin allows orientation of the ß-lactam ring in thje inclusion complex in a reactive conformation. Thus, the reaction proceeds relatively unhindered via attack of a CD hydroxyl on the



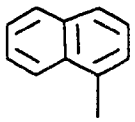
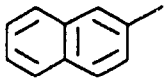
Penicillins	R-group	k_2/k_u	K_S (10^{-3} M)
Methylpenicillin	CH_3	37	33
1-naphthylpenicillin		31	16
2-naphthylpenicillin		89	75

Figure 10. Penicillin cleavage catalysed by CD under basic conditions

β-lactam carbonyl, eventually yielding penicilloic acid.[1] With a more rigid substrate, such as a nitrophenylacetate, the substitution effect is more pronounced as will be discussed later.[1]

In a similar manner, CDs covalently catalyse the breakdown of other amides, esters, organophosphates, sulfates and carbonates (see Table 2).[1] The types of covalent catalysis in these reactions are categorized as: a) Nucleophilic catalysis; b) general base catalysis; c) general acid catalysis.[3] In nucleophilic catalysis the CD molecule is the nucleophile as a result of its ionized secondary hydroxyls. General base catalysis involves enhancement of the nucleophilicity of water by abstraction of

Table 2. Reactions accelerated by cyclodextrins.[3]

Reaction factor	Substrate	Acceleration
Cleavage of esters	Phenyl Esters	300
	Mandelic acid esters	1.38
Cleavage of amides	Penicillins	89
	N-acylimidazoles	50
	Acetanilides	16
Cleavage of organophosphates	Pyrophosphates	>200
	Methyl Phosphonates	66.1
Cleavage of carbonates	Aryl carbonates	7.45
Cleavage of sulfates	Aryl sulfates	18.7
Intramolecular acyl migration	2-Hydroxymethyl-4-NPTMA	6
Decarboxylation	Cyanoacetate anions	44.2
	α-ketoacetate anions	3.95
Oxidation	α-hydroxyketones	3.3

a proton. Examples of reactions proceeding by general acid catalysis alone with CD have not been elucidated. However, in the hydrolysis of p-nitrotrifluoroacetanilide, general acid catalysis aids in the departure of the aniline leaving group.[3]

Cyclodextrin catalysts exhibit some of the features characteristic of enzymes: 1) Substrate specificity; 2) the formation of catalyst substrate complexes prior to chemical transformation; 3) use of different forms of catalysis (see above); 4) use of hydrophobic binding; 5) large accelerations.[3] Because of the fact that the only potential catalytic group on a CD is a hydroxyl group, there are limited possibilities for covalent catalysis. It is therefore not surprising that researchers began modifying the CD molecule itself, in an attempt to increase its catalytic efficiency.[1-6] The advantages of modifying the CD are many-fold: replacement of OH by a better nucleophile that can operate at lower pH; more effective positioning of the substrate for reaction; more rapid deacylation of the intermediate to ensure overall true catalysis; a possible improvement in stereoselectivity for some reactions.[3]

Researchers have functionalized CDs with imidazoyl groups in an attempt to

provide a nucleophile which is more reactive and has a better position for attack on the substrate.[1,3] The incorporation of such a group on CD also increased the rate of the deacylation step since in this case the intermediate is a labile N-acylimidazole. Following this encouraging result, many attempts have been launched to produce better catalysts.[22]

Cyclodextrins have also been modified so as to change their binding properties. For example, Emert and Breslow[2] prepared derivatives of ß-CD with pendant formamido groups in place of the primary hydroxyl groups (Figure 11). This modification increased the affinity for 1-adamantane carboxylic acid 20-fold, but had little effect on catalytic rates.

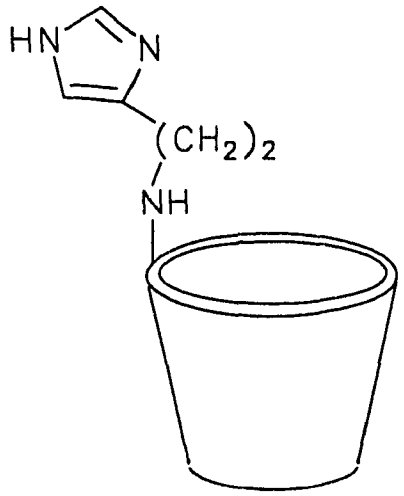


Figure 11. α-Cyclodextrin-histamine, one of the many CD derivatives engineered to improve the catalytic effect for specific substrates.[2]

The other main approach to better catalysis has been to "improve" the substrate; to design a substrate that is more appropriate for the CD catalyst. Using this approach, Breslow and coworkers[13] have achieved rate accelerations of 10^6 to 10^7. The same study also demonstrated an enantioselectvity of 60:1.

Cyclodextrin-Mediated Aryl Ester Cleavage

m- versus p-Substituted Phenyl Acetates

The most striking specificity with respect to substrates is found in the cleavage of phenyl acetates by α- and β-CD.[1] As shown in Table 3, the magnitudes of the acceleration by α-CD, as measured with respect to the rate for the reaction in the absence of CD (k_{cat}/k_{un}) are consistently larger for the meta-substituted esters.[1,3]

Table 3. Acceleration for phenyl acetate cleavage in α-CD solution at pH 10.6.[3]

Acetate	k_{cat} (10^{-2} s^{-1})	k_{cat}/k_{un}	K_d (10^{-2} M)
Phenyl	2.19	27	2.2
m-Tolyl	6.58	95	1.7
p-Tolyl	0.22	3.3	1.1
m-tert-Butylphenyl	12.9	260	0.2
p-tert-Butylphenyl	0.067	1.1	0.65
m-Nitrophenyl	42.5	300	1.9
p-Nitrophenyl	2.43	3.4	1.2
m-carboxyphenyl	5.55	68	10.5
p-carboxyphenyl	0.67	5.3	15.0

The greater ease with which *m*-substituted substrates undergo cleavage is believed to be due to a smaller activation enthalpy for the reaction.[3] It was hypothesized that meta substituents position the substrate in the CD cavity in a geometry that is more appropriate for reaction (Figure 12). Studies of the structure of the ester inclusion complexes, conducted by [^{1}H] NMR spectroscopy, supported

this hypothesis for the *meta-para* specificity. They revealed a correlation between the magnitude of acceleration and internuclear distance for CD hydroxyls and the ester carbonyl in the complex (Table 4). "The order of the increase in the acceleration, mNPA > phenyl acetate > pNPA is identical with that of the decrease in distance between the carbonyl atom of the substrate and the O-2 atom of the CD."[3]

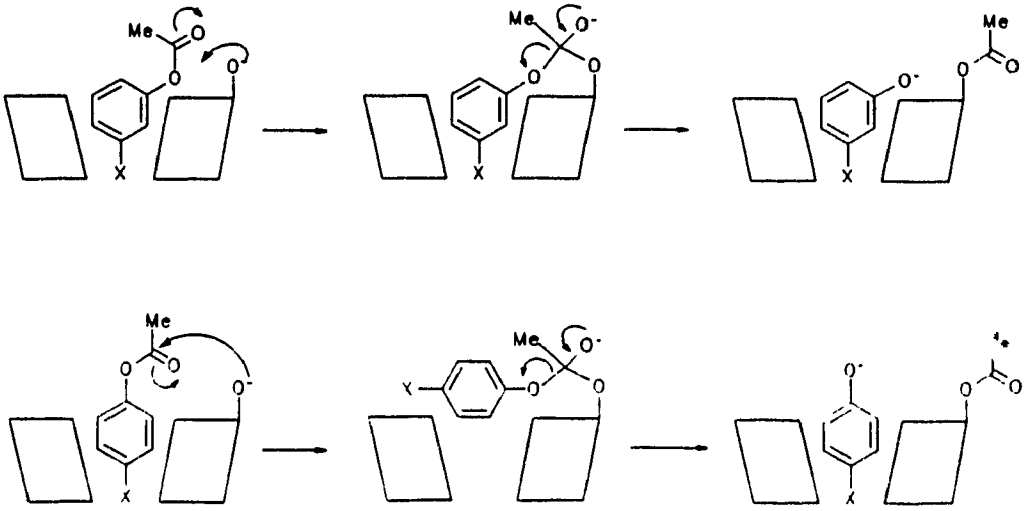


Figure 12. The structural differences between mNPA and pNPA make the former a better substrate for CD catalysed cleavage due to better positioning of the ester carbonyl near the ring hydroxyls.

More proof that the secondary hydroxyls are responsible for the cleavage reaction was obtained when it was observed that the 2',3'-dimethoxyderivative of α-CD are devoid of the accelerating effects customarily observed.[4b] On the other hand, blocking the primary hydroxyls caused no diminution of the rate acceleration. In fact, in some instances, the acceleration is increased, for example with the heptamesyloxy derivative of ß-CD,[4b] and the derivatives shown in Figure 11.[3]

Table 4. Distances between the carbonyl carbon atoms of the phenyl acetates and the O-2 atoms of α-CD and the magnitudes of acceleration of reaction in a 1:1 (v/v) mixture of pH 8.5 buffer and DMSO.[3]

Substrate	Distance (Å)	Acceleration
p-Nitrophenyl acetate	6.0	4.4
Phenyl acetate	5.8	14
m-Nitrophenyl acetate	3.4	220

Evidence was provided that penetration of the cavity by the substrate occurs from the wide face of the CD cavity. Docking calculations revealed that in both syn and anti complexes of a typical substrate, the ferrocenylacrylate ester shown in Figure 13, a cyclodextrin 2'-hydroxyl is well-positioned for attack on the carbonyl group.[6]

The strong dependance of the acceleration is proportional to the difference in structure between the initial state and the transition state.[3] Thus a substrate that can form the transition state while retaining the optimum initial state binding geometry would be expected to exhibit a high rate of reaction. This is the case with the cleavage of p-nitrophenyl ferrocenylacrylate (Figure 13) in 60% (v/v) aqueous DMSO with ß-CD. The acylation of ß-CD by this ester is 750,000 times faster than the background reaction,[13] and the rate is comparable to that achieved in the acylation of chymotrypsin by pNPA.[3]

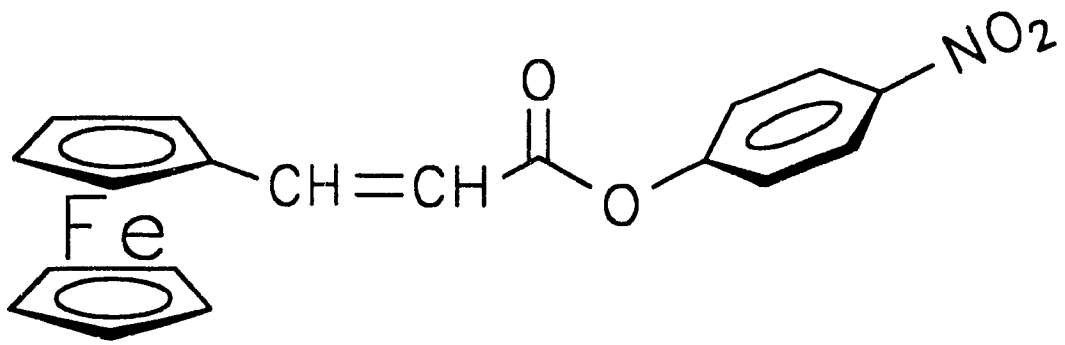


Figure 13. The structure of ferrocene acrylate, a substrate which requires little movement from the ground state complex with CD to achieve TS geometry.

Spectator Catalysis

All the work dealt with to this point has focused on the amelioration of substrate or CD structure to increase reaction rate. The next logical step is to determine whether other compounds which interact with CD are capable of affecting rates. Originally, the mechanism by which ester cleavage was believed to occur was via inclusion of the aryl moiety of the ester into the relatively hydrophobic cavity of the cyclodextrin molecule, followed by the attack of a secondary hydroxyl group (Figure 12).[4,8] One would normally expect that any other species capable of competing with the binding site in CD should cause a disruption of catalysis (competitive inhibition). This expectation is true for α and ß-CD when *m*-nitrophenyl acetate (mNPA) is the substrate. However, it is not the case when the substrates are either *p*-nitrophenyl acetate (pNPA) or *p*-nitrophenyl hexanoate (pNPH) (Figure 14), as shown in this laboratory.[10]

Hoeven[10] found that the cleavage of pNPA and of pNPH by ß-CD is not inhibited by various species which do inhibit the cleavage of mNPA. This finding was taken as further evidence of the two mechanisms in Figure 12, and the difference

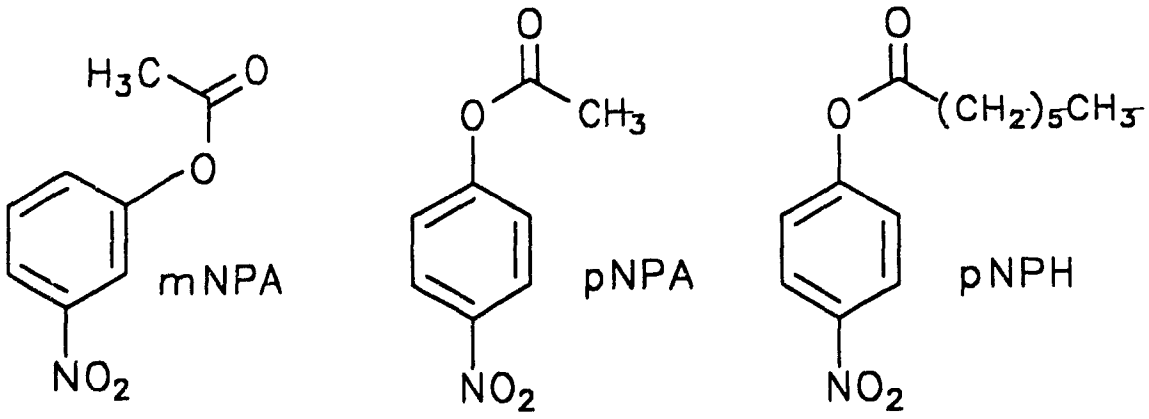


Figure 14. The three substrates used in this work.

between them, namely, that with mNPA the phenyl group is in the CD cavity in the transition state but with pNPA it must come out. Therefore, with pNPA as substrate other species ("potential inhibitors") can reside in the CD cavity while acyl transfer occurs. Thus, with pNPA the potential inhibitors are acting as inert spacers or "molecular spectators" which prevent full complexation of the substrate with the CD cavity, but still allow acyl transfer to take place. In some cases, the potential inhibitor might facilitate the positioning of the substrate in such a manner that it reacts more quickly with the CD. For this to be true, it would necessitate that cleavage of pNPA occur with the aryl moiety largely displaced from the ring.[10]

Objectives of this Work

The experiments performed herein had as their first objective consolidating proof for the existence of cocatalysis by molecular spectators in the cleavage of pNPA by ß-CD.[10] It was believed that potential inhibitors of reaction actually serve to loosen substrate binding enough to cause a more facile attainment of the

transition state. At the outset, evidence seemed to indicate that the cocatalytic effect is not contingent upon nucleophilicity or acidity of the inhibitors.[10] Rather, it is a direct result of the affinity that the spectators have for the CD cavity in aqueous solution. Consequently, it was of interest to investigate the variability of the cocatalysis with the structure of the spectator. To substantiate former conclusions, a large number of inhibitors of the reaction of mNPA with CD have been tested. The mNPA reaction was used as a gauge of the inhibitor's affinity of CD where literature sources failed to provide reliable dissociation constants. These values of K_i (constants of inhibition) were used to predict the effect expected when pNPA was used as the substrate. Three different classes of inhibitor with varying chain lengths have been tested: alkanols, alkanoate ions, and sulfonate ions. This was done to remove any doubt that rate variation was due to the nucleophilicity of the inhibitor and also to be able to construct linear free energy relationships based on length of the alkyl chains, if they exist.[16] At the same time, T.A. Gadosy, working on an undergraduate thesis, investigated the effect of potential inhibitors on the cleavage of pNPA by α-CD. His results provide a useful complement to the discussion of the results obtained for this thesis.

The second part of the research deals with the effect of potential inhibitors, such as those already mentioned, on the cleavage of *p*-nitrophenyl hexanoate (pNPH) by ß-CD, and to a lesser extent α-CD. This work showed more significant catalysis and provided evidence of the involvement of ternary complexes (ester.CD.inhibitor). Some of the work has been published as a communication.[11]

Experimental

Materials

The alcohols, organic acids, sulfonates, and cyclodextrins used in the experiments were as obtained from Aldrich, except for t-butanol and c-hexanol purchased from A&C chemicals, and n-butanol obtained from Fisher.

Most of the experiments involving straight chain alcohols were problem free. However, it was necessary to test branched alcohols for the formation of hydroperoxides. This was achieved by exposing the alcohol to a saturated aqueous mixture of KI. The formation of yellow color, seen as a result of the oxidation of iodide ion to triiodide was indicative of hydroperoxides. The presence of peroxides in the alcohols causes a very large rate enhancement of ester cleavage due to the direct attack of the hydroperoxide anion on the substrate esters. The first time we used hydroperoxide contaminated alcohol a spectacular rate enhancement was observed for the cleavage of pNPA in the presence of ß-CD. Without the alcohol the observed rate constant, k_{obs}, was 0.317 s^{-1}; with 200mM 2-butanol added it rose to 1.471 s^{-1}. *Without cyclodextrin* the rate constant was still 1.269 s^{-1}! This problem stresses the importance of testing reagents before their use in kinetic measurements. Following this experience, alcohols were obtained from new bottles of certified high purity and when the necessity arose, the alcohol was distilled under reducing conditions and used immediately.

The p-nitrophenyl esters (pNPA and pNPH) that were used as substrates were obtained from Sigma and used without further purification. These esters (and

mNPA) were dissolved in methanol to form concentrated stock solutions to be stored in a freezer at -4° C (to safeguard against significant methanolysis over several weeks). The stock solutions prepared in this manner were then used in the preparation of the mixtures of cyclodextrin with phosphate buffer and potential inhibitor with substrate to be used in the stopped-flow reaction. A micropipette was used to aliqout the ester solutions into the volumetric flasks used in preparation to ensure the uniformity of substrate concentration. The (substrate) ester concentration in the volumetric flasks ranged from 0.1 to 0.4 M, depending on its solubility in water. The usual aliqout size was 50 μL of the stock ester solutions in a 50 mL volumetric flasks to give a final (post-mix in the stopped-flow apparatus) substrate concentrations of 2.5×10^{-5} M (pNPH), 5×10^{-5} M (pNPA), and 2.5×10^{-4} M (mNPA).

The concentration of the esters in solution is crucial to the validity of kinetic results due to the possibility of aggregate formation, particularly with regard to longer chain esters. Guthrie has stressed the fact that clear solutions may still have most of the solute present in aggregates if the solute is a large hydrophobic compound.[25] Because of aggregation, some difficulty was encountered in the acquisition of reliable absorbance values at $t = \infty$, when pNPH was the substrate, suggesting that the reaction was not strictly adhering to pseudo-first-order kinetics. Presumably, as the reaction proceeded more aggregated substrate underwent dissolution with the rate of solvation proving to be a rate limiting step. When this occurred, better correlation coefficients resulted using an A_∞ value obtained by Swinbourne analysis (detailed later).

Because it is not available from suppliers, the m-nitrophenyl acetate was synthesized as follows: Acetic anhydride (0.0287 mol) was added to 12.5 mL of 10% NaOH, m-Nitrophenol (0.0178 mol), and 25g of crushed ice. The mixture was shaken vigorously until the ice melted. The resulting precipitate was filtered off and redissolved in methanol. Following dissolution of the product, water was again added to the mixture with chilling until a precipitate formed. Alternatively, to isolate a purer product, the original reaction mixture could be filtered and resuspended in low boiling (30-60°C) petroleum ether. The mixture was heated with repeated removal of aliquots of the dissolved product present in the petroleum ether. Concentration by evaporation of the ether then resulted in precipitation of white mNPA needles, which were be filtered off and blotted dry on fresh filter papers. Repeating the procedure until no mNPA precipitates provides a reasonable yield. It is a good idea to keep the last aliqouts extracted from the reaction mixture separate from the main batch until they have been tested for impurity. To ensure that the product is mNPA, addition of a small amount to dilute aqeous base should result in the *gradual* appearance of the characteristic yellow color. If the color change is immediate, dilute the base solution more to differentiate between hydrolysis of mNPA and ionization of m-nitrophenol impurities. This diagnostic experiment was carried out on the mNPA product isolated for the purposes of this study with. The gradual change in color that was observed indicated that it was not an ionization reaction.

All the experimental results were obtained in a phosphate buffer of pH 11.6± 0.05, with or without cyclodextrin. Buffer concentration was 0.4 M of dibasic sodium

phosphate with the addition of 1M NaOH (or the equivalent amount of NaOH pellets) and cyclodextrin to arrive at the proper pH and CD concentration (typically 20mM). This pH was chosen to match that of earlier studies; it had been arrived at as a compromise between obtaining a fairly quick reaction, while keeping the pH below the pK_a of the CD. The large buffer concentration is to counteract the acidity of the CD.

To prepare the buffer solution, 21.44g of disodium hydrogen phosphate heptahydrate was mixed with 51.84 mL of 1M NaOH, followed by the addition of 4.54 g of ß-CD and dilution to 200mL[b] with distilled water.[3] The mixture resulted in an effective ß-CD concentration of 10 mM, after mixing 1:1 with substrate and inhibitor-containing solution in the stopped-flow apparatus.

For experiments with variations of CD concentration, a primary stock solution of phosphate buffer was prepared without CD, and divided in two. One half served to prepare stock CD/buffer and the other half was used as the dilution buffer.

Following mixing, the reactions were monitored for a minimum of 10 half-lives to ensure > 99.9% completion. If the absorbance traces were noisy, as with some pNPH experiments, then more measurements were made to increase confidence in the rate measurements.

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[b] Larger batches of buffer are ill advised unless a number of experiments are to be run in quick succession. It is also good practice to avoid excessive air exposure of buffer during the course of the experiment to prevent CO_2, attracted by the high base concentration, from being incorporated into the solution.

Apparatus

All the kinetic experiments were run by monitoring the appearance of the product nitrophenolate anion at 405 nm, through a 5.0 nm bandpass, using an Aminco DW-2 UV-vis spectrophotometer, equipped with an Aminco-Morrow stopped-flow apparatus. The injection port is fed by two metal cylinders, the pistons of which are actuated by a single block to ensure that equal quantities of reagent reach the mixing chamber. The block which presses on the pistons to fill the reaction chamber of the stopped-flow is pressurized to 60 psi. Compressed air (or Nitrogen) was used to supply the pressure so that the solutions could be mixed rapidly and reproducibly.

The solutions used for the reaction were tested in order of ascending concentration of analyte (CD or potential inhibitor) to avoid errors caused by adsorption of chemicals onto the walls of the stopped-flow cylinder. One cylinder was always used for buffer solution (+ CD) whereas the other was always used for substrate with inhibitor. Each time a new solution was used, he parts of the machine coming into contact wit. :t were rinsed twice with aliquots of the reaction mixtures. The third refill of the cylinders was used for kinetic acquisition. Maximum speed of mixing was ensured by removing all the bubbles from refill syringes before introducing them into the stopped-flow. If an air bubble is swirled into the mixing chamber it can also cause diffraction of the light passing through with a concomitant reduction of transmission intensity.

Initially, data was acquired using an Apple IIe microcomputer interfaced to

a Cyborg Isaac 91a digitizer. Software for data acquisition in this set-up was written in BASIC by B. Takasaki and O.S. Tee. Analysis software was written using Turbo Pascal by the same. Later on, data acquisition was accomplished using an Olivetti M24 microcomputer with a Metrabyte 16F A/D card connected to the output voltage of the spectrophotometer. Data acquisition software for the computer that was used to collect the data from the A/D card was written by Dr. O.S. Tee and Mr. T.A. Gadosy in BASIC.

The components of the stopped-flow which come into contact with the solutions are double-walled so that they may be thermostatted by water flowing through them. A constant temperature of 25±0.1° was maintained in all kinetic experiments by a water-jacketed observation cell connected to a constant temperature bath. Furthermore, prior to conducting the reactions, all the volumetric flasks containing reagents were immersed in the constant temperature bath for 15 min. The pH of solutions was checked, when necessary, with a Corning ion analyses 250 pH meter. The control of pH was particularly important when dealing with alkanoates as inhibitors because of the significant rate changes that could occur due to their incomplete neutralization during preparation from the alkanoic acid.

Method of Data Acquisition

Data points were obtained by measuring the increase in absorbance of the reaction mixtures at 405 nm. Pseudo first-order kinetics were established by using low concentrations of the esters so that concentrations of the other reactants remain

relatively unaffected by reaction with the substrates. During a stopped-flow experiment the absorbance was sampled 100 times per run by the electronics of the stopped-flow set-up. The preferred sampling rate during most of experiments conducted for the purposes of this study was "medium" on the Aminco model d636-70067 detector. The medium sampling rate represents a compromise between a noisy baseline and an unacceptable lag in measurement time. The resulting absorbance trace was submitted to analysis by two techniques, descibed later, to obtain the observed rate constants.

Any reaction in which a change of absorbance occurs may be measured by the use of the Beer-Lambert's law, $A=\epsilon bc$. It is useful for the determination of analyte concentration i.e. the nitrophenoxide anion. Through this relationship the rate of change in absorbance can be used to monitor the change in concentration of product or reactant. For a first-order reaction during which absorbance increases with time: $kt = \ln (A_\infty-A_0)/(A_\infty-A)$, which may be rewritten as $\ln(A_\infty-A) = \ln(A_\infty-A_0) - kt$. Thus, the value of the rate constant can be obtained by least-squares analysis of $\ln(A_\infty-A)$ versus time. This treatment is dependent on a value of A_∞. When determination of A_∞ became difficult due to complications such as aggregation of the substrate, more weight was given to the k_{obs} values obtained by using the Swinbourne treatment of data.[55]

The Swinbourne method utilizes two sets of data points which are displaced in time with respect each other by a constant amount (ΔT) as the basis for determining the slope of a line. For the first set of points: $(A_\infty - A) = (A_\infty - A_0)e^{-kt}$

for times t_1, t_2, etc.; for the second set: $(A_\infty - A') = (A_\infty - A_0)e^{k(t+\Delta T)}$, where A' is an absorbance datum collected at time $t_1+\Delta T$, $t_2+\Delta T$, etc.. From rearrangement of these two equations we get: $(A_\infty - A)/(A_\infty - A') = e^{k\Delta T}$. Thus, $A = A_\infty(1 - e^{k\Delta T}) + A'e^{k\Delta T}$. From the least-squares slope and intercept obtained from the analysis of A vs. A' one can estimate values of A_∞.

Ordinarily, the recorded k_{obs} was taken from the average of five or more kinetic runs. Each experiment consisted of determining values of k_{obs} at six inhibitor concentrations. To conduct an experiment two solutions were prepared for mixing 1:1 in a stopped-flow apparatus. One solution contained the substrate (mNPA, pNPA, or pNPH) with a particular concentration of inhibitor (I), whereas the other solution contained CD solution in alkaline phosphate buffer (pH 11.6).

Four different types of experiment were conducted to collect data pertaining to this study. Three different kinetic analyses, outlined in the next section, were used according to the behaviour being studied. To determine the dissociation constants for potential inhibitors, K_i, the inhibition of mNPA cleavage in cyclodextrin was studied. The results from this type of experiment were analyzed by a computer program called INHIB. The analysis of "spectator" catalysis by these same inhibitors, but with pNPA as substrate was quantified by using PIANALYSIS. When the substrate was pNPH both PIANALYSIS and a program that makes allowances for saturation behaviour, CDFIT, were used to analyze data. The last program, which has several options, was also used to calculate the results of experiments run to verify the kinetic behaviour of ester cleavage under conditions of varying initial [CD], with

no inhibitors. However, few experiments of this type were conducted as Du[19] had already procured data in this area.

Kinetic Analysis Programs

Kinetic analyses made use of a number of programs, written by Dr. O.S. Tee and Mr. B.K. Takasaki. They are: EDIT, to store, plot and edit data; PIANALYSIS, which provides scaled treatment of data plots, was used for the analysis of experiments with pNPA + I + CD to obtain the rate constants (k_a, k_b); INHIB was used for the analysis of inhibition experiments in the presence of mNPA + PI + CD and CDFIT for the analysis of saturation kinetics. The last provided analysis of curved plots for the pNPH, I (PI), CD experiments, where ternary binding seems to be important.[11]

Results

Substrate and Cyclodextrin

A nitrophenyl ester (S), undergoing reaction in cyclodextrin-containing medium reacts via two pathways. The first, represented by equation 1 is the "uncatalyzed" reaction, due to nucleophilic attack of hydroxide on the ester. The second, equation 2, relates the contribution due to substrate complexed with CD (S·CD). Summing the two contributions yields the overall observed rate (eq 3).

$$S \xrightarrow{k_u} R \tag{1}$$

$$S + CD \underset{K_s}{\rightleftharpoons} S{\cdot}CD \xrightarrow{k_c} P \tag{2}$$

$$v = k_{obs}[S]_o = k_u[S] + k_c[S{\cdot}CD] \tag{3}$$

The data plots were obtained under pseudo-first-order conditions by keeping the ester (substrate or S) concentration low and were analyzed using either Eadie-Hofstee or Lineweaver-Burke treatment of results. Assuming that [CD] ≫ [S], then [CD] will remain practically unchanged due to its large excess when compared to [S], thus allowing us to treat it as such mathematically. It can be shown that,

$$k_{obs} = \frac{k_u K_s + k_c[CD]}{K_s + [CD]} \tag{4}$$

With this equation,[c] of the same general form as the Michaelis-Menten equation, we can account for the two most significant rate processes for the

[c] The derivation of this equation can be found in Appendix A.

cyclodextrin catalysed cleavage of phenyl esters in basic aqueous solution.[1]

Inhibitors and Cyclodextrin

Occasionally, due to the discrepancies between values of dissociation constants (K_i's) reported in the literature for a variety of potential inhibitors, (I),[26-30] it became necessary to test their validity under conditions which more closely resembled those of our experiments. This way, we could use the K_i values, that were extrapolated from the observed inhibition of mNPA cleavage, in the calculation of expected rate constants for pNPA cleavage in the presence of inhibitors.

To illustrate the range of uncertainty in literature sources, Servé cites the K_i for the dissociation constant of I^- with α- and ß-CD in aqueous solution.[30] The values were obtained by different researchers using a variety of methods, i.e. conductivity, potentiometry, electrometry, spectrophotometry and others. For α-CD, values ranged from 31 to 125 mM and from 56 to 690 mM for ß-CD. The distribution of results however, was not as discouraging as it may appear, since some clustering was noted among the different methods, albeit not enough to justify the use of literature sources alone.

The approach used to ascertain the dissociation constants of CD and I relied on the inhibition of the CD-catalysed cleavage of mNPA.[4] For a potential inhibitor (I) binding to a CD:

$$I + CD \underset{K_i}{\rightleftharpoons} CD \cdot I$$

$$K_i = \frac{[I][CD]}{[CD{\cdot}I]} \quad (5)$$

Since, for mass balance, $[CD]_o = [CD{\cdot}I] + [CD]$ and $[I]_o = [CD{\cdot}I] + [I]$, we can elaborate:

$$K_i = \frac{[CD]([I]_o - ([CD]_o - [CD]))}{([CD]_o - [CD]} \quad (6)$$

Expansion of eq 6 yields the quadratic relationship:

$$[CD]^2 + [CD]([I]_o + K_i - [CD]_o) - K_i[CD]_o = 0$$

Solving this quadratic is possible only if K_i is known. In such cases, solution using the quadratic formula yields the concentration of free CD which is necessary for data analysis for the reactions involving pNPA and pNPH:

$$[CD] = \frac{-([I]_o+K_i-[CD]_o)+(([I]_o+K_i-[CD]_o)^2+4K_i[CD]_o)^{1/2}}{2} \quad (7)$$

When K_i was not known, experiments on the inhibition of mNPA cleavage were carried out and analysis of the data was based on the following approach.[4] If $[I]_o > [CD{\cdot}I] = [CD]_o - [CD]$, as it was in these experiments, with $[CD]_o$ being kept at 1mM to guarantee a low concentration of complex, then equation 6 simplifies to,

$$K_i = \frac{[CD][I]_o}{([CD]_o - [CD])} \quad (8)$$

which rearranges to,

$$[CD] = \frac{[CD]_oK_i}{(K_i + [I]_o)} \quad (9)$$

Substitution of eq 9 into eq 4 yields the following:

$$[I]_o = \frac{(k_c - k_{obs})}{(k_{obs} - k_u)} X \frac{[CD]_o K_i}{K_s} - K_i \qquad (10)$$

Thus, a plot of $[I]_o$ vs. $(k_c - k_{obs})/(k_{obs} - k_u)$ should give a straight line with slope = $([CD]_o K_i)/K_s$ and intercept = $-K_i$.[5]

Figure 15 is a typical inhibition plot, based on eq 10. It is useful to note that this technique of determining K_i is prone to the limitations imposed by extrapolation back to the y-axis of the graph. If, for example, the inhibition effect is measured over a small range of concentrations and if the terms on the x-axis which represent $(k_c - k_{obs})/(k_{obs} - k_u)$, end up relatively far from the origin, the inaccuracy of extrapolation tends to be amplified. Duplicate experiments and careful choice of $[I]_o$ helped to ensure the reliability of results.

Table 5 documents the chain length dependence of K_i values from three sources; they are also plotted in Figure 16. The three curves represent the results of different stu s. Satake's results, determined by equivalent conductivity measurements[27,28] closely parallel values determined in the present work by inhibition. Okubo's[29] results disagreed with Satake's and he also criticised Satake's method claiming their own kinetic conductivity method was more accurate. As mentioned before, it was necessary for us to conduct inhibition measurements in the range corresponding to the dissociation constant of the inhibitor to avoid problems such as micellization, pH fluctuation and ionic strength variation. To preserve accuracy, reactions were carried out as much as possible, over the same range of inhibitor concentrations for both mNPA and pNPA or pNPH experiments.

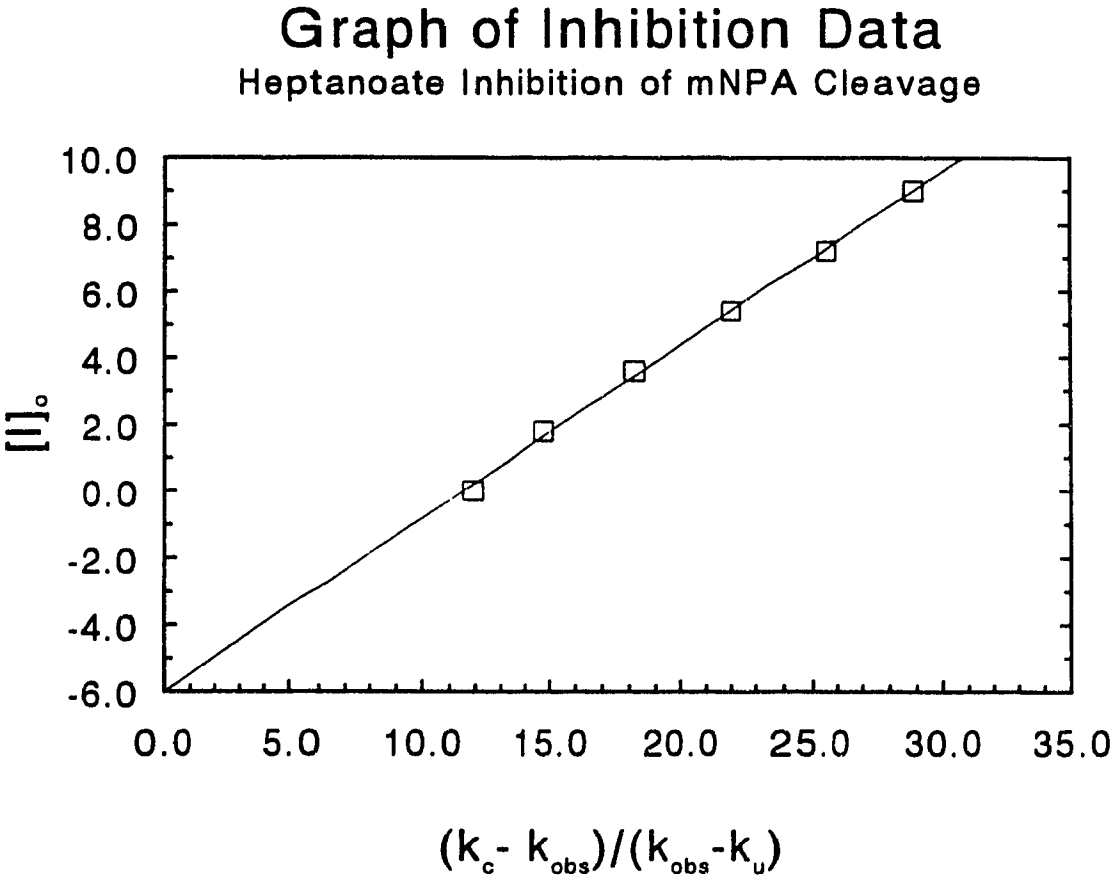

Figure 15. The INHIB program we used provided a linearised plot of inhibition data acquired with mNPA as substrate. INHIB creates plots according to equation 10 where the negative y-intercept corresponds to the extrapolated K_i value in mM. The plot above corresponds to the effect of heptanoate on the system.

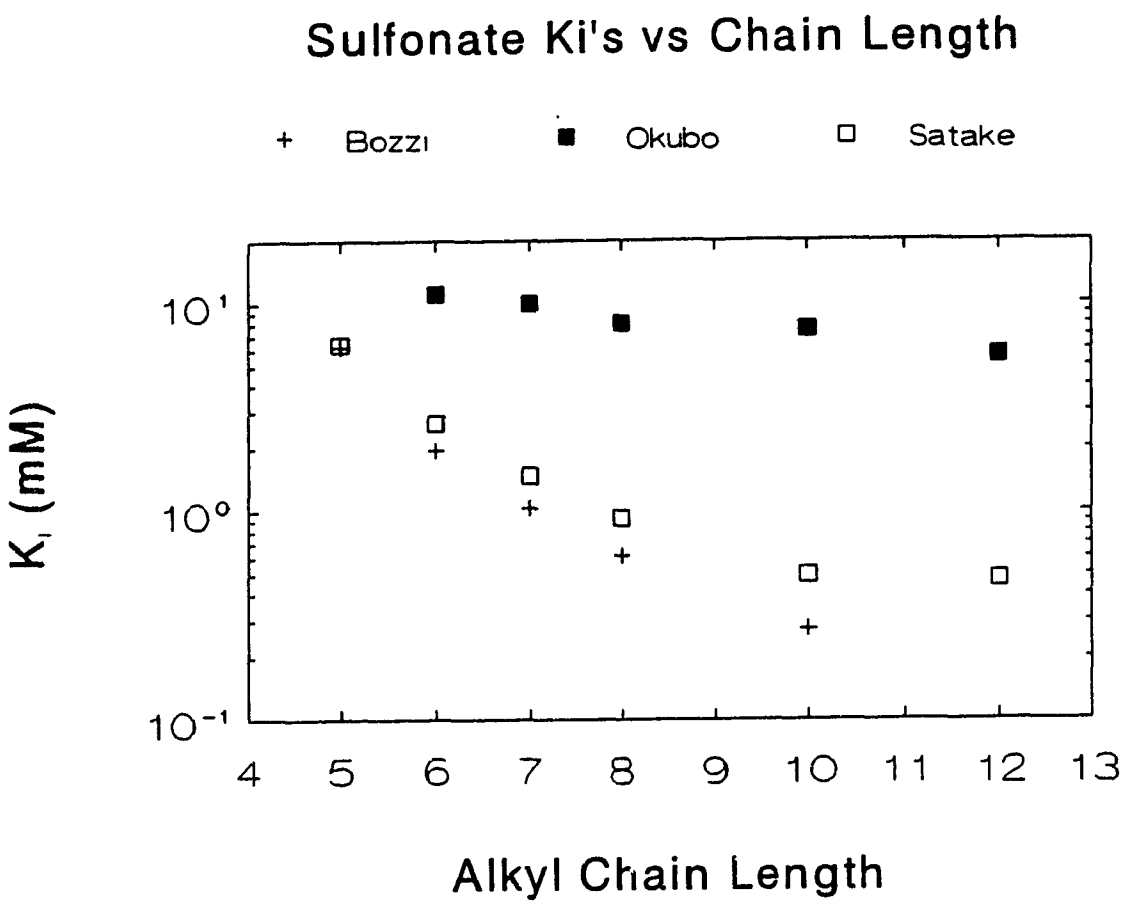

Figure 16. Dissociation constants versus chain length of sulfonates in α-CD at 25°C, obtained by our kinetic methods disagreed with those of Okubo, but were similar to Satake's

Table 5. K_is of several alkanesulfonate ions as obtained in this work and by others for α-CD at 25°C. Graphed above in Fig. 16.

Sulfonate	Bozzi mM	Okubo mM	Satake mM
C_5	6.22	-	6.37
C_6	1.95	11.1	2.64
C_7	1.34	10.0	1.48
C_8	0.60	8.0	0.93
C_{10}	0.28	7.5	0.50
C_{12}	-	5.6	0.47

pNPA and pNPH Cleavage in the Presence of Potential Inhibitors

Literature K_i values, or measured values obtained in previously described manner, were applied to predict the inhibition of reaction when pNPA was a substrate. Kinetic experimental results showed, however, that another rate process must be occurring, as reaction rates are not as slow as predicted for normal inhibition.[10] This required further modification of equation (3) to account for the rate due to a process involving the potential inhibitor, (PI).

$$v = k_{obs}[S]_o = k_u[S] + k_c[S{\cdot}CD] + k_a[S{\cdot}CD][PI]$$

The added term, containing k_a, represents the rate constant for a process involving both the substrate.CD complex and the PI going to products.

$$CD + S + PI \underset{K_s}{\longrightarrow} CD{\cdot}S + PI \xrightarrow{k_a} P \qquad (11)$$

When the extra term is added to equation 4 we get:

$$k_{obs} = \frac{k_uK_s + k_c[CD] + k_a[CD][PI]}{K_s + [CD]} \qquad (12)$$

Upon rearrangement this becomes:

$$k_{corr} = \frac{k_{obs}(K_s + [CD]) - k_uK_s}{[CD]} = k_a[PI] + k_c \qquad (13)$$

Equation 13 is plotted in the form $y = mx + b$ with the second term of eq. (13), $(k_{obs}(K_s + [CD]) - k_uK_s)/[CD]$, above, represented as "k corrected." The values of k_{corr} were scaled to obtain easily comparable results,[d] and to account for the

[d] See Appendix C for scaling experiments.

inevitable slight pH variations. The slopes of the lines obtained from the plots mentioned above correspond to the k_s values for the respective inhibitor, whereas the numbers plotted on the x-axis, as in Figure 17, represent the inhibitor concentration corrected for association with CD.

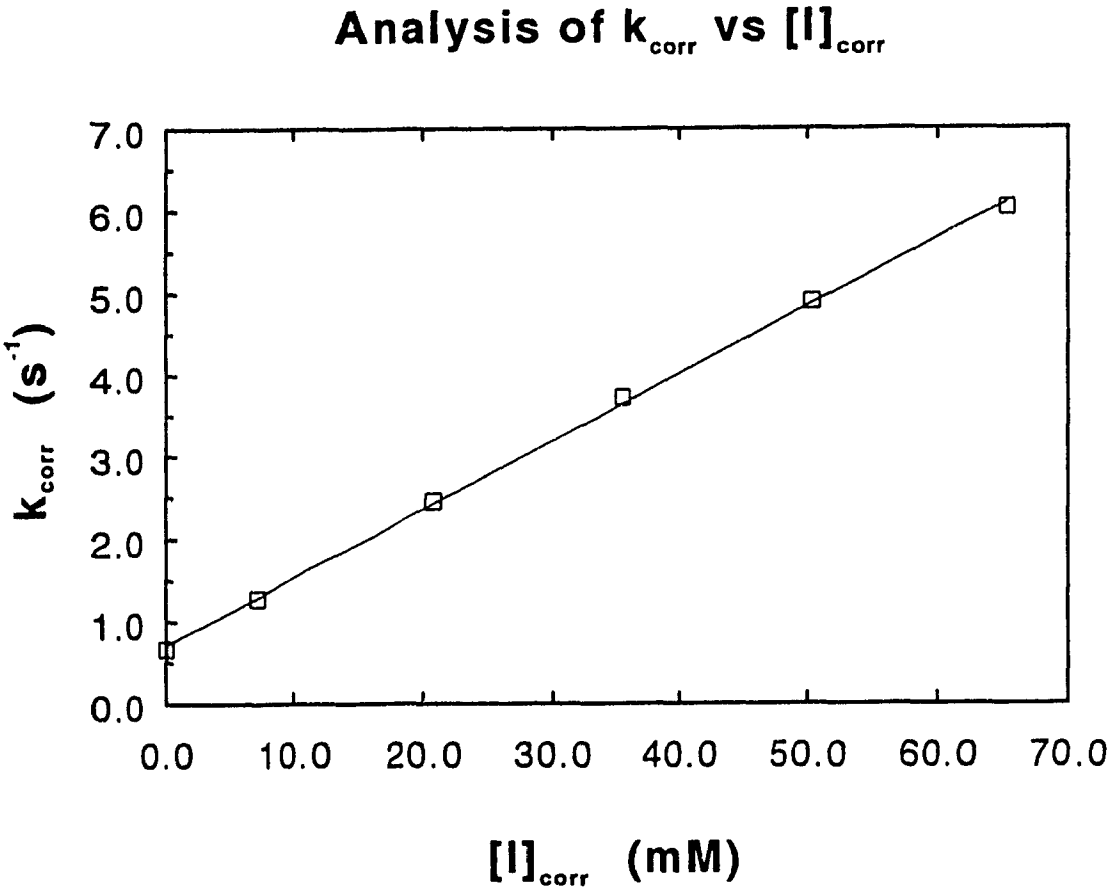

Figure 17. PIANALYSIS plot of data according to eq 13, for neo-pentanol as the potential inhibitor of CD catalysed pNPA cleavage. See Appendix C; experiment #20 (p.119) for data.

Solving the quadratic in [CD], using equation 7, and use of the appropriate mass balance equation: $[PI] = [PI]_o - ([CD]_o - [CD])$, to obtain the free equilibrium concentrations of PI and CD, leads to a good fit of data obtained by this method, especially with pNPA as substrate. Figure 17 is an example of data graphed according to eq 13. analysed through a computer program called PIANALYSIS.

As discussed more fully later, it is possible that reactants come together in another way.[10] In this scheme (eq 14), the potential inhibitor binds to CD first, and the CD.PI complex reacts with the substrate:

$$CD + PI + S \underset{K_i}{\longrightarrow} CD{\cdot}PI + S \xrightarrow{k_b} P \qquad (14)$$

It is important to note that the processes in equations 11 and 14 are kinetically indistinguishable. For eq 11 the third-order rate constant $k_1 = k_a/K_a$ whereas for eq 14 $k_3 = k_b/K_i$. Thus, values of k_b can easily be calculated from k_a (obtained from PIANALYSIS plots) through: $k_b = k_aK_i/K_a$. In later discussion the observed results are considered in terms of both processes; it turns out that they are most easily understood in terms of eq 14 when a series of inhibitors are compared. From the trend it is possible to infer the relative positions of the reactants as they near the transition state.

Ternary Complexes

When the PIANALYSIS program was utilized in the analysis of data acquired with pNPA as substrate and any one of the three types of inhibitors, i.e. alkanols,

alkanoates or alkanesulfonate ions, satisfactory results were obtained, the best results being with alkanols and sulfonate ions. Alkanoates also provided reasonable results if neutralization of the alkanoic acid was carefully carried out. However, with pNPH plots according to eq 13 clearly showed saturation behaviour with increasing concentration of PI. Moreover, the k_{obs} values also increased and showed saturation. This enzyme-like behaviour led us to consider the pre-equilibrium formation of ternary complexes: PI·CD·S.

The formation of crystalline ternary complexes has been observed since 1972 when Takeo and Kuge observed what they termed "Einshleppeffekt." This term is used to describe the situation of molecules that form stable crystalline complexes only when incorporated into the CD cavity with another molecule.[2] For example, diethyl ether does not give a crystalline CD complex on its own, yet a ternary complex is obtained if anthracene is present. Likewise, indomethacin, DMF and ß-CD form a ternary complex of regular structure.[2] These observations reinforce the notion that led us to consider the possibility of systems that would optimize the structure of the transition state through a similar synergistic effect.

The equation depicting the formation of a ternary complex is:[11]

$$PI + CD{\cdot}S \underset{K_t}{\rightleftharpoons} PI{\cdot}CD{\cdot}S \xrightarrow{k_t} P \qquad (15)$$

If reaction proceeds in this fashion then eq 15 replaces eq 11, and $k_s = k_t/K_t$. Correspondingly, the rate law is then given by:

$$v = k_{obs}[S]_o = k_u[S] + k_s[S{\cdot}CD] + k_t[S{\cdot}CD{\cdot}PI] \qquad (16)$$

Therefore, the overall observed rate constant according to this scheme would be,

$$k_{obs} = \frac{k_u[S] + k_c[S\cdot CD] + k_t[S\cdot CD\cdot PI]}{[S]_o} \quad (17)$$

where $$[S]_o = [S] + [S\cdot CD] + [S\cdot CD\cdot PI]. \quad (18)$$

When $[S]_o \ll [CD]$ and $[PI]$ the following reasonable approximation is allowable:

$$[S\cdot CD] \ll [CD] \quad \text{and} \quad [S\cdot CD\cdot PI] \ll [CD] \text{ and } [PI]$$

and the concentrations of CD and PI are not really affected by the presence of the ester S.

Dividing equation 18 on both sides by [S], and using the definitions of K_s and K_t leads to,

$$\frac{[S]_o}{[S]} = 1 + \frac{[CD]}{K_s} + \frac{[CD][PI]}{K_sK_t}$$

Thus, $$\frac{[S]}{[S]_o} = \frac{K_sK_t}{K_sK_t + [CD]K_t + [CD][PI]} \quad (19)$$

Likewise, $$\frac{[S\cdot CD]}{[S]_o} = \frac{[CD]K_t}{K_sK_t + [CD]K_t + [CD][PI]} \quad (20)$$

and $$\frac{[S\cdot CD\cdot PI]}{[S]_o} = \frac{[CD][PI]}{K_sK_t + [CD]K_t + [CD][PI]} \quad (21)$$

Substituting 19, 20 and 21 into 17 yields

$$k_{obs} = \frac{k_uK_sK_t + k_cK_t[CD] + k_t[CD][PI]}{K_sK_t + [CD]K_t + [CD][PI]} \quad (22)$$

Since the uncatalysed reaction ($k_u = 0.045\ s^{-1}$)[19] is slower than the catalysed one ($k_c = 0.14\ s^{-1}$),[19] the terms containing [CD] dominate top and bottom of eq 22 when [CD] is high. Then, it is reasonable to express the observed rate constant as

$$k_{obs} = \frac{k_c K_t + k_t[PI]}{K_t + [PI]} \qquad (23)$$

The accuracy of this approximation tends to increase as the binding affinity of the potential inhibitor increases, due to the fact that less PI is necessary to elicit a measurable rate enhancement (see later), which in turn allows us to maintain low [PI] and so less CD is complexed. If, due to the weak binding of the inhibitor it was necessary to have high [PI], a higher $[CD]_o$ of 15mM (vs. 10mM) was used to offset the lowered concentration of the CD.S complex.

In summary, the ternary complex can best be visualized as being formed from the binary CD.S complex and the potential inhibitor (eq 15). For this scheme, and according to eq 23, the observed rate is the sum of the rates due to CD accelerated cleavage and reaction via a ternary complex. Figure 18 is an example of data analyzed using the computer program (CDFIT), which makes use of an iterative calculation to fit eq 23 to the data. Following analysis via Eadie-Hofstee and Lineweaver-Burk methods to estimate K_t and k_t values, the resulting average values of the rate constant were used as initial values for non-linear least squares fitting of eq 23. From comparisons of these constants with estimated pseudo-dissociation constants of the transition state, derived in the next section, we can speculate on the structural similarity of the ternary complexes PI.CD.S and the proposed TS.

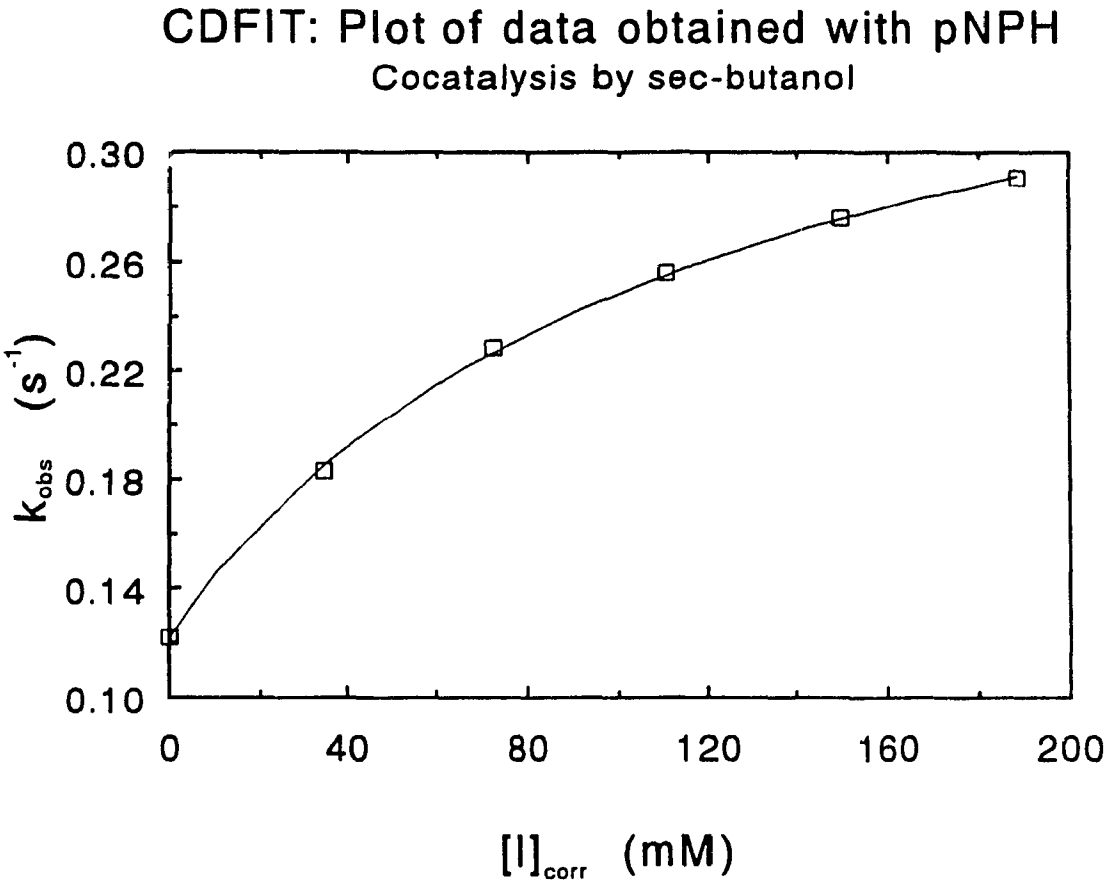

Figure 18. Analysis of data for pNPH as substrate yielded curved plots when PIANALYSIS was used. Therefore, a scheme invoking the pre-equilibrium formation of a reactive ternary complex was employed. Satisfactory fits to the data were obtained with the program CDFIT.

Transition State Binding

Tight binding of the altered substrate in the transition state is not, as formerly believed, one of the many ways for catalysis to be achieved. Rather, it is required in catalysis and applies whatever the detailed mechanism might be. Wolfenden and Frick[14] have reported that the rate acceleration for a reaction is directly and exclusively proportional to the tightness of the enzyme's grip on the transition state (TS) versus its ground state binding of the substrate, represented by K_x= [CD][S]/CD·S]. In other words, the enzyme tightens its grip on the substrate as its conformation approaches that of the TS and then loosens up again when it has passed it.[16,31,32,46]

Since rate constants for reaction are directly related to free energies of activation, it follows that if a catalyst.substrate complex decomposes more rapidly than the free substrate in solution, then its activation free energy is considerably less. For significant rate acceleration, stabilization of the transition state by the catalyst must be greater than that of the substrate.[31] This relationship can be quantified using equilibrium constants K_{TS} and K_S, where K_{TS} is the virtual dissociation constant of the catalyst.transition state complex and K_S is the dissociation constant of the catalyst.substrate complex in the ground state.

According to transition state theory[31] the rate constant for the reaction in the medium:

$$S \xrightarrow{k_u} P \; ; \quad \text{is} \quad k_u = Q[TS]/[S] \text{ and } Q = \kappa(k_B T/h) \qquad (24)$$

where κ represents the transmission coefficient. The introduction of κ arises from the

possibility that not all activated complexes will necessarily form products. If some peculiarity exists in the potential energy surface the reacting system may be reflected back with the result that the reaction does not take place. Usually, however, it is believed that κ is close to unity.[33] The treatment which follows circumvents the problem of transmission coefficients by assuming that they are the same for the catalyzed and uncatalyzed reactions; in this case they cancel in the derivation of K_{TS}, leaving only the constituent concentrations.

Comparable to eq 24, we may elaborate on the second order rate constant for reaction of S with a "catalyst", here a CD:

$$CD + S \xrightarrow{k_2} P \; ; \quad \text{where } k_2 = Q[CD \cdot TS]/[CD][S] \tag{25}$$

According to equation 25, the rate constant k_2 is directly related to the concentration of free substrate and CD in solution *and* to the concentration of transition state bound to CD. If S reacts with CD through a complex CD.S then eq 25 becomes.

$$CD + S \underset{K_s}{\rightleftharpoons} CD \cdot S \xrightarrow{k_c} P \; ; \quad \text{where } k_c = Q[CD \cdot TS]/[CD \cdot S] \tag{26}$$

In this case, the rate constant k_2 is equal to k_c/K_s.

From the above definitions of k_u and k_2 one may define a pseudo-equilibrium constant, K_{TS}:[16]

$$\frac{k_u K_s}{k_c} = \frac{k_u}{k_2} = \frac{Q[TS][CD][S]}{Q[CD \cdot TS][S]} = \frac{[CD][TS]}{[CD \cdot TS]} = K_{TS} \tag{27}$$

The constant K_{TS} is the apparent dissociation constant of the transition state of the

catalyzed reaction (symbolized as TS.CD) into transition state of the normal reaction (TS) and CD.

Similarly, for the reaction involving a potential inhibitor (eq 11), we can define a constant K_{TS}', for dissociation of PI from the termolecular transition state. Again, using transition state theory:

$$CD \cdot S + PI \xrightarrow{k_a} P \; ; \; k_a = Q[CD \cdot TS \cdot PI]/[CD \cdot S][PI] \qquad (28)$$

From this definition, and the corresponding definition of k_c (eq 26), we may elaborate:[e]

$$\frac{k_c}{k_a} = \frac{Q[CD \cdot TS][PI][CD \cdot S]}{Q[CD \cdot S][PI \cdot CD \cdot TS]} = \frac{[CD \cdot TS][PI]}{[PI \cdot CD \cdot TS]} = K_{TS}' \qquad (29)$$

The dissociation constant of the potential inhibitor from the transition state (K_{TS}') provides a measure of how effectively the inhibitor binds to the CD-containing transition state. Likewise, the dissociation constant of CD from the CD-containing transition state (K_{TS}) may provide insight into the role of CD in the reaction. The use of these principles, derived from transition state theory, can therefore help to clarify the involvement of each component of the reaction. Similarly, the information gathered from studying different series of potential inhibitors helps to disclose the relative position of the reactants during the reaction. For example, by comparing K_{TS}' values with the K_i values for the potential inhibitors we may obtain information

[e] See Appendix B for a comprehensive list of the constants we measured and how they interrelate.

about the binding of PI in the transition state in relation to that in the initial state. Where a series of inhibitor is available, such as the series of alcohols discussed later, we can establish if correlations exist between these parameters.

It is important to note that the constants K_{TS} and K_{TS}', defined above, can be used to calculate free energy differences, using the normal type of equation:[14]

$$\Delta G°_{TS} = -RT \ln K_{TS} \qquad (30)$$

Thus, for standard conditions one can calculate the relative energies of the reactants and transition states of various compositions. Such relationships are schematized in Figure 19, which also shows the various constants that can be used to calculate the free energy differences. Equation 30 also serves to emphasize that $pK_{TS} = -\log K_{TS}$ is directly proportional to free energy, and so any linear correlations between such values and other appropriate parameters constitute linear free energy relationships.[16]

Linear free energy relationships can be useful in the determination of mechanism. For example, if a plot of pK_t and pK_{TS}' versus pK_t for a series of inhibitors is made, as is discussed later, the binding of the inhibitor PI in the proposed ternary complexes and in the transition state containing PI can be related to the binding of PI to the CD, for a variety of shapes and sizes of inhibitor. From such comparisons it may be possible to state whether the proposed mechanism is feasible or not. Thus, the information afforded by transition state theory is a useful supplement to the normal terms used in enzymology, discussed briefly below.

"The catalysis or inhibition of reactions by CDs is usually discussed in terms of k_c/k_u, K_s, and, less frequently, k_c/K_s. The ratio k_c/k_u is emphasized since it

measures the limiting rate acceleration (or retardation) due to the CD."[16] The constant $k_2 = k_c/K_s$ is useful as a measure of the selectivity of CD for different substrates comparable to k_{cat}/K_m for enzymes.[16]

Note that in the free energy diagram, Fig. 19, the uncomplexed TS has been placed at the highest free energy, as expected for a reaction in the absence of CD catalysis. Below this is TS complexed to CD since a catalyzed reaction proceeds with smaller free energy of activation. The TS complexed to both CD and a potential inhibitor was placed lower still on the free energy diagram to illustrate the cases where the potential inhibitor actually catalyses reaction.

The diagram emphasizes that stabilization of the ground-state by complexation will decrease the rate of reaction by making the transition state less accessible, unless the transition state is stabilized by the catalyst. For example, only if the free energy difference between S·CD and TS·CD is smaller than that between S and TS of S, will the reaction be accelerated by CD ($k_c/k_u = K_s/K_{Ts} > 1$). Likewise, there will only be a rate increase upon addition of PI if the difference between S·CD·PI and TS·CD·PI is less than that between S·CD and TS·CD.

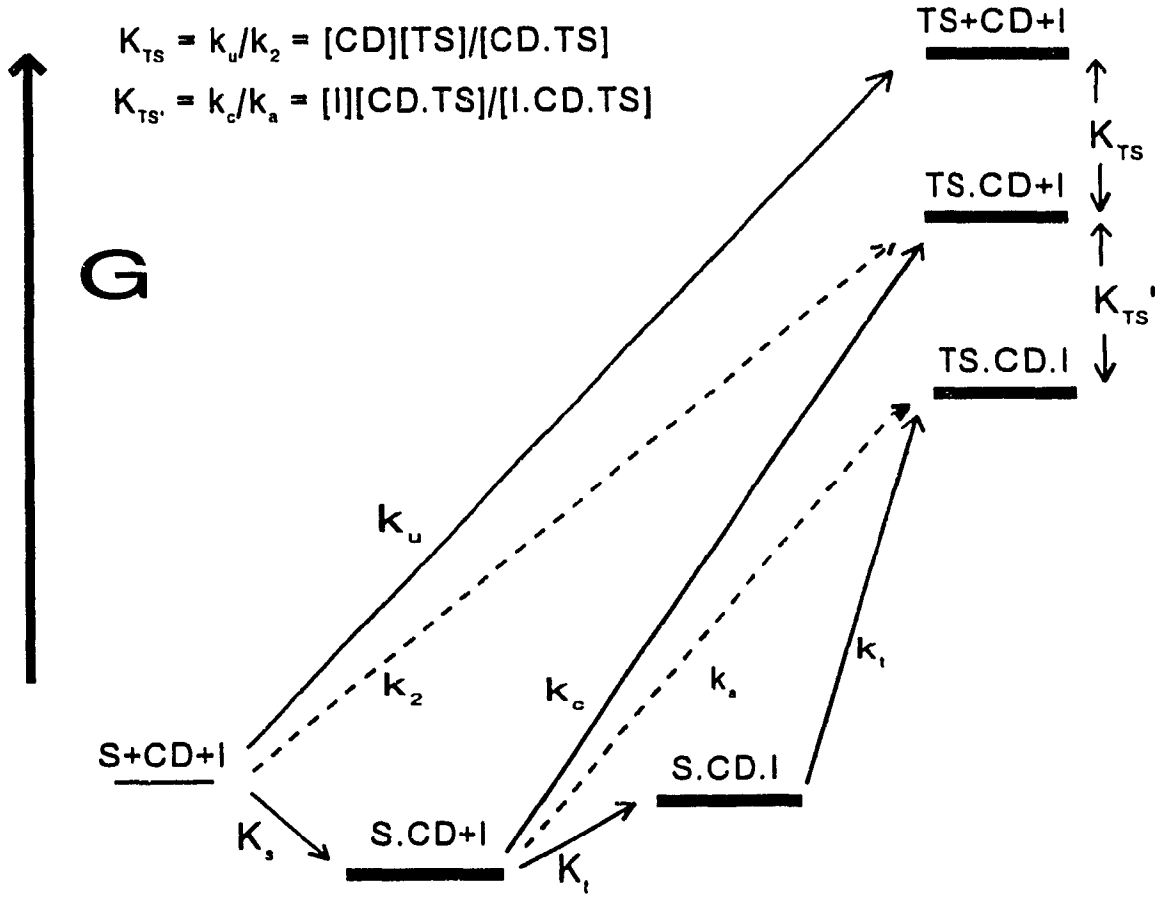

Figure 19. The relative Gibbs energies for some of the species mentioned in equations 24-29. See Appendix B for a more complete portrayal.

Discussion

CD Catalysed Cleavage of Esters

Part of this project was intent on extending experimental evidence for the mechanism proposed by Tee and Hoeven.[10] They found that p-nitrophenyl acetate

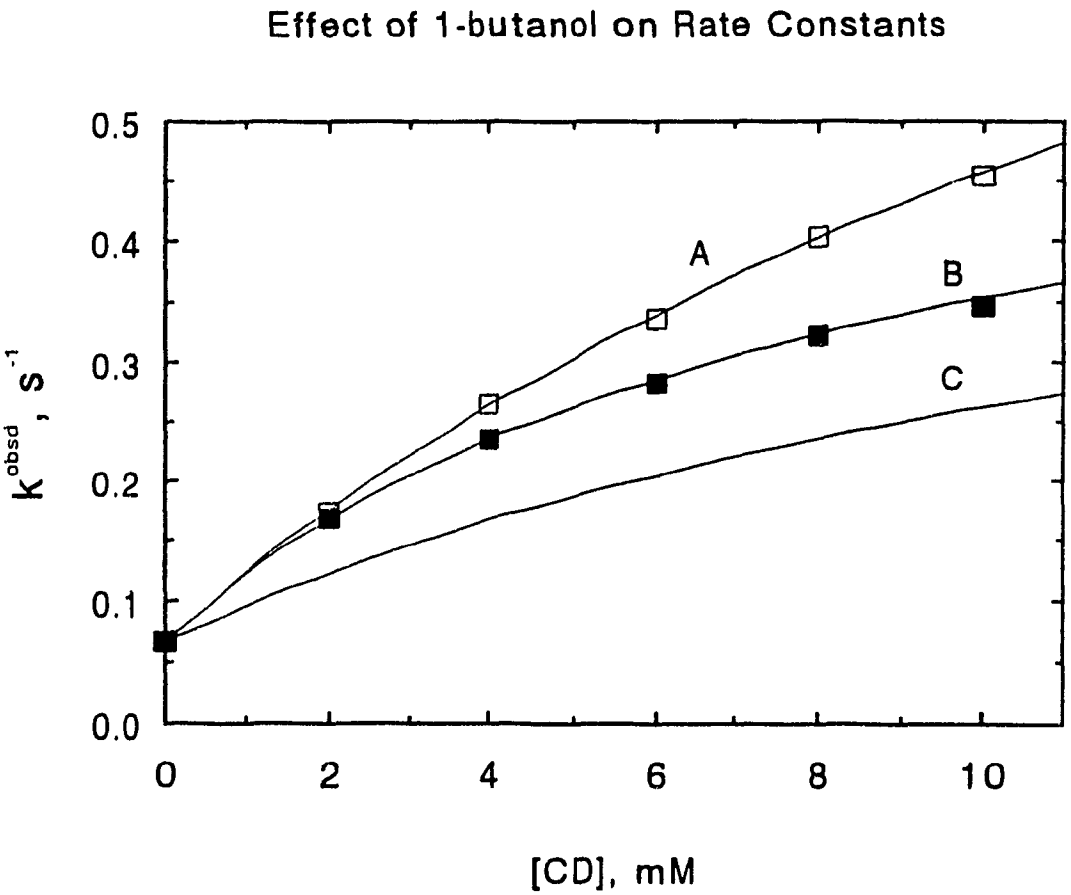

Figure 20. The unexpected rate enhancement for the cleavage of pNPA in β-CD due to the presence of a competitive inhibitor of complexation. A) Observed data for 60 mM n-butanol B) No n-butanol C) Expected inhibition by n-butanol modeled after mNPA.

cleavage undergoes modest rate enhancement or only partial inhibition under conditions that cause normal inhibition in the rate of MNPA cleavage. The graph

above (Fig. 20) is a comparison of pNPA cleavage under identical pH, CD content, and ionic strength conditions except for the presence or absence of a potential inhibitor, n-butanol.[10]

As mentioned earlier, *meta* substituted phenyl acetates undergo cleavage more readily in the presence of ß-CD than do similarly substituted *para* compounds due, presumably, to the smaller distance between the carbonyl of the ester and the attacking hydroxyls in the ester-CD complex.[3] To account for the reduced inhibition, it was proposed that "inhibitors" may act as inert "spacers" which can improve the binding of pNPA in the transition state for acyl transfer by restricting its degree of freedom and positioning it during the reaction,[10] in much the same way that residues at an enzyme's active site may aid in the accommodation of transition state structure.[40] (see Fig. 21) Thus, the *meta-para* effect[1] leads to divergent behaviour under conditions of expected inhibition: with mNPA, a competitive inhibitor prevents catalysis, whereas with pNPA it can promote it.

A variety of inhibitors were tested with pNPA to see if the rate enhancement (sometimes referred to as "cocatalysis") or reduced inhibition occur independently of any chemical property of the spacer other than its affinity for ß-CD. A large number of straight, branched, and cyclic alcohols that could be dissolved in aqueous solution were found to mediate the cleavage reaction. 1,6-Hexanediol, and the adipate and suberate dianions were the only compounds found to inhibit pNPA cleavage to the same extent as they inhibited the reaction of mNPA. It is arguable that because of the bulk of the hydration shell, the second hydroxyl or carboxylate group of these

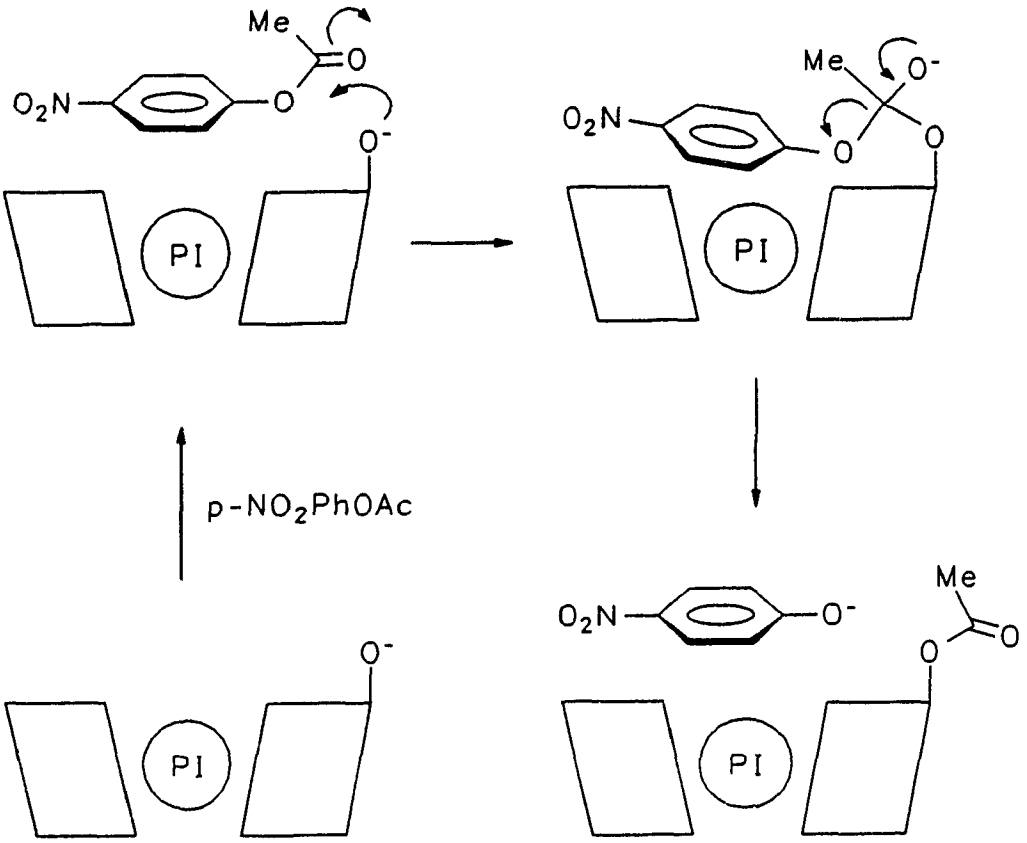

Figure 21. The inert spacer orients the substrate into a favourable position for nucleophilic attack by the ring hydroxyls when the substrate is a linearly substituted ester, i.e. pNPA. PI = potential inhibitor.

difunctional inhibitors ($X-(CH_2)_n-X$) prevents adequate approach of pNPA to the hydroxyls of ß-CD.

Many straight and branched chain alcohols were found to mediate the reaction. Attack on the substrate esters by alkoxides was ruled out as a variable because it was demonstrated that the reactivity of strongly basic oxygen anions towards pNPA is relatively insensitive to the basicity of the anion.[35] To ensure that cocatalysis was occurring as a result of the positioning of substrate by the potential inhibitor, and not as a result of nucleophilic attack of the inhibitor on the ester, two

other types of inhibitors with much lower nucleophilicities were tested: alkanoate ions and sulfonate ions, both of which gave similar results. In addition, as already mentioned, the structures of the alkanols were varied.

Before continuing, an explanation of the method used to analyze the rate constants for the reaction of pNPA in CD with inhibitors is in order. The observed rate constant for the reaction of pNPA in solution with CD and potential inhibitors of complexation is satisfactorily described by:

$$k_{obs} = \frac{k_u K_s + [CD](k_c + k_n[PI])}{K_s + [CD]} \qquad (31 = 12)$$

The derivation of this equation is described earlier in the Results Section. Rearrangement of eq 31 gives a linear form that is useful for analysis.

$$k_{corr} = \{k_{obs}(K_s + [CD]) - k_u K_s\}/[CD] = k_c + k_n[PI] \qquad (32 = 13)$$

This equation, using the equilibrium concentrations of CD and PI obtained from the quadratic formula (eq 7), allows us to plot k_{corr} values vs [PI] to obtain k_n from the slope. At very low [PI] equation 31 reduces to the normal expression employed to describe the observed rate constant (eq 4). By measuring k_u, the rate constant for the uncatalyzed reaction, K_s, the dissociation constant of the substrate and k_c, the rate constant at saturation, it is possible to determine k_n values by the above method. The rate constant k_n is that used to describe the CD·S complex reacting with the potential inhibitor and proceeding to products.

In Figure 22 the effect of 2-butanol and 2-pentanol on the cleavage of pNPA by ß-CD is compared to that expected for inhibition. The rate constants for the

reaction, calculated for competitive inhibition, decrease substantially as the concentrations of the alkanol inhibitors increase. In contrast, the observed rate constants are significantly higher. From these observations, and comparable behaviour of many other potential inhibitors,[10] we conclude that, while the alkanols are competing for binding with the cyclodextrin, they are also aiding in the achievement of reaction in some way. In the case of 2-butanol this cocatalytic effect causes not only a reduction in the expected inhibition, but a net increase in the overall rate of reaction (Fig. 22, curve **a**).

In Figure 23, some data for alcohols (2-pentanol, t-butanol, 2-hexanol, iso-pentanol) are plotted according to equation 32. The concentration of the inhibitors have been corrected for complexation with ß-CD to depict the increasing cocatalysis of pNPA cleavage by inhibitors. Note that rate accelerations increase linearly with the amount of added PI. What is not obvious from observation of the data plotted in this way is the deviation from linearity that occurs if the concentration of the inhibitor in solution greatly exceeds the K_i value of the PI. If the experiment is conducted with too much inhibitor, the catalytic effect levels off. The latter is a significant observation because if the alcohol were attacking the esters, with no assistance from CD, then the "catalysis" would not exhibit saturation. Note also the large difference in slope (k_a) made by the addition of one methylene group to the inhibitor, eg. the effect of 2-hexanol is larger than that of 2-pentanol (cf. lines **c** and **a** in Figure 23). If the potential inhibitor were somehow helping the reaction from outside of the CD cavity then one would not expect such a large difference to be

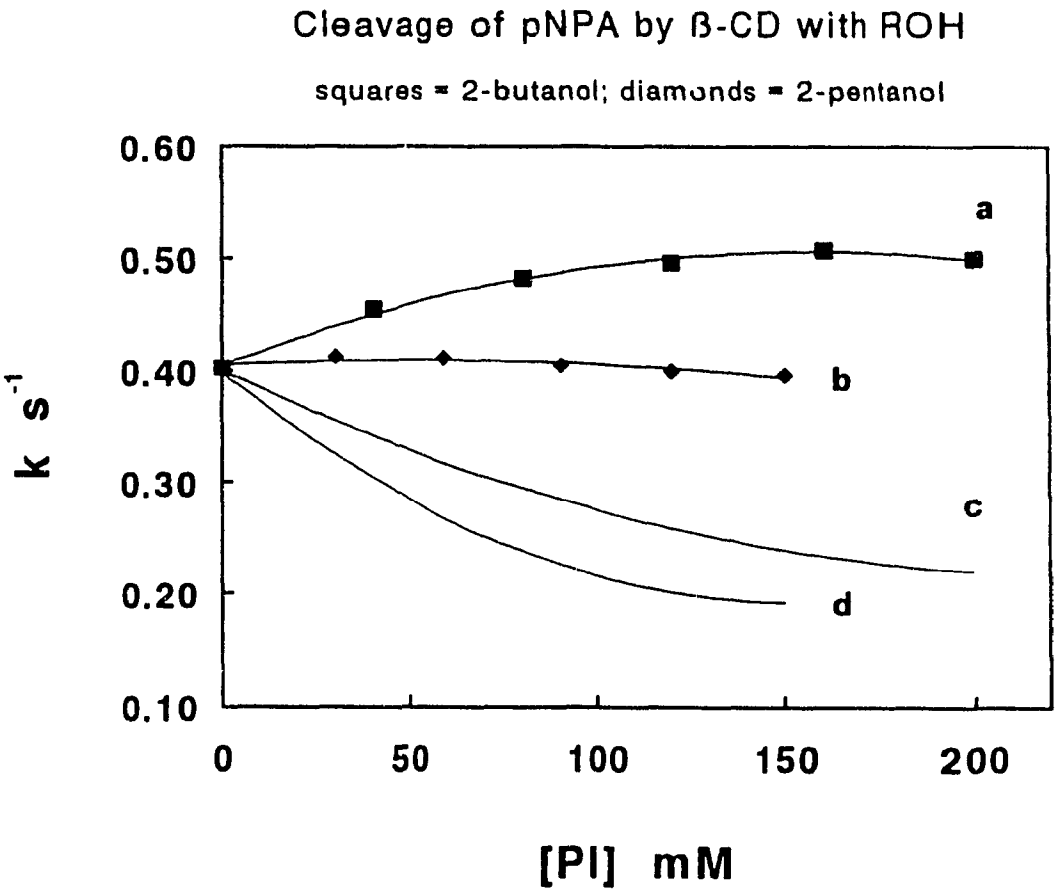

Figure 22. Plots **a** and **b** are plots of the observed rate constants for the cleavage of pNPA in 10 mM ß-CD in the presence of 2-butanol and 2-pentanol, respectively. Plots **c** and **d** represent the rate constants expected for normal, competitive inhibition. The differences between **a** and **c**, and between **b** and **d**, indicate the presence of processes mediated by the potential inhibitors.

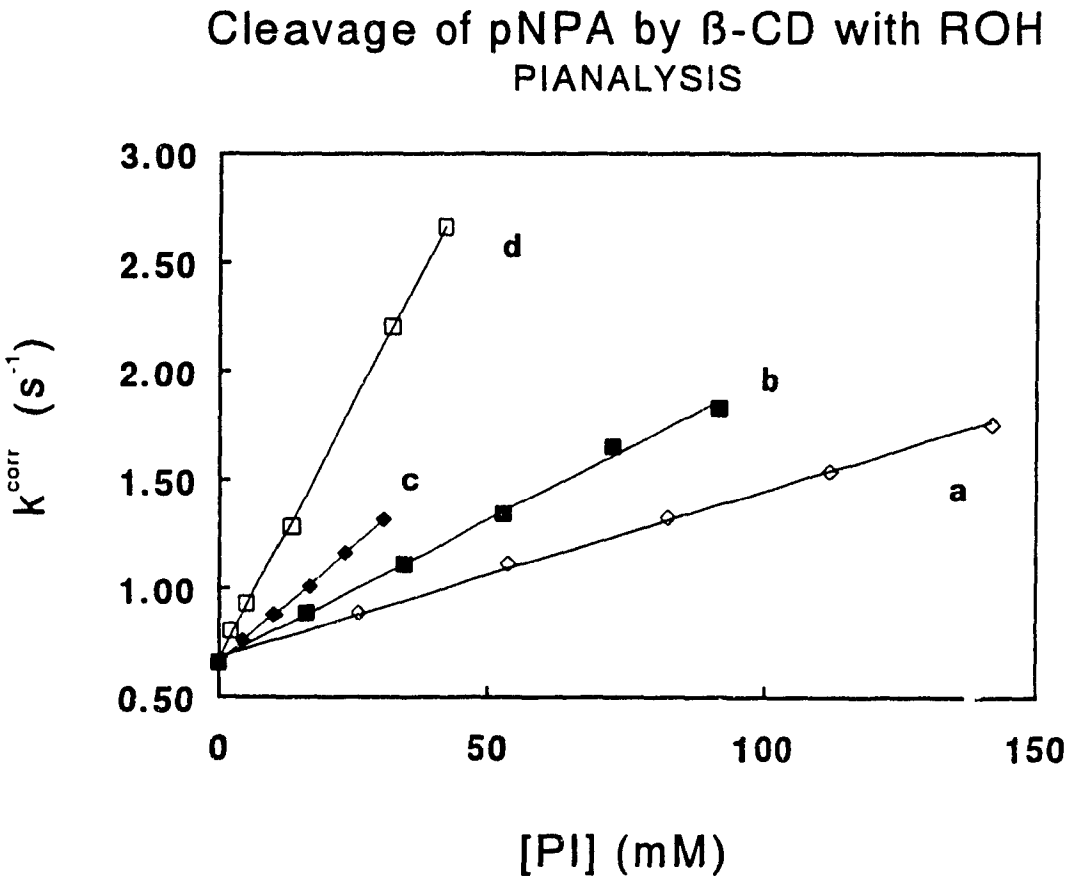

Figure 23. Analysis of the experimental data using eq 32 afforded the k_s values for: a) 2-pentanol, b) t-butanol, c) 2-hexanol, d) cyclohexanol from the slopes of the lines.

made by one methylene group.

Whereas, up to now, the inhibitor-mediated reaction has been ascribed to the steps in equation 33, there is another way for these species to interact: The CD·PI complex may form first, followed by encounter with the substrate, leading to reaction, as in equation 34. Referring to Figure 22, if all the differences between observed and expected rate in this depiction were attributable to the process described by equation 34, then the rate constant for this pathway is k_b. The two schemes discussed so far are summarized below.

$$CD + S \underset{K_s}{\rightleftharpoons} CD{\cdot}S + PI \xrightarrow{k_a} P \qquad (33)$$

$$CD + PI \underset{K_i}{\rightleftharpoons} CD{\cdot}PI + S \xrightarrow{k_b} P \qquad (34)$$

The processes in equations 33 and 34 are kinetically indistinguishable. For eq 11 the third-order rate constant $k_3 = k_a/K_s$, whereas for eq 14 $k_3 = k_b/K_i$. Thus, values of k_b can easily be calculated from k_a through: $k_b = k_a K_i/K_s$. From the trends in k_a and k_b it is possible to infer the relative positions of the reactants in the transition state. Values of these rate constants for various alcohols are in Table 6.

In view of the mechanism proposed by Tee and Hoeven,[10] it is more logical to view the occurrence of the reaction given in eq. 34 as it represents a smaller number of microscopic steps. From the point of view of an uninitiated observer, equation 33 represents a circuitous route to the transition state proposed in Figure 21, since it is impossible for the proposed tetrahedral adduct to form when a linear ester such as pNPA is complexed to CD. For a kineticist, however, the dissociation

that must occur for reaction to proceed represents a clever diagnostic tool. A series of experiments with different potential inhibitors can uncover how much the individual properties of each inhibitor are contributing to achievement of transition state structure. For example, when log k_a and log k_b are plotted against pK_i for the results of experiments with ß-CD and pNPA with alcohol inhibitors (Table 6) we obtain the graph in Figure 24. Note the difference in the variation of k_a's and k_b's with K_i's. The value for the rate constants k_a vary more than, and in a manner inversely proportional to, the values of k_b. As justified below, this is consistent with the substrate being partially dislodged from the CD cavity during reaction.

Table 6. Constants for the Basic Cleavage of p-Nitrophenyl Acetate by ß-CD in the Presence of Alcohols

Inhibitor R-OH	K_i mM	pK_i	k_a $M^{-1}\ s^{-1}$	k_b $M^{-1}\ s^{-1}$	K_{TS} mM	pK_{TS}
n-Pr	269	0.57	2.8	94	240	0.63
i-Pro	263	0.58	2.9	95	230	0.64
s-Bu	65	1.19	6.1	50	110	0.97
n-Bu	60	1.22	6.3	48	105	0.98
2-Pen	32	1.49	7.7	31	86	1.07
i-Bu	24	1.62	10	31	66	1.18
t-Bu	21	1.68	13	34	51	1.29
n-Pen	16	1.80	15	30	44	1.36
2-Hex	10.5	1.98	22	29	30	1.52
c-Pen	8.3	2.08	26	27	25	1.60
i-Pen	5.6	2.25	48	34	14	1.85
n-Hex	4.6	2.34	36	21	18	1.74
c-Hex	2.0	2.70	81	21	8.1	2.09
neo-Pen	1.74	2.76	83	18	8.0	2.10
n-Hep	1.41	2.85	58	10	11.0	1.94

Values of k_a are for: $CD.S + PI \longrightarrow P$
Values of k_b are for: $CD.PI + S \longrightarrow P$

To clarify what is happening it may help to visualize the substrate forming a tight complex with CD. In this form of encounter, the cyclodextrin ring serves to protect the linear pNPA from nucleophilic attack by its own ring hydroxyls. If pNPA were to bind in an essentially irreversible manner to ß-CD, the ester carbonyl would be protected from attack by the secondary alcohol groups lining the mouth of the ring. Rate enhancement may seem incongruous with the latter situation, but still occurs because, of course, the system is *not* static and the CD and pNPA may adopt a configuration of higher energy which is reactive. Partial dissociation of the mNPA·CD complex is less necessary than with pNPA prior to reaction because the carbonyl group is closer to the 2° hydroxyl groups, even with the substrate fully included.

A potential inhibitors cause pNPA to encounter competition for the CD pocket. The stronger the affinity of the inhibitor for the CD pocket, the more competitive force it exerts upon the substrate. Therefore a high affinity inhibitor (lower K_i) affords larger k_a values (Table 6). The potential inhibitor helps to position pNPA for more favourable attack by preventing its full entry into the cavity. If time averaged measurements were made to discern the position of the various components of the reaction mixture during reaction, high affinity inhibitors would be found to spend most of their time in the CD cavity, whereas the lower affinity ones would be found mostly in the bulk solvent, providing little assistance to transition state stabilization. Thus, k_a measurements show primarily which inhibitors *participate* as cocatalysts but not necessarily which ones are providing the most efficient "help."

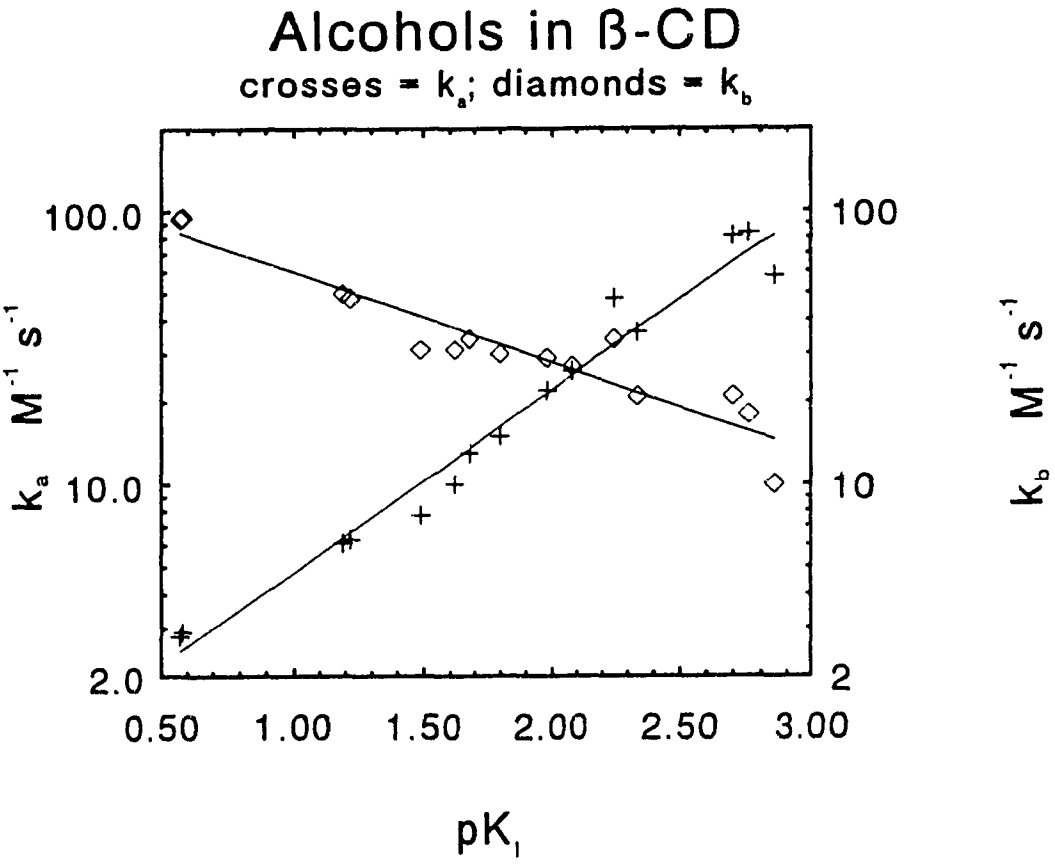

Figure 24. The reactions defined by equations 33 and 34, though kinetically indistinguishable, help to elucidate the mechanism of the reaction when a number of potential inhibitors are plotted. The increase of k_a values with increase in affinity confirms the cocatalytic effect of alcohols acting as spacers and the decrease of k_b values with increase in size of the inhibitors reveals that a small inhibitor is best suited to position the substrate.

A study of the trends in k_b values helps bring into focus the significance of other factors which are not detected with k_s. The smaller variation of k_b values represents a low sensitivity of the reaction to the structure of the inhibitor. The trend observed for k_b values is again consistent with the proposed mechanism of ester cleavage, because it confirms the potential inhibitor's role as a molecular "spectator". Once the inhibitor is complexed to CD, factors other than its affinity for CD should play a more important role in affecting the outcome of the reaction, such as its steric bulk. Hence, a trend of diminishing cocatalysis is observed as the size of inhibitor increases.

The cocatalytic effect of inhibitors and the trends of k_s and k_b values for the reaction of pNPA with ß-CD are not restricted to alcohols, but are also manifested by alkanoate ions and alkanesulfonate ions. These findings substantiate the claim that inhibitors are aiding reaction simply by acting as spacers and not by participating as nucleophiles. The experimental results for these ions are documented in Table 7 and plotted in Figures 25 and 26.

When Bender and coworkers investigated the CD-accelerated cleavage of phenyl esters with respect to the geometric requirements of reaction, it became clear that with acetate esters such as pNPA the fully complexed substrate's electrophilic carbonyl is not as accessible as that of mNPA. (the *meta-para* effect).[14] Molecular models of the reaction involving pNPA suggest that reaction can only occur with the substrate partly lifted out of the ring (Figure 12). Clearly, the energy expenditure required to unseat the complexed substrate would contribute to the activation energy

for reaction. This situation is avoided with esters which do not need to move from the CD cavity during reaction. Various designed substrates have been synthesized in an attempt to maximize the rate acceleration and large values (up to 6 million) have been obtained.[6,13,21,22]

If pNPA were to remain fully complexed, in the CD cavity, the tetrahedral intermediate formed in ester cleavage would be too strained for it to be on the chosen pathway. The results (Tables 6 and 7, Figures 24-26) strongly suggest that the presence of a "molecular spectator" in the CD pocket can facilitate the reaction by preventing full entry of the substrate into the cavity. From inspection of space-filling molecular models of ß-CD and pNPA, it seems likely that a small amount of lifting of the substrate out of the ring is necessary for attainment of maximal activity. With this consideration in mind, the only impediment to the reaction in eq 34 that could occur would be as a result of steric hindrance due to excessive length of the potential inhibitor. If, indeed, a large displacement of the substrate was necessary to achieve transition state geometry, then k_h values would continue to increase as the size of the molecular spectator increased, and the k_a values would increase even more than they do. Instead, there is a steady decrease in the k_h values. Because the maximum k_h values are attained with small inhibitors the conclusion reached is that maximization of the cocatalytic effect would be achieved by a short high affinity inhibitor. The high affinity would predispose it to be in the CD cavity so that it would be present to participate in the reaction whereas its small size would moderate the effects of steric hindrance.

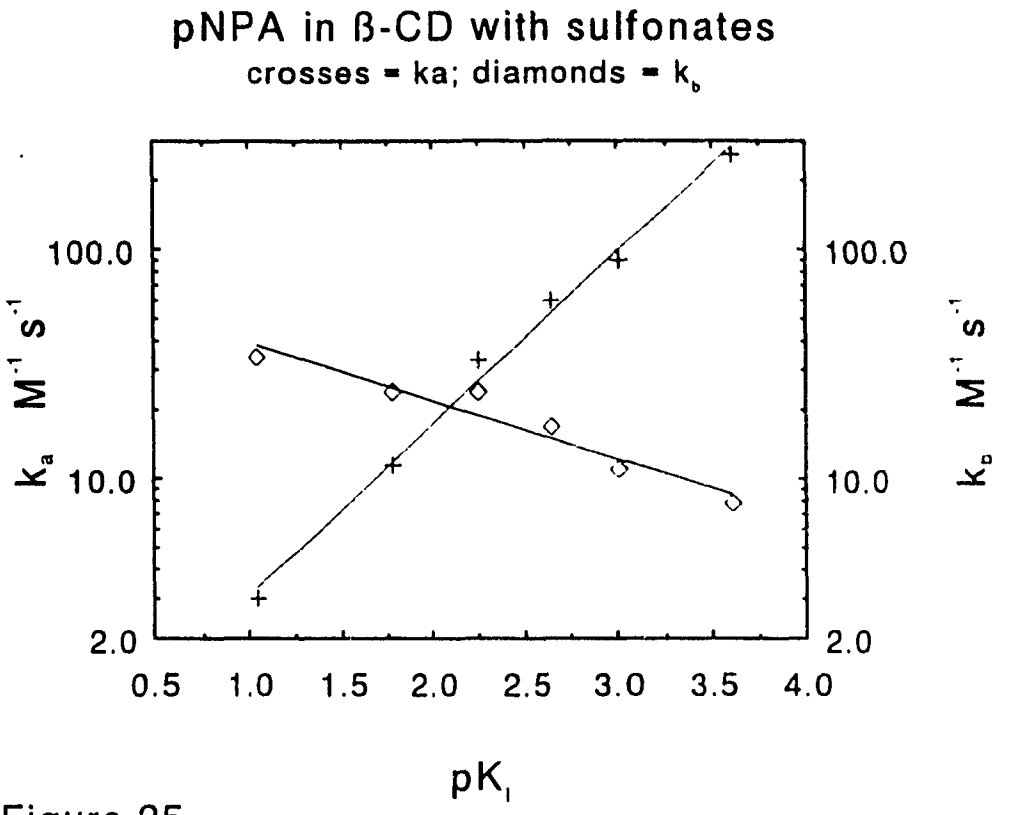

Figure 25.

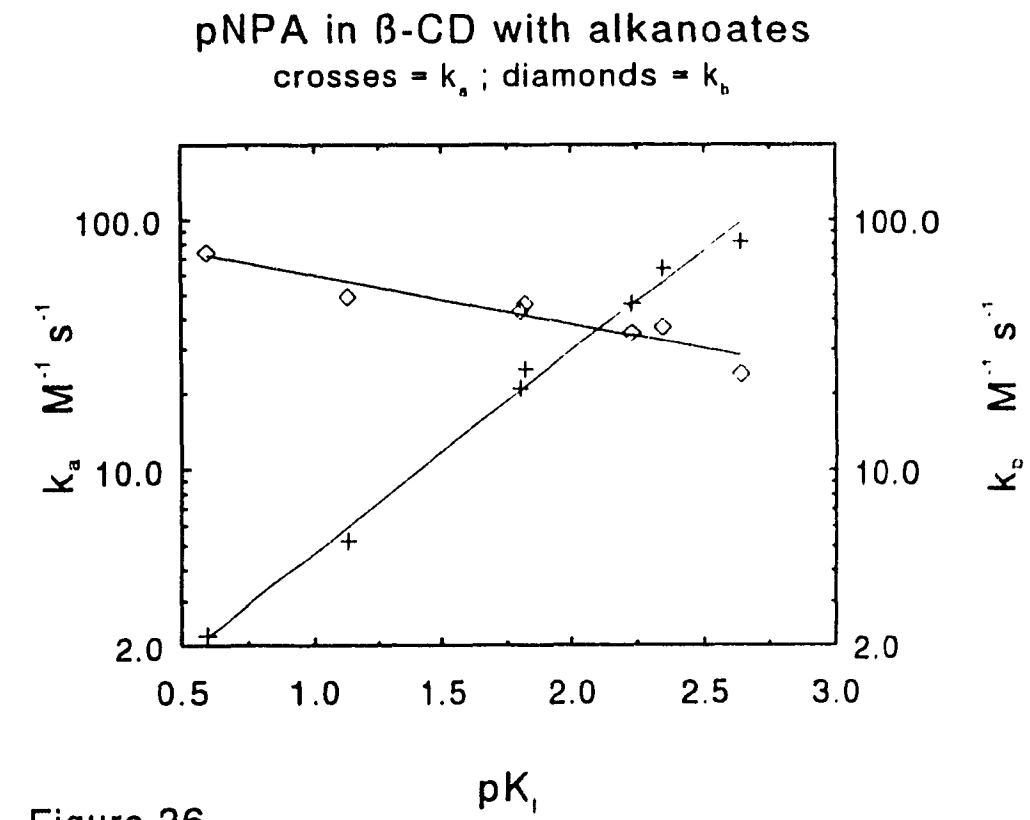

Figure 26.

Table 7. Constants for the Basic Cleavage of p-Nitrophenyl Acctate by ß-CD in the Presence of Potential Inhibitors (PI): others.

PI	K_i, mM	pK_i	k_a M^{-1} s^{-1}	k_b M^{-1} s^{-1}	K_{TS}' mM	pK_{TS}'
(a) alkanesulfonate ions:						
C4	89	1.05	3.0	34	220	0.66
C5	16.7	1.78	11.5	24	57	1.24
C6	5.6	2.25	33	24	20	1.70
C7	2.3	2.64	60	17	11	1.96
C8	0.97	3.01	90	11	7.3	2.13
C10	0.244	3.61	260	7.8	2.5	2.60
(b) alkanoate ions:						
C4	260	0.59	2.2	74	300	0.52
C5	74	1.13	5.2	49	130	0.89
C6	16	1.80	21	43	31	1.50
C6*	15	1.82	25	46	26	1.59
C7	5.9	2.23	46	35	14	1.84
C7*,*	4.6	2.34	64	37	10	2.00
C8	2.31	2.64	82	24	8.0	2.09
(c) others:						
ClO_4^- *	48	1.32	2.4	15	280	0.55

Values of k_a are for: $CD.S + PI \longrightarrow P$;
Values of k_b are for: $CD.PI + S \longrightarrow P$; k_b ($= k_a.K_i/K_s$).
C6* is 4-methylvalerate. C7* is cyclohexane carboxylate.
* This data was obtained by J.J. Hoeven.

Results of pNPA with α-CD

The effect of potential inhibitors on the cleavage of pNPA by α-CD will be discussed briefly for comparative purposes, even though the data were obtained by another member of the laboratory.[9] Broadly speaking, with α-CD the results, summarised in Table 8, were of a similar nature to those discussed above for ß-CD.

Table 8. Constants for the Basic Cleavage of p-Nitrophenyl Acetate by α-CD in the presence of Potential Inhibitors.[f]

PI	K_i mM	pK_i	k_a M^{-1} s^{-1}	k_b M^{-1} s^{-1}	K_{TS}' mM	pK_{TS}'
(a) alcohols						
i-Pr	200	0.69	0.33	6.7	800	0.10
n-Pr	43	1.37	1.8	7.4	150	0.82
s-Bu	38	1.42	1.9	7.1	140	0.85
i-Bu	36	1.44	1.5	5.2	180	0.74
c-Pen	22	1.66	2.2	4.8	120	0.92
c-Hex	15	1.81	2.5	3.8	110	0.96
i-Pen	13	1.87	4.5	6.1	59	1.23
n-Bu	11	1.95	6.0	6.7	44	1.36
2-Pen	7.4	2.13	9.1	6.7	29	1.53
n-Pen	3.1	2.51	22	6.7	12	1.91
2-Hex	2.8	2.55	28	7.9	9.5	2.02
n-Hex	1.1	2.95	75	8.3	3.6	2.45
n-Hep	0.44	3.36	145	6.3	1.8	2.74
(b) alkanesulfonate ions:						
C4	22.9	1.64	1.28	2.89	209	0.68
C5	6.37	2.19	5.90	3.72	45.3	1.34
C6	2.64	2.57	13.2	3.45	20.2	1.69
C7	1.48	2.83	26.2	3.80	10.2	1.99
C8	0.93	3.03	29.2	2.68	9.14	2.04
(c) alkanoate ions:						
C3	596	0.225	0.190	11.2	1410	-0.15
C4	90.5	1.04	1.81	16.2	148	0.83
C5	16.3	1.79	2.85	4.60	93.7	1.03
C6	3.80	2.42	12.1	4.56	22.1	1.66

[f] Data obtained by Gadosy.[9]

The observed range of variation in k_a was greater however, than that seen with ß-CD, although the absolute magnitude of the rate constants was not. Furthermore, the values for k_b were almost constant, notwithstanding the structure of potential inhibitor used. This assertion, as we shall see, provides yet more evidence for cleavage of pNPA occurring with the aryl ring lifted out of the pocket.

Molecular models reveal the smaller cavity size of α-CD does not allow the simultaneous occupation by substrate and inhibitor. As a result, if the substrate is in, the inhibitor has no entry. By the same token, if the PI is already sequestered by α-CD, approach of the ester carbonyl is very likely to lead to a favourable orientation for reaction for the same reasons mentioned with ß-CD. Figure 27 shows the data obtained for alcohols interacting with α-CD and pNPA. Notice that as a result of the smaller cavity size of α-CD an accentuation of the effects observed with ß-CD occurs. That is, a linear dependence of log k_a values on pK_i is apparent (slope = 1.03; r = 0.992), whereas log k_b varies inversely, if at all, with respect to pK_i. It is important to note that the cavity of α-CD is narrower than that of ß-CD, although both have the same depth. As a result, there is a tighter fit of the inhibitors in the α-CD cavity and because of this the spacer effect contributes more efficiently to achievement of the TS. This is observed experimentally as an increase of reaction rate over that which would be expected for simple inhibition. When the mechanisms suggested by eq 33 and 34 are analyzed in relation to the effect α-CD and potential inhibitor interactions have on the reaction, we observe structure-reactivity relationships (Figures 27-29) similar to those which were observed with ß-CD. The trend of k_b

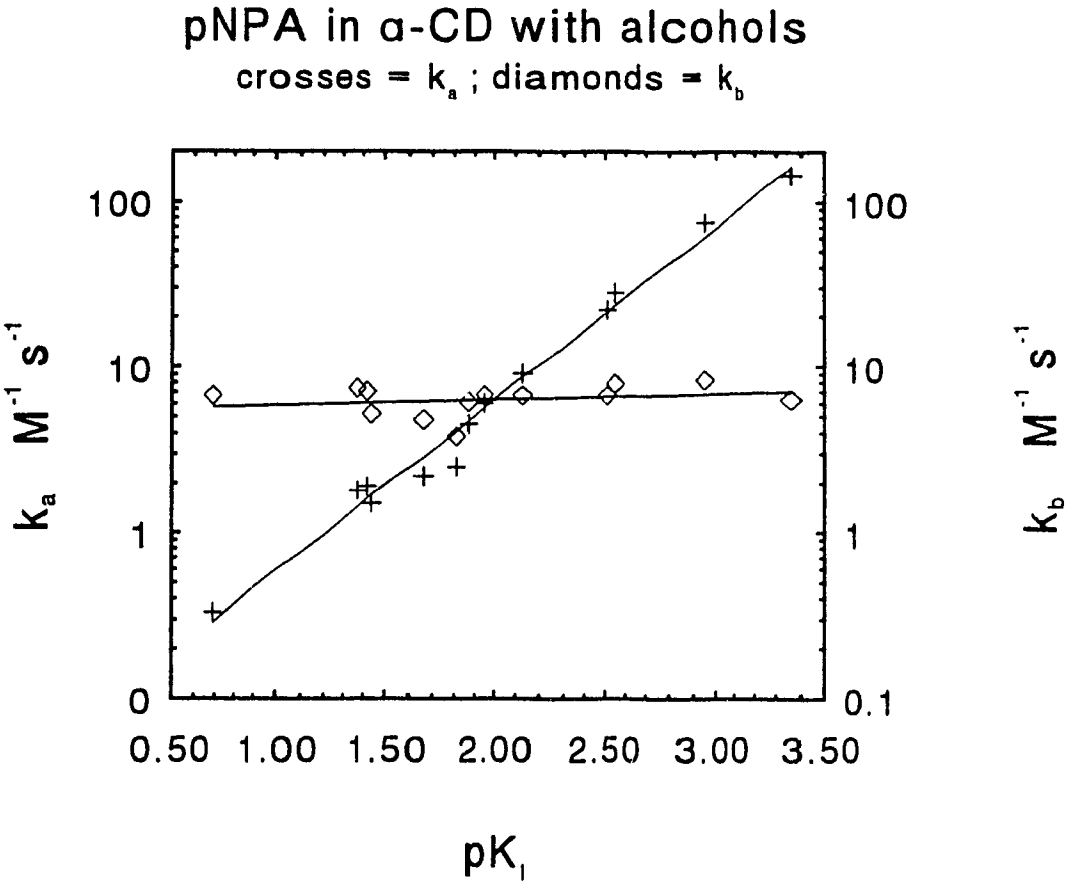

Figure 27. This plot reveals the effect of a smaller CD pocket on the cleavage of pNPA. Comparison with the trend of k_b values in Figure 24 suggests that a more complete dislodging of the substrate is necessary with α-CD.

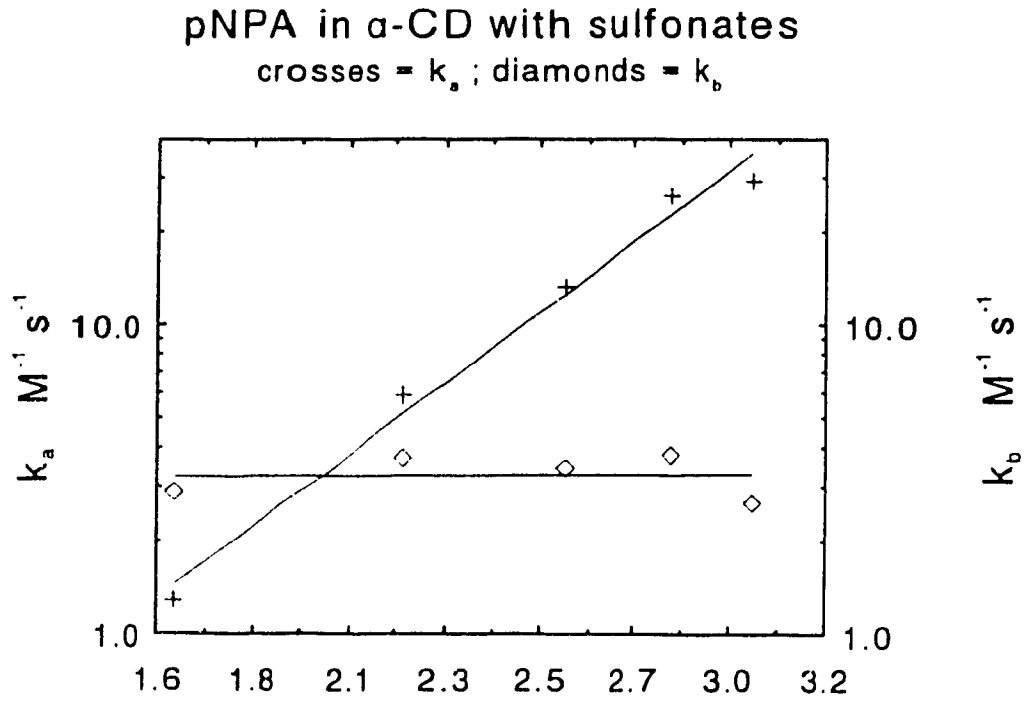

Figure 28.

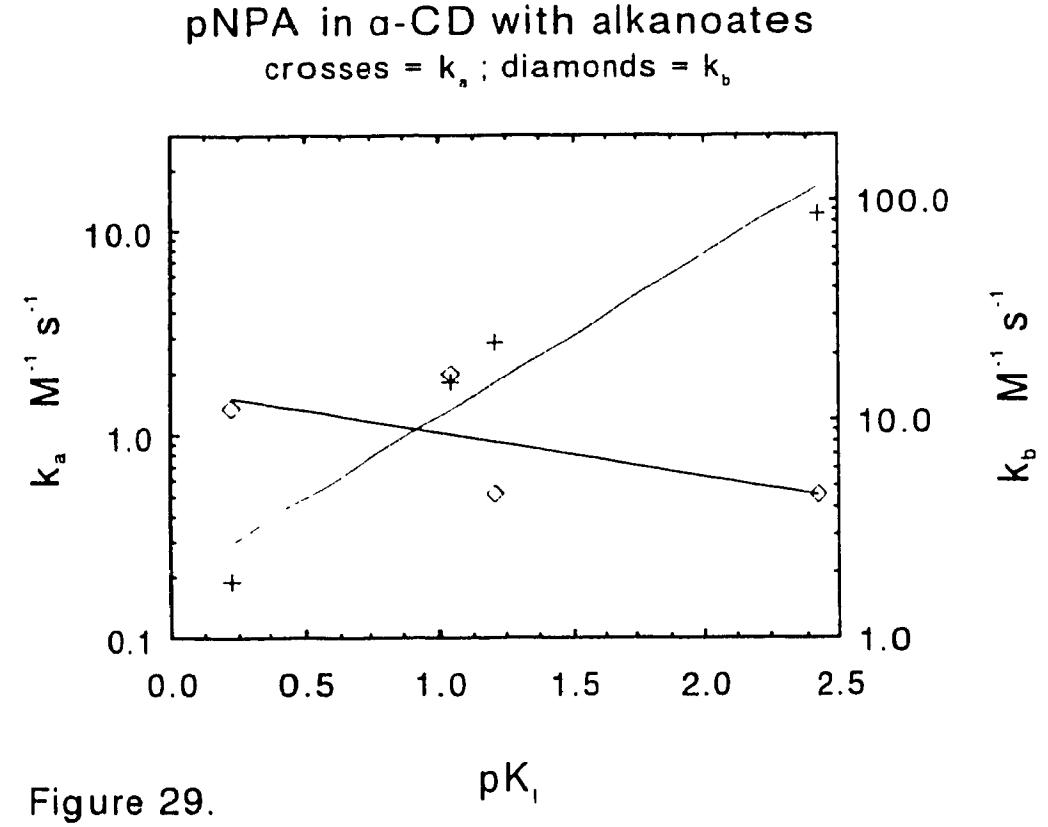

Figure 29.

values does not vary as much whereas the variation of k_s values is accentuated (compare Table 8 to Table 7). The steeper slope of the log k_s vs. pK_i data plotted in Figure 24 versus Figure 27 indicates that a more complete dislodging of substrate is necessary in the reaction with α-CD as host. Roughly the same is observed with other inhibitors, a range of alkanesulfonate and alkanoate ions (Table 9, and Figures 28 and 29).

Inspection of molecular models of the reaction between pNPA and α-CD also suggest that the ester must be largely clear of the cavity for it to have easy access to the 2'-hydroxyls on the lip of the CD. This notion is supported from the point of view of the k_b values collected: since the substrate must be well clear of the α-CD during the reaction, the size of the potential inhibitors, within reasonable constraints, should not be a limiting factor in the reaction. The constancy of k_b values (around 6 M^{-1} s^{-1}) obtained for the reaction of pNPA with α-CD·alcohol complexes attests to the conclusions drawn from the molecular models. Because the reaction of pNPA in α-CD is taking place with the substrate outside of the α-CD cavity the size of the inhibitor does not interfere sterically with the approach of the substrate. The one exception to this behaviour was observed with the results obtained by using alkanoate ions as the potential inhibitors. The k_b values decreased slightly with increasing size of the inhibitor. Perhaps it is due to the bulky solvation sphere that ionized alkanoates have. The reordering of water of solvation incurred by the approach of substrate may repress the occurrence of a suitably energetic collisions with substrate.

Figure 28 exhibits the same pattern as the alcohols and alkanoates with

respect to cocatalysis, albeit of reduced magnitude. Because the longer alkanesulfonate ions tend to form micelles in aqueous solution[27-29,37] there is a limit on the amount that can be dissolved into solution without introducing another variable.

Transition State similar to Pre-Equilibrium Structure

The results for the effect of inhibitors on the cleavage of pNPA by CDs can be explained equally with the schemes in equations 33 or 34, providing the constants in Tables 6 through 8. However, the minimal variation of k_b with structure suggests that eq 34 represents the more likely pathway taken by the reaction. A rational explanation for this conclusion is provided by analysis of the positioning of the nucleophilic hydroxyls on the cyclodextrin relative to the bound ester. A *p*-substituted ester which has fully penetrated the cavity will not be in reach of the 2'-hydroxyls (Figure 12). In the scheme where substrate enters CD first, as represented by eq 33, a high inhibitor affinity aids in stimulating reaction rates by dislodging the substrate. On the other hand, in the scheme where the inhibitor enters the CD ring first (eq 34), there is less interdependence between binding affinity of the inhibitor and reaction rate than there is between the steric bulk of the spacer and the resulting rate enhancement.

On the basis of the observations discussed in the previous two sections it is proposed that the pre-equilibrium structure in the initial state (CD.PI + S), with respect to positioning of the inhibitor, closely resemble the structure of the proposed

transition state for ester cleavage assisted by CD and potential inhibitors (see Figure 21). This conclusion is supported by the relative uniformity of k_h values for each CD, regardless of the nature of spacer used.

Ternary Complex analysis of pNPH Cleavage Data

As described earlier, the analysis of data according to the method used for pNPA cleavage (PIANALYSIS) resulted in non-linear plots which exhibited saturation when *p*-nitrophenyl hexanoate (pNPH) was used as substrate. Accordingly, the program CDFIT was used for analysis of the saturation behaviour of the pNPH data, from which were obtained values of K_t and k_t (see eqs 15 - 23) which correspond to the formation and reactivity of ternary pre-equilibrium complexes (eq. 15):

$$PI + CD{\cdot}S \underset{K_t}{\rightleftharpoons} PI{\cdot}CD{\cdot}S \xrightarrow{k_t} P \qquad (15)$$

The ternary complexes can be imagined as a logical step towards the reaction geometry which exists somewhere between the pre-equilibrium suggested by eq. 34 and the proposed transition state for reaction with the potential inhibitor inside the CD cavity and substrate atop (Figure 21). The ternary complex is probably close enough in structure to the actual structure of the transition state to make comparisons in the energetics of formation of the proposed ternary complexes (proportional to $pK_t = -\log K_t$) and the binding of PI in the transition state (proportional to $pK_{TS}' = -\log K_{TS}'$; K_{TS}' is defined in eq 29).

The use of K_{TS}' values aid the understanding of what is occurring during

reaction because the values represent the stability of the transition state as it is inferred by measurable parameters:[g] k_u, k_c, k_a, k_b, and K_i. Tee has applied this method, developed by Kurz,[31] and used by enzymologists, to the reactions mediated by cyclodextrins.[16] The premise of this method is that the most important factor in catalysis is the stabilization of the reaction transition state by the CD, or any other catalyst. From the above constants one can calculate the pseudo-dissociation constants K_{TS}, and K_{TS}'. With these constants, the extent of involvement of the CD catalyst and the potential inhibitor with the transition state for ester cleavage can be revealed (see equations 25 - 29)

This application of transition state theory can provide us with constants that can be used in linear free energy correlations when plotted according to suitable parameters, as we shall see later.[16] This approach has been used before as a method of showing the chain-length dependance of reaction rates in CD for certain substrates. For example, when $pK_{TS} = -\log K_{TS}$ for the cleavage reaction of *m*- and *p*-nitrophenyl alkanoates by CD is plotted versus the acyl chain length (Fig. 30) there is a notable difference in sensitivity of K_{TS} values, reflecting different mechanisms for the two series of substrates.[16,18] Chain length is chosen as one of the variables because size and hydrophobicity parameters both increase linearly with chain length.[16] Thus, as the length of the acyl chain increases, it exerts a more energetic demand for sequestration by CD. It can be deduced from observing the graph below that with

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[g] Appendix B represents a compilation of the salient constants and their interrelations.

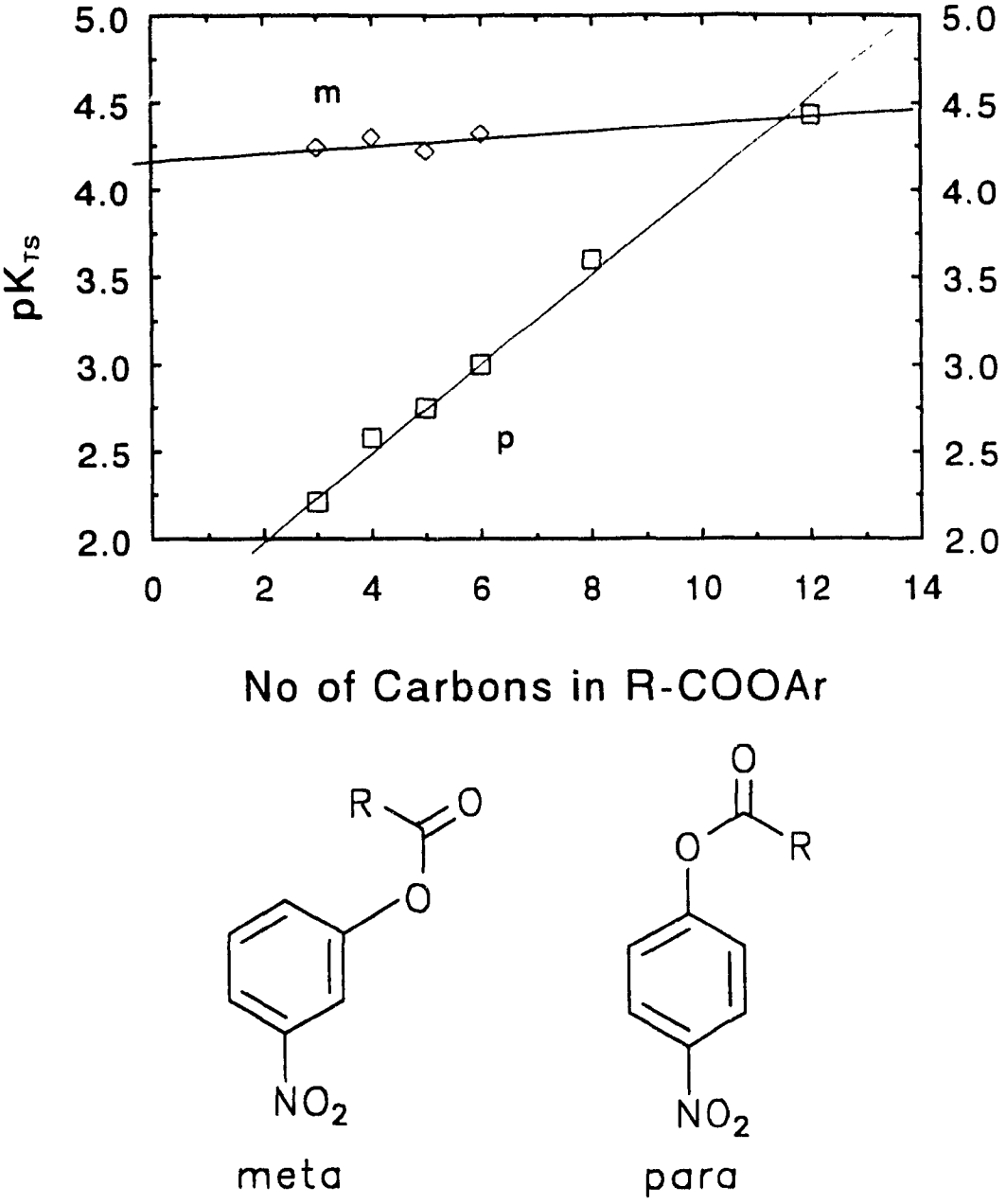

Figure 30. The dependence of pK_{TS} values upon alkyl chain length,[16] for the *para* isomers, signals that the preferred orientation for this substrate is with the alkyl chain in the CD cavity, whereas for the *meta* isomers the preferred orientation is with aryl ring in the cavity. Presumably, there is a switch-over in preferred geometry for the *meta* substituted substrates at a chain length of approximately 11 carbons.

p-nitrophenyl esters, alkyl chain binding in the cavity predominates, whereas with the *m*-nitro isomers the aryl group remains in the ring until a critical length is reached by the chain, at which point, binding of the alkyl chain will predominate.

The dissimilar behaviour of the two series of substrates in Figure 30 is a product of transition state geometry. *m*-Nitrophenyl alkanoates, because of their shape, stringently meet the geometric requirements of the transition state imposed by acyl transfer via aryl inclusion when complexed to CD. Therefore they are quite insensitive to changes in the length of the acyl chain. On the other hand, *p*-nitrophenyl alkanoates benefit from an increase in the acyl chain length because inclusion of the *p*-substituted aryl group into the CD cavity does not lead to efficient cleavage, whereas the floppy alkanoate chain can provide more productive binding. If the graphs of pK_{TS} vs chain length truly represent linear free energy relationships, the switch-over in reactivity should occur where the curves intersect, thus, the *meta-para* effect should vanish, and the point of cross-over on the linear free energy relationship should predict the chain length at which the mechanisms of reaction become identical.[16,18] The value of a linear free energy relationship such as the latter is demonstrated by its use as a probe of mechanism. The relative energy difference between the "binding",inthe transition state, of a substrate that reacts readily and one that does not, can be compared to the effect of potential inhibitors on the reaction with less favourably shaped substrates to see if the proposed mechanisms are reasonable.

Parallels With Enzyme Behaviour

As will be shown in the next section, when pK_{TS}' values for the transition states incorporating PI are plotted against pK_i of the inhibitors, linear relationships are obtained, demonstrating how the affinity of the inert spacer serves to "lever" the substrate into place during the reaction transition state. The higher the affinity of the spacer for the CD, the more effectively it accommodates the transition state configuration.

The use of potential inhibitors in a reaction which is accelerated by the presence of CD can be a useful model of enzyme behaviour. An enzyme helps achievement of the transition state by manoeuvring its substrate into place with the active site functional groups, or by providing nucleophilic and electrophilic moieties in close proximity to areas where electron movements are necessary to the reaction.[23,24] In much the same way, the substrate.CD complex can be assisted towards the transition state by the intrusive presence of an inert spacer, as seen for the cleavage of pNPA and pNPH by CDs in this study.

Many of the attributes of enzyme-catalysed reactions have been mimicked using CDs, eg. substrate specificity,[1] complexation,[2] stereochemical selectivity,[51] saturation kinetics,[1,4] high rate of reaction and accommodation of transition state structure.[13,21] The accommodation of the transition state is a recurrent theme in discussions involving enzymes[14,15] and study of it by use of CD models may provide the necessary simplification to gain better understanding of biochemical reactions.

An example of the effect afforded by steric hindrance at the active site of a

carrier protein, or perhaps even the binding site of an enzyme, is reminiscent of the strategy that nature has evolved to decrease the preferential binding of carbon monoxide to deoxyhemoglobin compared to oxygen. The active site of this oxygen carrier is a sterically hindered cavity, which discourages the linear bonding of carbon-monoxide, yet provides a snug fit for the obliquely bound oxygen molecule. Because oxygen binds at an angle in the active site, it is not disturbed by the low clearance; whereas the linearly bound CO is. Any alteration of the geometry by amino acid substitutions at the active site inevitably leads to a loss of selectivity between the two gases.[24,36]

Similarly, with cyclodextrins the weakening effect that a potential inhibitor exerts upon ground-state complexation of the reactant, effectively increases the concentration of favourably oriented substrate. In this way, more of the available free energy is available to fuel the breakage and formation of new bonds, which translates into a faster rate for the reaction.

Applications of Transition State Theory

The linear free energy relationships that can be plotted making use of the transition state theory, as in Figure 30, serve as useful probes of mechanism. The likelihood of a given mechanism occurring can be deduced by analyzing data pertaining to it with data from a similar reaction. In this way, researchers can elucidate the most likely path the reaction is going to take, which will often be the one with the tightest binding of the transition state. Another facet of this approach

allows us to establish whether or not a series of modifications to a given substrate is effective in achieving the transition state structure.

For example, the *para-meta* effect is revealed as a relative insensitivity of transition state binding to *p*-substitution of a phenyl acetate as compared to the effect of *m*-substitution (Figure 31). The plot suggests that *p*-substituted substrates do not benefit greatly from an increased ground-state complexation, due, presumably, to the widening of the gap between the geometry of the activated state and the ground-state complex. On the other hand, for the *m*-substituted substrates there is an increase in transition-state binding because of the positioning in the ground state being closer to that of the transition state.

Correlations of transition state-binding with initial state binding for various potential inhibitors used in this study, with both α- and ß-CD, yield further evidence for the proposed mechanism of pNPA cleavage by cyclodextrins (Figure 12). Earlier in this discussion it was concluded that it is necessary for the substrate to lift out of the CD cavity for reaction to take place and that the reaction mediated by α-CD requires more complete elevation of the substrate because the α-CD cavity is narrower, the presence of spacer molecules may help to hold the substrate in a geometry which is more appropriate for reaction despite being a competitive inhibitor for complexation.

The effect of the smaller α-CD pocket is demonstrated in Figure 32 by the greater slope of the line of the plot of pK_{TS}' against pK_i for the data obtained with α-CD than with ß-CD. Transition state-binding is improved more by the potential

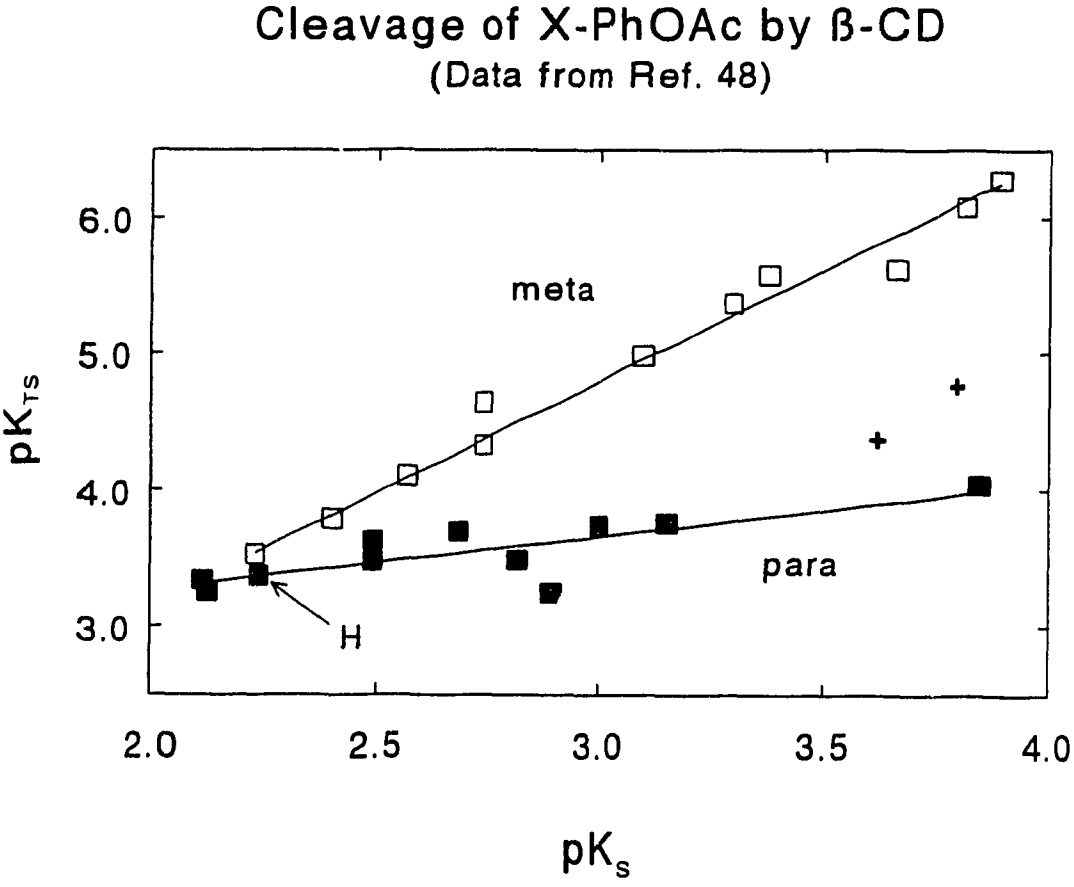

Figure 31. Comparison of the effects of *m*- and *p*-alkyl and halo substituents on the cleavage of phenyl acetate by ß-CD. The plot reveals a greater similarity between transition state binding and ground-state binding for the *m*-substituted compounds.[34]

inhibitors that are larger and have greater affinities for cyclodextrin, especially for the reaction in α-CD. The cleavage of pNPA by ß-CD is not as responsive to an increase in the affinity of the inhibitor because the looser cavity of ß-CD allows more free movement of both substrate and inhibitor, and therefore steric factors play an attenuated role when compared to α-CD.

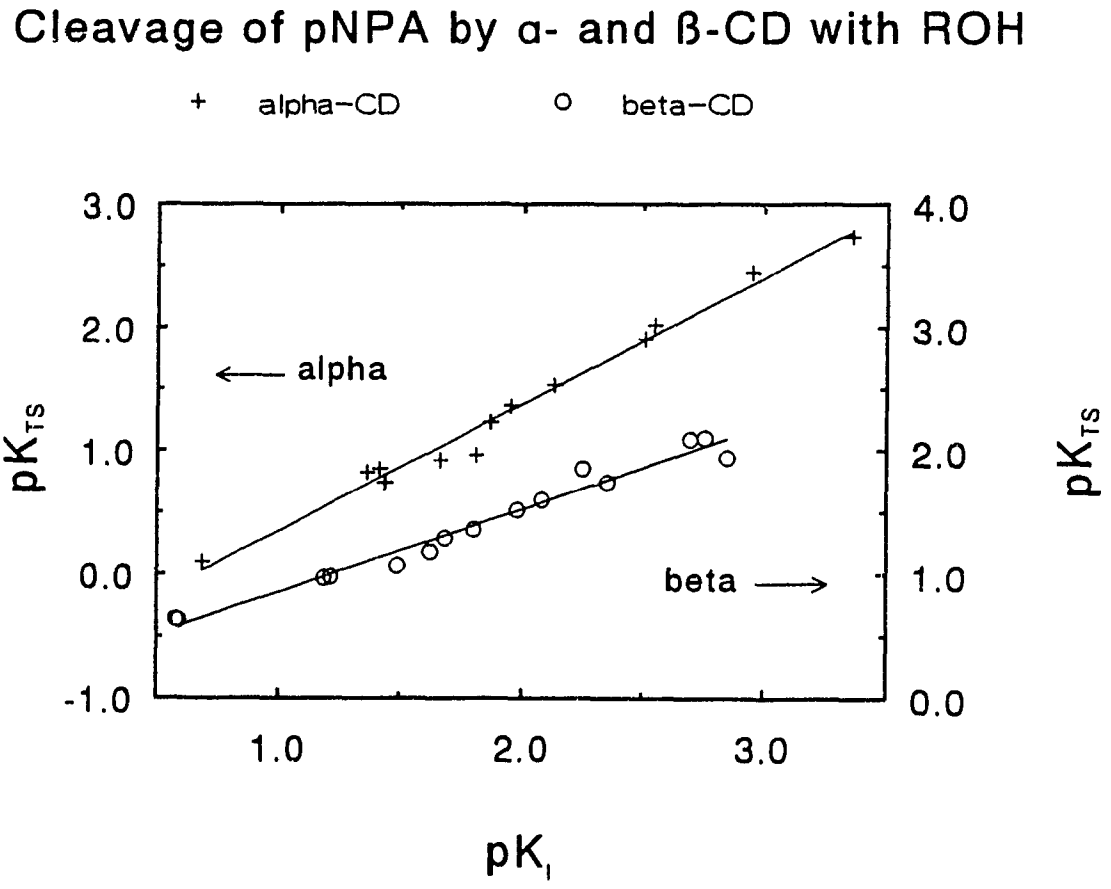

Figure 32. Transition state binding of pNPA cleavage benefits more from an increase in the affinity of the potential inhibitor if its fit is tighter. The slope of the line for α-CD is 1.03; that for ß-CD is 0.67.

Limitations of the Transition State Theory

It is hard to provide *direct* proof of transition state theory due to the short life of the transition state (on the order of $h/k_BT \approx 10^{-13}$ sec) and because the signal that would be observed has to compete with more populous species.[11] Nevertheless, the concepts provide a framework for the analysis of rate constant data that allows us to "observe" the behaviour of the transition state. Briefly, the assumption is made that to define the rate for any single step reaction, it suffices to give the structure of only one intermediate which is at the saddle point of the potential energy surface for reactants and products. "The concentration of activated complexes in that transition state is further assumed to be maintained at its equilibrium value with respect to the concentration of reactants."[31] This condition is necessary so that it becomes possible to manipulate (mathematically) the concentrations of activated species in the same manner that those of stable compounds are treated. In other words, transition state theory relates the concentration of reactants, the concentration of the activated complex and the frequency with which the activated complex passes through the transition state to the rate of reaction.

However, caution must be employed in the application of transition state theory to enzyme-catalyzed reactions. Wolfenden[32] has stated that the observed rate acceleration (k_c/k_u) provides only a minimum estimate of the rate enhancement that the enzyme might produce if both the catalyzed and non-catalyzed transition state structures were the same. We may represent this affirmation in terms of the rate and association constants, as follows:

$$\frac{K_S}{K_{TS}} \geq \frac{k_c}{k_u} \qquad (35)$$

The reason for this reservation can be ascribed to the fact that it is possible for the enzyme to catalyze the reaction through a pathway that does not resemble the mechanism taken by the uncatalyzed reaction. In other words, k_c may refer to an entirely different mechanism from that to which k_u applies. For example, an enzyme might stabilise the formation of a carbonium ion whereas the non-enzymatic reaction proceeds through a carbanion intermediate.[14] If there is a significant difference in the two mechanisms, then the non-enzymatic reaction (k_u process) must have a lower free energy of activation than that of the uncatalyzed version of the enzyme-catalyzed reaction (k_u', say), otherwise the enzyme would not be needed for that particular transition state to occur, nor would the selective pressure necessary to its evolution exist.[14] Another factor contributing to inequality of the above relationship (35) is proffered by Wolfenden.[32] Even if the two mechanistic pathways are the same in form, this will not guarantee the same timing or concertedness in the bond breaking and making processes. An extreme case of this phenomenon would result if the enzymatic rate were dependent on some step such as protein isomerization or product release from the enzyme, with no counterpart in the non-enzymatic reaction.[32] This particular problem should not greatly affect acyl transfers to CDs due to the small size of the components involved. Furthermore, it has been established that both the uncatalyzed and catalysed reactions proceed via a tetrahedral intermediate.[1]

Parallelism of Ternary Binding with Transition State Binding

As mentioned earlier, one of the applications of transition state theory derived by Kurz[31] and expanded upon by others,[14-16,32] serves as a useful probe of mechanism. A comparison of the variation in transition state binding to changes in the substrate structure or its binding affinity for CD, as in Figures 30 and 31, may shed light on the mechanism of reaction. If the mechanism is known, such as in the case of acyl transfer to cyclodextrin, then it may be helpful to probe the role of spectator molecules in catalysis with respect to the structure of the "catalyst" as in Figure 32.

As discussed earlier, the formation of ternary complexes (S·CD·PI) was suspected upon the observation of saturation kinetics (eg. Figure 18) for the reaction of pNPH with ß-CD catalyzed by alcohols. For these cases, the normal equation for the observed rate constant (eq 4) had to be modified to include ternary complex formation (K_t) and the rate constant (k_t) for reaction of these species (eqs 15-23). Table 10 summarizes the experimental results obtained with pNPH, ß-CD and a series of alcohols, and analyzed in terms of this model. Because earlier work had concluded that pNPH binds to CDs through its acyl chain[18,20] it was anticipated that inhibition of the reaction would occur. Inhibition was, in fact, observed with 1,6-hexanediol, suberate dianion, and perchlorate anion but simple alcohols, alkanoate ions, and alkanesulfonate ions showed catalysis thus necessitating the formulation of a new approach to analyze the data. A reasonable approximation was settled upon with eq 23. From this equation were calculated the values of K_t, which vary in same manner as does the affinity of the inhibitors for CD (K_i). Conversely, k_t values vary

little, from 0.60 to 0.20 s^{-1}, with the structure of the alcohols (Table 10). The small variation of k_t values confirms the role of alcohols as spacers. The same arguments can be applied to the relative invariance of k_t values, as were applied to the near constancy of k_b values for the reaction of pNPA with CDs in the presence of PIs.

Table 9. Constants for Cleavage of pNPH in Presence of ß-CD and Alcohols.

R-OH	pK_t[h]	K_t mM	k_t s^{-1}	k_t/k_u[i]	K_{TS} mM	pK_{TS}
n-Pr	0.57	368	0.61	12.3	99.5	1.00
i-Pro	0.58	415	0.52	10.5	95.3	1.02
s-Bu	1.19	116	0.40	8.10	38.3	1.42
n-Bu	1.22	85	0.42	8.50	26.9	1.57
2-Pen	1.49	81	0.32	6.48	36.9	1.43
i-Bu	1.62	44	0.31	6.28	16.7	1.78
t-Bu	1.68	45	0.30	6.17	17.9	1.75
n-Pen	1.80	38	0.35	7.09	13.6	1.87
c-Pen	2.08	16	0.37	7.49	6.0	2.22
n-Hex	2.34	9.8	0.22	4.45	5.6	2.25
c-Hex	2.70	5.8	0.26	5.26	2.9	2.54
neo-Pen	2.76	3.3	0.20	4.05	2.3	2.65

h. Values of K_t are contained in Table 6.
i. The value of k_u in this calculation is 0.0494 s^{-1} as in Appendix C for [ß-CD] = 0.

The next application of transition state theory will be used to quantify the energy difference between the pre-equilibrium ternary complexes (S·CD·PI) discussed above and the transition state for ester cleavage that incorporates CD and the potential inhibitor, PI. In the next graph (Figure 33) there are two telling correlations involving the constants for transition state binding (pK_{TS}) and ternary complex binding (pK_t) with the affinity of alcohols for ß-CD (pK_t). The similarity

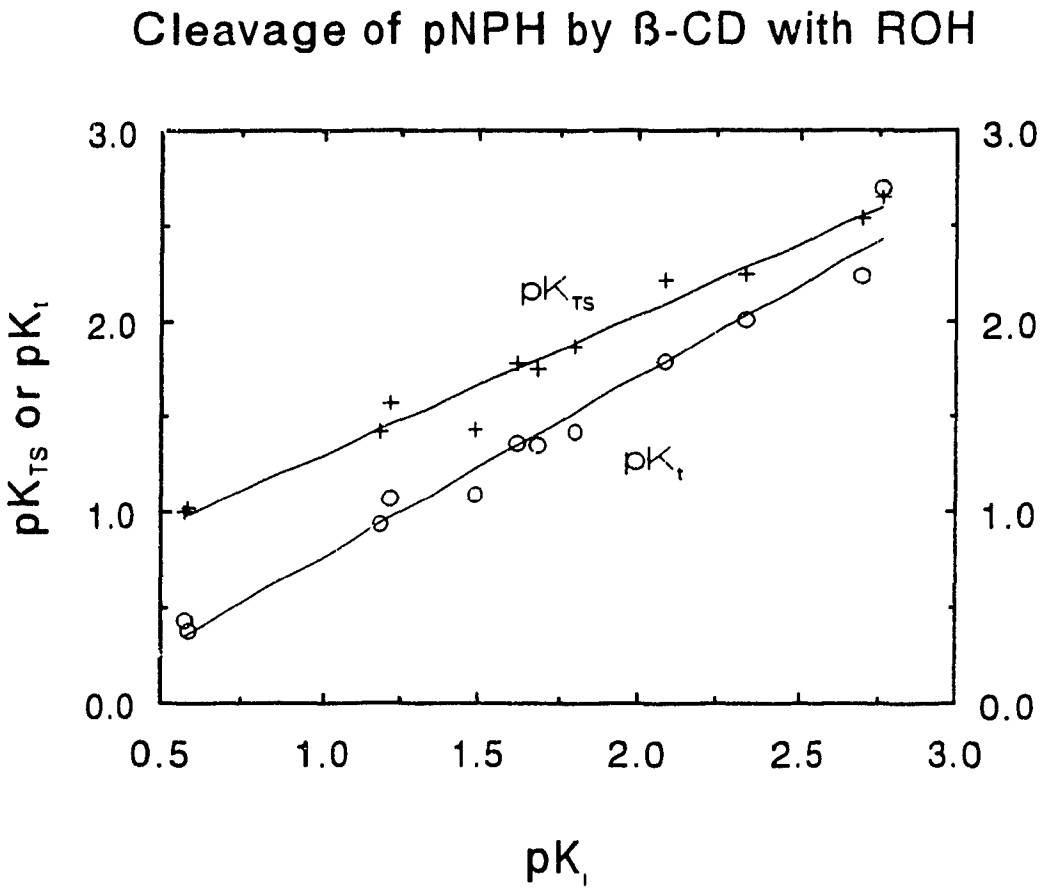

Figure 33. The two remarkable linear free energy relationships above suggest that the termolecular transition states and the ternary complexes in eq 15 have similar structures, at least in respect of the binding of the alcohols.

of the two plots, with slope approaching one (0.74 and 0.90), are consistent with modes of binding of the alcohol in the transition state and the ternary complex that are not too dissimilar from those in the ß-CD·alcohol complexes.[11] The convergence of the lines as the size and affinity of the inhibitor increases arises because k_t diminishes (Table 10) and approaches $k_c = 0.14\ s^{-1}$. The lines terminate where they do as a result of solubility limitations of the inhibitors.[1] The data may also be plotted as in Figure 34 which shows that there is a good linear relationship between the pK_{TS} and pK_t values. This finding also supports the notion that the structures of the transition states and the ternary complexes are similar as regards the binding of the alcohols.

The conclusion we may draw from Figures 33 and 34 is that as the affinity of the alcohols for ß-CD increases, the binding of PI in the ternary complexes PI·CD·S and in the related transition states becomes stronger. However, as the size of the alcohols increases, the magnitude of the catalytic effect that they exert decreases. This is reflected by the downward trend of k_t values in Table 10. From the latter observations it is plausible that the reaction would be catalyzed most efficiently by a small high affinity inhibitor, if such exists.

[1] When 1-octanol was added to aqueous CD solutions a white flocculent precipitate was seen to form at all but the lowest concentrations. i.e >1mM.

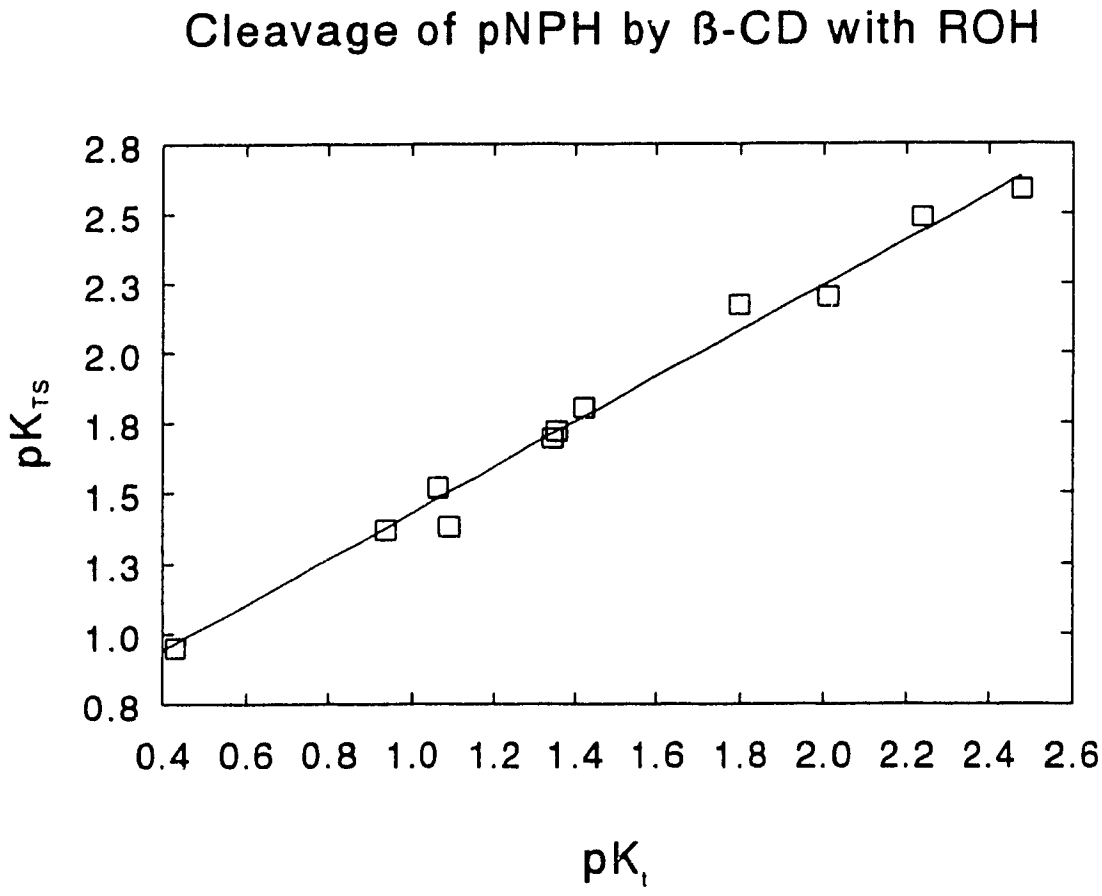

Figure 34. Data from Table 10. A linear relationship exists between the binding of the alcohol inhibitor in the cleavage transition state and in the ternary complex preceding it. The slope of the line is 0.81 and the correlation coefficient is 0.994.

Effects of Alkanoate and Alkanesulfonate ions on pNPH in ß-CD

The results from experiments with alkanesulfonate ions in this section were analyzed according to the binary pre-equilibrium model (eqs 11 and 14) and the ternary pre-equilibrium model (eq 15). The alkanoate ions yielded satisfactorily to analysis with PIANALYSIS (eq 11) and so were analyzed only by that method. To avert 2:1 binding, the concentrations of potential inhibitors were kept low. Table 11 documents the k_a, k_b and k_t values obtained.

Table 10. Constants for the Basic Cleavage of p-Nitrophenyl Hexanoate by ß-CD in the Presence of Inhibitors (PI): Sulfonates and Alkanoates

PI	K_i mM	pK_i	k_a $M^{-1}s^{-1}$	k_b $M^{-1}s^{-1}$	k_t s^{-1}	k_t/k_u [k]
(a) alkanesulfonate ions:						
C5	16.7	1.78	2.88	36.2	0.31	6.28
C6	5.65	2.25	7.50	31.9	0.29	5.87
C7	2.30	2.64	13.62	23.4	0.26	5.26
C8	0.97	3.01	41.2	30.1	0.30	6.07
C10	0.244	3.61	112.1	20.6	0.24	4.86
(b) alkanoate ions						
C4	260	0.59	0.32	62.2	-	-
C5	74.4	1.13	1.95	108.8	-	-
C6	16.0	1.80	.24	87.2	-	-
C7	5.86	2.23	7.00	30.9	-	-
C8	2.31	2.64	21.1	36.6	-	-

k. $k_u = 0.0494 s^{-1}$

Figures 35 and 36 show plots of log k_a and log k_b versus pK_i for the data summarised in Table 11.

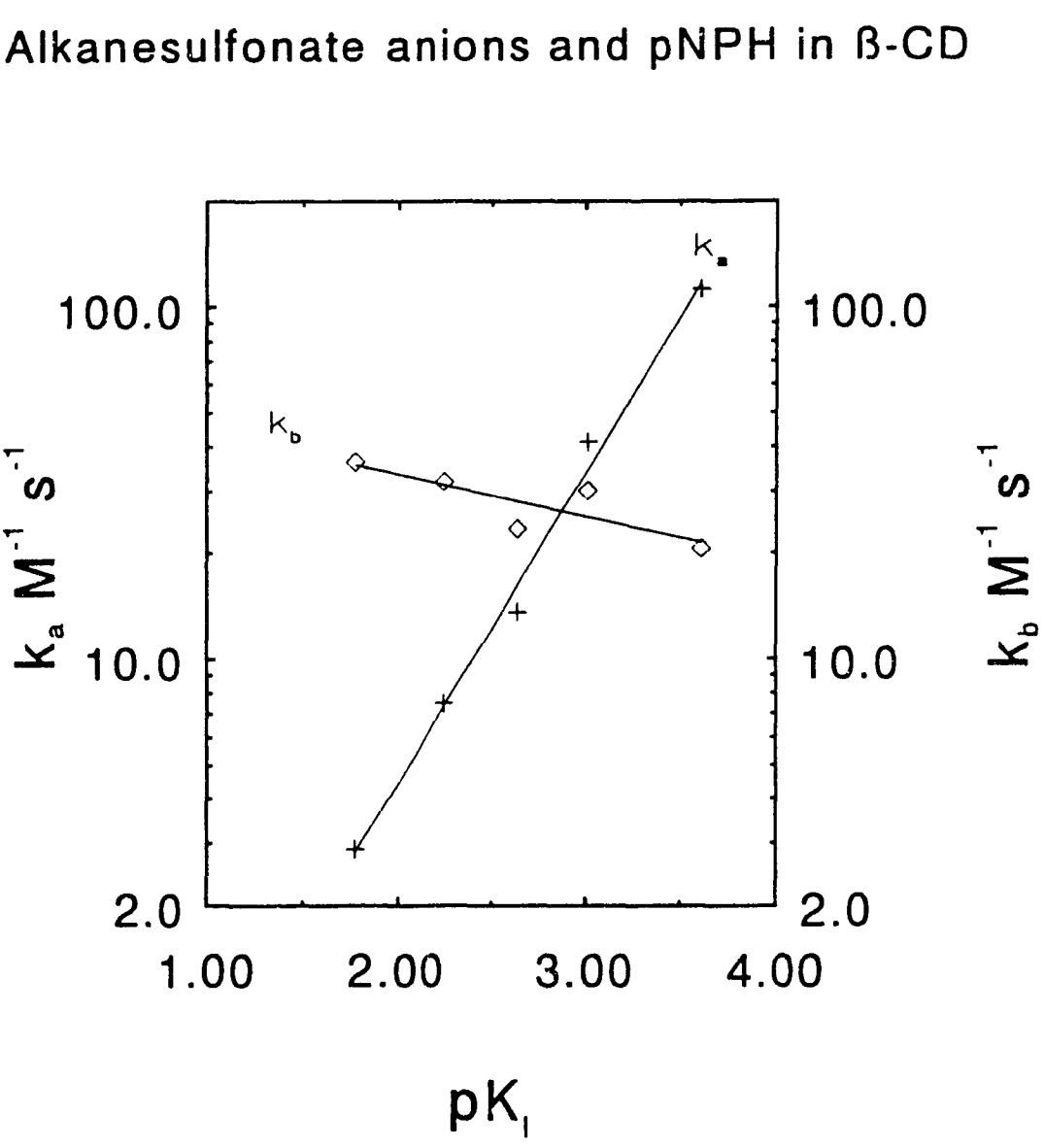

Figure 35. Plot of k_a and k_b against pK_i, for the alkanesulfonates from the data in Table 11. These results are similar to those obtained with pNPA as the substrate. See Figures 24-26 and the accompanying discussion.

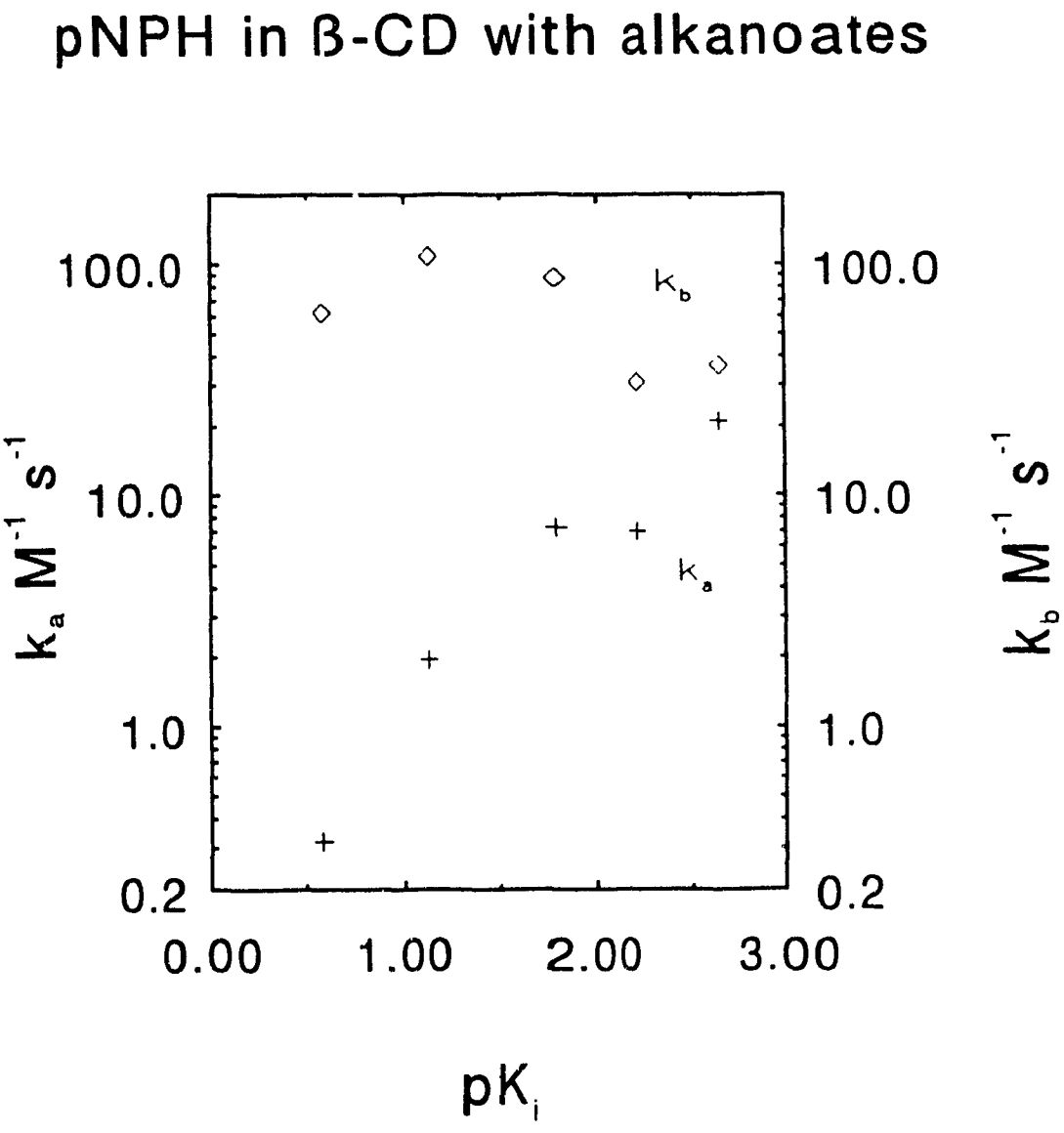

Figure 36. In the ß-CD catalysed cleavage of pNPH, the shorter alkanoates seem to possess the most efficient geometry for catalysis (note the high k_b values). However, the high water solubility of alkanoates seems to limit their clathration with ß-CD, which limits their participation in the reaction. For the k_a plot m = 0.82 and correlation = 0.97 whereas for the k_b plot m = -0.18 and correlation = 0.65.

The conclusions we may draw from observing the effects of the two types of ions on the cleavage of pNPH by ß-CD are that the reaction is quite sensitive to the affinity of the potential inhibitors for ß-CD (Figures 35 and 36). The increasing values of k_a indicate this quite clearly. Values of k_b, which allow for the prior binding of the inhibitor to the CD (eq 14), vary little but there is a definite decreasing trend as the inhibitors become larger. In consequence, similar conclusions may be drawn for pNPH as were drawn earlier for pNPA: the substrate is enabled to interact more effectively with the catalytic hydroxyls on the lip of the ß-CD if it is prevented from entering the ring completely. If, however, the molecular spectator is too long, it begins to interfere with the approach of the substrate, which results in a decrease of cocatalysis.

For the alkanesulfonate ions, values of k_a increase regularly with the binding ability of the the ion (Figure 35). Correspondingly, values of k_b, which assume prior complexation of the ion, vary much less, but drift downward as the chain of the ion becomes longer, as discussed in the previous paragraph.

The results obtained with alkanoate ions appear atypical, when analyzed according to eq 14, in that the cocatalytic effect (k_b) peaked at the valerate (C5) ion (Table 11, Figure 36). However, caution must be used in interpreting these values because the K_s values for alkanoate ions may not be accurate as one would like. It is probably safe to say, as for alcohols and RSO_3^-, that smaller alkanoates are more effective in catalyzing the cleavage of pNPH by ß-CD.

Valerate ion seems to fulfil the geometric requirements of the transition state

most effectively. Anything larger (or smaller) causes a precipitous drop of k_h values. Given these considerations, it is quite probable that ß-CD catalysed cleavage of pNPH is occurring with inclusion of the *alkyl* chain of the substrate in the CD cavity, and not the aryl group. Perhaps, with pNPH as substrate, the carboxylate group of valerate with its relatively voluminous solvation sheli can act as an efficient plug for the ß-CD cavity. The alkyl moiety of the potential inhibitor might be helping to secure the fit of the sequestered alkyl chain of the ester while at the same time, the carboxylate group would prevent too deep an entry into the ß-CD cavity. This orienting and depth controlling effect could help pNPH achieve a transition state geometry that decreases the barrier height.

Another credible explanation for the unusually high k_h values observed with alkanoates, pNPH and ß-CD, is to consider that hydrogen bonding may exist between the carboxylate group of the potential inhibitor and an unionized hydroxyl on the cyclodextrin. If hydrogen bonding *is* functioning to aid the achievement of transition state geometry, it is reasonable to suppose that the effect would peak at the length of potential inhibitor which is proportional to the depth of the ß-CD cavity. Valerate ion has the proportions that would lend credence to this contention.

Of course, the above arguments would apply only if the alkyl chain of pNPH was inserting into ß-CD. There is ample evidence for cleavage of long-chained esters via inclusion of the alkyl group in CD.[16-20]

Effects of potential inhibitors on pNPH in α-CD

The next section should help to clarify what kind of binding is occurring in the α- and ß-CD catalysed cleavage of pNPH by comparing the experimental results obtained with ß-CD to those with the smaller α-CD molecule, in the presence of various potential inhibitors.

Space-filling (CPK) models of the molecules involved in the reaction of pNPH, potential inhibitors, and α-CD reveal that the tighter fit in α-CD prevents the simultaneous binding of two alkyl chains, side-by-side. The tighter fit of α-CD with linear alcohols and alkane sulfonate ions is well-documented, as a comparison of K_i values discloses.[17,48] Using Tables 10 and 11 the reader can compare K_i values of any given potential inhibitors with α- or ß-CD. Both cyclodextrins are 7.8 Å deep, and ß-CD is 7.0 Å across but α- is only 4.5 Å wide,[2] so that α-CD does not allow as much "play" to molecules complexed in its cavity.

In Table 11 are summarised the results of the experiments conducted with α-CD and pNPH, and various potential inhibitors. With the alcohols, a moderate rate enhancement was observed, as compared to the reaction with α-CD alone (see footnote 1 of Table 11). A slight upward trend can be detected in the range of k_a values, whereas the k_b values seem to remain constant within the uncertainty for these experiments. Thus, the same pattern of behaviour is detected as in previously discussed reactions with pNPA, albeit considerably diminished.

As pertains to the alkanesulfonate and alkanoate ions, the catalytic effect of all these potential inhibitors, except one, is essentially zero. The pentanesulfonate

Table 11. Constants for the Basic Cleavage of p-Nitropheny[l] Hexanoate by α-CD[l] in the Presence of Inhibitors (PI)

PI	K_i mM	pK_i	k_a $M^{-1}\,s^{-1}$	k_b $M^{-1}\,s^{-1}$
(a) alcohols				
EtOH	178	0.75	0.0728	3.68
PrOH	42.7	1.37	0.430	5.22
BuOH	11.2	1.95	1.45	4.62
PenOH	3.10	2.51	7.91	6.95
HexOH	1.12	2.95	3.39	1.08
(b) alkanesulfonates[m]				
C4	20.7	1.68	0.37	2.94
C5	7.78	2.11	9.49	21.0
C6	2.03	2.69	-	-
C7	0.72	3.14	6.12	2.33
C8	0.36	3.44	0	0
C10	0.05	4.30	0	0
(c) alkanoates[m]				
C3[n]	333	0.478	0	0
C4	64.3	1.19	0	1.51
C5[n]	16.0	1.80	0	0
C6	3.94	2.40	1.90	5.26

l. For the cleavage of pNPH in the presence of α-CD $k_c = 0.107\,s^{-1}$, $k_u = 0.0467\,s^{-1}$ and $K_s = 3.52$ mM as compared to β-CD with $k_c = 0.137\,s^{-1}$, $k_u = 0.0451\,s^{-1}$ and $K_s = 1.60$ mM.

m. Some of these experiments were repeated two or three times so that the K_i values reported are the average of more than one set of results.

n. Apparent negative results for both k_a and k_b indicate that no catalysis is occurring as result of the potential inhibitors.

was found to cause a comparatively substantial increase in the observed rate constant as well as the corrected rate constant. These data are viewed with diffidence, since no such effect was observed in the other experiments with pentanesulfonate. Repeat experiments should be made before a trustworthy conclusion can be made.

Overall, the experimental results seem to indicate that pNPH does not achieve a significant improvement of transition state binding by being dislodged from the α-CD cavity with potential inhibitors. If the constants describing the rate of pNPH cleavage in ß-CD are compared to those in α-CD, the values of k_a and k_b observed in α-CD pale by comparison. This effect is believed to be due to the proposed mechanism of alkyl insertion. Bonora *et al.* have observed that the catalytic effects of CDs, measured as the ratio of k_c/k_u, first decrease then increase with increasing chain length of the substrate. This is mainly due to changes in k_c since values of k_u are insensitive to chain length (beyond C4), as long as aggregation is not a problem [25,52,53] The increase in cleavage efficiency with chain length is attributed to the mode of insertion of the substrates into the CD. As the acyl chain length and hydrophobicity of the alkanoate esters increases the likelihood of its insertion into CD during a reaction in aqueous solution increases. According to two previous studies of the cleavage of pNPH[18,20] acyl chain insertion is dominant with chains the size of hexanoate and larger. In the case of substrate binding, this was determined by the absence of induced circular dichroism, which is evident with the pNPA, being absent for longer esters.[20] Also, K_s values decrease significantly with chain length, as do kinetic parameters which reflect the transition state binding.[16,18,20]

Evidently the system involving pNPH and α-CD involves more variables than have been taken into account. For example, it is known that due to their lack of solubility, longer chain nitrophenyl esters are subject to aggregation without any observable turbidity occurring in aqueous solution. This imposes a severe limit (0.1mM) on the amount of pNPH that can be dissolved into solution without engendering disreputable results.[25,o] Furthermore, the low concentrations enforced by low solubility of the esters lowers the signal to noise ratio of spectrophotometric measurements. This problem was overcome by Guthrie who established "critical concentrations" for the long-chain esters. Measurement of the very dilute solutions was facilitated by using a 10 cm cell instead of the customary 1 cm path-length. Unfortunately, we did not have this luxury, thus constraining us to use solutions of pNPH which were close to the critical concentration despite the presence of cyclodextrin. The most obvious effect of using too high a concentration of the ester is a tailing of kinetic data, as explained earlier in the Results section.

° Guthrie studied this problem. He observed the kinetics of a second order reaction involving pNPH, but in the absence of CD. The [CD] in our system exceeded substrate concentration 10:1; hopefully destroying this aggregation enough to consider the results valid.

Conclusions

The geometry of the transition state for ester cleavage of three alkanoate esters as it occurs in the cyclodextrin catalysed reaction was elucidated by observing the kinetic results of potential inhibitors on the system. The three substrates studied: mNPA, pNPA and pNPH, are known to undergo complexation with cyclodextrin and all three undergo an acceleration of cleavage in aqueous solution with CD. However, mNPA exhibits inhibition of reaction as a result of the competitive inhibition of complexation, whereas the substrates pNPA and pNPH behave anomalously under similar circumstances in that there is a lack of inhibition under conditions where it would be observed with mPNA. With some of the experiments, using pNPA and pNPH actual catalysis by potential inhibitors is observed.

The analysis of results indicates the transition state for reaction with both pNPA and pNPH must take place with aryl ring lifted out of the CD pocket. Conversely, mNPA is not substantially displaced from the geometry of the ground-state complex during the transition state for ester cleavage because of the proximity of its ester carbonyl to the catalytic hydroxyls on the periphery of the CD ring. Because of the similarity which the mNPA-CD complexes bear to the transition state for the CD-catalysed reaction, the introduction of any compounds with affinity for the CD ring are strictly inhibitory. Because of the foregoing observation, it was decided to make use of rate retardation measurements using mNPA-CD-PI experimental data to measure the binding constants of potential inhibitors whose binding constants could not be found in the literature.

Our experiments indicate the cyclodextrin accelerated cleavage of aryl alkanoate esters proceeds in a manner which is consistent with transition state theory, that is: catalysis of reaction is due to tighter binding of transition states to the catalyst than of ground state substrate.[15] Since, unlike enzymes, cyclodextrins do not alter their shape to tighten around the transition state, our experiments had the aim of introducing molecules which would loosen ground-state binding in an attempt to make the energy jump from ground state complexation to transition state smaller. The presence of these molecular spectators, potential inhibitors, or cocatalysts, as they were referred to, had the two-fold effect of hindering ground state complexation of substrate with CD while optimising positioning of substrate during the transition state. In some cases the above phenomenon had the effect of increasing the rate of reaction in apparent contradiction of competitive inhibition conditions. The increase in the rates of reaction was found to be proportional to the affinity of the cocatalyst (PI) for the CD host, although the rate increases were mitigated by the excessive length or steric bulk of certain inhibitors.

The chemical properties and nature (other than binding affinity) of the inhibitors were demonstrated to be of relatively little consequence to the rate stimulating effect when alcohols, and sulfonates were analyzed as inhibitors with respect to α- and ß-CD with pNPA as the substrate. It is believed that, because of their bulky hydration sphere, alkanoate ions had a more easily observable effect on the reaction, and tended to diminish the cocatalytic effect if oversized. Also, analysis of results seems to support the inference obtained from CPK models, that due to the

stiffer geometric requirements of α-CD, more decomplexation of the substrates, particularly pNPA, is required for the reaction to proceed.

The analysis of experiments with pNPH according to the scheme utilized for pNPA generally did not yield a satisfactory result because of the saturation kinetics they displayed. However, this complication was overcome by considering an additional equilibrium prior to the rate-limiting step. Analysis of the kinetic results with respect to the formation of a ternary complex involving host, substrate, and inhibitor yielded an acceptable fit to data with pNPH. The equilibrium constants (K_t) obtained in this manner and the "quasi-equilibrium" constants calculated by using the transition state theory (K_{TS}) correlate strongly with equilibrium constants for the binding of potential inhibitors (K_i). Since the apparent dissociation constants of the transition state K_{TS} were found to vary in the same manner as those of the ternary complexes K_t, the conclusion was drawn that the structure of the transition state is similar in structure to the ternary complexes.

The present research conducted on the cleavage of the substrates as it is affected by CDs and other additives may provide a useful paradigm for simple allosteric effects. With the results reported herein we have demonstrated how binding and reactivity of a substrate with a catalyst can be improved by the concomitant binding of a third, inert species, leading to what has been termed spectator catalysis.

While some medicinal chemists and molecular pharmacologists are sceptical about the practical value of such studies, the advent and accessibilty of computers

with sufficient power to help conduct preliminary modelling studies is changing attitudes towards the application of structure-activity correlations to drug design. By combining the use of spectator catalysis phenomena with computer modelling, to focus on the areas of interest, it may eventually become feasible to design drugs in a more calculated manner. For example, it would be much faster and cheaper to calculate the required properties of novel compounds from a large pool of data on their analogues than to synthesize and screen all such new compounds in the classical fashion.[57] Likewise with catalysts, such as enzymes, which have binding regions that are much more complex than CD, the use of spectator catalysis, combined with computer modelling studies, may one day be used to remedy the function of mutant enzymes.

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Appendix A

Derivation of the Rate Equation for Substrate Cleavage in CD

The overall rate $v = k_{obs}[S]_o = k_u[S] + k_c[S\text{-}CD]$ (1)

$$f_c = \frac{[S\text{-}CD]}{[S]_o} \quad \text{and;} \quad 1 - f_c = \frac{[S]_f}{[S]_o} \tag{2}$$

where f_c is the fraction of substrate complexed. From 1:

$$k_{obs} = k_u\frac{[S]_f}{[S]_o} + k_c\frac{[S\text{-}CD]}{[S]_o} \tag{3}$$

Therefore, from the definitions in 2:

$$k_{obs} = k_u(1 - f_c) + k_c f_c \tag{4}$$

Since, $$K_s = \frac{[CD][S]}{[S\text{-}CD]} \quad \text{where} \quad [S]_o = [S] + [S\text{-}CD] \tag{5}$$

and, [CD]>[S], then it is reasonable to assume that the small amount of substrate will not appreciably affect the cyclodextrin concentration.

From 5:

$$K_s = \frac{[CD]([S]_o - [S\text{-}CD])}{[S\text{-}CD]} \tag{6}$$

$$K_s[S\text{-}CD] = [CD][S]_o - [CD][S\text{-}CD] \tag{7}$$

$$[S\text{-}CD](K_s + [CD]) = [CD][S]_o \tag{8}$$

therefore:

$$\frac{[CD]}{(K_s + [CD])} = \frac{[S\text{-}CD]}{[S]_o} = f_c \text{ (from 2)} \tag{9}$$

Substitution of eq 9 into eq 4 yields:

$$k_{obs} = k_u\{1 - ([CD]/K_s + [CD])\} + k_c([CD]/K_s + [CD]) \quad (10)$$

multiplying top and bottom by $K_s + [CD]/K_s + [CD]$ yields:

$$k_{obs} = \frac{k_u(K_s + [CD]) - k_u[CD] + k_c[CD]}{K_s + [CD]} \quad (11)$$

$$k_{obs} = \frac{k_u K_s + k_c[CD]}{K_s + [CD]} \quad (12)$$

This equation represents the most significant rate processes contributing to cleavage of phenyl esters in the presence of basic cyclodextrin solution. The other equations described in this study are modifications to equation 12. The program INHIB is set up for analysis where competitive inhibition is taking place by allowing for the reduction in free [CD]. The program PIANALYSIS provides for an additional rate process (k_u[S-CD][PI]), so that there is an extra term in the numerator of equation 12 (see eq 12 in the Results section).

Appendix B

Transition State Binding[15,16,31,34]

The equations developed in this Appendix are based upon the approach originated by Kurz.[31]

For reaction of a substrate in the medium:

$$S \xrightarrow{k_u} P \quad ; \qquad k_u = Q[TS]/[S], \text{ where } Q = \kappa k_b T/h \tag{1}$$

and for its reaction mediated by CD:

$$CD + S \xrightarrow{k_2} P \quad ; \qquad k_2 = Q[CD \cdot TS]/[CD][S] \tag{2}$$

$$\text{or} \quad CD + S \underset{K_s}{\rightleftharpoons} CD \cdot S \xrightarrow{k_c} P \quad ; \qquad k_c = Q[CD \cdot TS]/[CD \cdot S] \tag{3}$$

$$\text{Definition:} \quad K_{TS} = \frac{[CD][TS]}{[CD \cdot TS]} = \frac{k_u}{k_2} = \frac{k_u K_s}{k_c} \tag{4}$$

Using this formulation one can discuss the binding of the transition state TS to CD, whatever the actual reaction mechanism. For a reaction mediated by the presence of a potential inhibitor (PI) there are various possible schemes:

$$CD \cdot S + PI \xrightarrow{k_a} P \quad ; \qquad k_a = Q[CD \cdot S \cdot PI]/[PI][CD \cdot S] \tag{5}$$

$$CD \cdot PI + S \xrightarrow{k_b} P \quad ; \qquad k_b = Q[CD \cdot S \cdot PI]/[S][CD \cdot PI] \tag{6}$$

$$CD \cdot PI \cdot S \xrightarrow{k_t} P \quad ; \qquad k_t = Q[CD \cdot PI \cdot TS]/[CD \cdot PI \cdot S] \tag{7}$$

These can be interrelated using the equilibrium constants:

$$CD + S \rightleftharpoons CD \cdot S \quad ; \qquad K_s = [CD][S]/[CD \cdot S] \tag{8}$$

$$CD + PI \rightleftharpoons CD\cdot PI \quad ; \quad K_i = [CD][PI]/[CD\cdot PI] \tag{9}$$

$$CD\cdot S + PI \rightleftharpoons CD\cdot PI\cdot S \quad ; \quad K_i = [CD\cdot S][PI]/[CD\cdot PI\cdot S] \tag{10}$$

$$CD\cdot PI + S \rightleftharpoons CD\cdot PI\cdot S \; ; \quad K_s' = [CD\cdot PI][S]/[CD\cdot PI\cdot S] \tag{11}$$

Note that $$K_s' = K_t K_s / K_i \tag{12}$$

For the termolecular process:

$$k_a = k_t/K_t \text{ and } k_b = k_t/K_s' = k_a K_i/K_s \tag{13}$$

By using the equations defined above, the constant which describes the stabilization of the transition state by PI is defined as follows:

$$K_{TS}' = \frac{[CD\cdot TS][PI]}{[CD\cdot PI\cdot TS]} = \frac{k_c}{k_a} \tag{14}$$

With the values of K_{TS}' and K_i it is possible to compare the binding of PI in CD·PI·TS to its binding in CD·PI. Values of K_s' versus K_s compare substrate binding to cyclodextrin in the presence and absence of potential inhibitor respectively. Where a series of inhibitors or substrates are tested under identical conditions, it is possible to verify if any linear free energy correlations exist between the logarithms of these constants.[16,34]

On the next page is a relative free energy diagram representing the possible pathways leading to cleavage of the substrates used in this study. All the important rate and equilibrium processes named in this project are represented in the diagram. The energy levels are based very roughly on the results obtained in the experiments carried out during this study.

Schematic Re:tive Free Energy Diagram

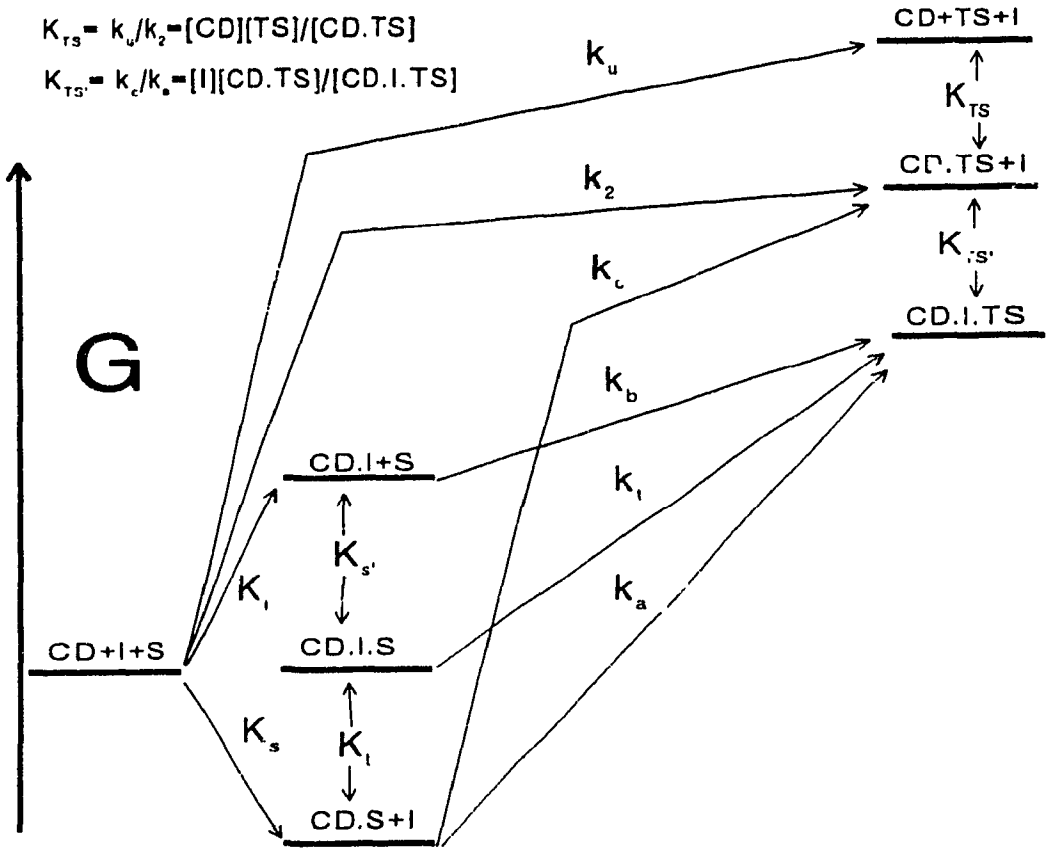

Figure 37. The reaction schemes discussed in this study are summarized above. The relative free energy of the reactive species however, do not necessarily occur as portrayed on the diagram.

Appendix C

The experiments conducted in this thesis were scaled to the rate constants summarised on the next two pages. The experimental results for ß-CD/pNPA/PI were scaled to Hoeven's earlier data, whereas those for ß-CD/pNPH/PI were initially scaled to the results of Du, and later to our own.

The scaling of the experiments was accomplished by setting the results obtained with potential inhibitors to the same value as was obtained in the set of scaling experiments recorded on the next two pages. For example, the zero [PI] point of experiment #19 is set equal to the 10 mM value of (k_{obs} = 0.403 s-1) in the scaling experiment conducted for pNPA and ß-CD (next page). The constant that is used to multiply k_{obs} to obtain $k_{obs'}$ for the first point is then used to multiply the other values of k_{obs} in the experiment to yield the other values of $k_{obs'}$. The point of this scaling is to correct for small fluctuations in pH, between different experiments.

The tables on subsequent pages summarise the best experimental results obtained during the course of this study. Often it was necessary to repeat experiments before obtaining the best concentration range of PI. This was particularly the case with the alkanoates which required careful neutralization before being used in the experiments to avoid pH fluctuations. Also, because certain reactions exhibited saturation, it was best to rerun certain experiments to obtain the region of largest change for best results.

Scaling Experiments

Constants For the Basic Cleavage of *m*-Nitrophenyl and *p*-Nitrophenyl Acetate in the Presence of Cyclodextrins[P]

	k_{obs}, s^{-1}	
[α-CD], mM	*m*-nitro*	*p*-nitro*
0	0.0858	0.0956
1.0	1.04	0.111
2.0	1.77	0.124
4.0	3.55	0.144
6.0	4.87	0.160
8.0	6.14	0.171
10.0	7.08	0.181
	k_c = 24.6 s^{-1} K_d = 25.0 mM	k_c = 0.267 s^{-1} K_d = 10.1 mM

[ß-CD], mM	*m*-nitro*	*p*-nitro‡
0	0.0858	0.0773
1.0	0.486	-
2.0	0.801	0.195
4.0	1.29	0.273
6.0	1.75	0.329
8.0	2.21	0.371
10.0	2.44	0.403
	k_c = 5.25 s^{-1} K_d = 12.3 mM	k_c = 0.0773 s^{-1} K_d = 8.00 mM

[P] By stopped flow technique @ 25 °C, in a 0.2M phosphate buffer of pH 11.6-11.7 containing 0.1% (v/v) methanol in both the α and ß experiments. The data marked with * was acquired by Du[1]; those marked with ‡ was collected by Hoeven.

Constants for the Basic Cleavage of *p*-Nitrophenyl Hexanoate in Cyclodextrins

a) Obtained by Du:

[CD], mM	k_{obs}, s^{-1} α-CD	k_{obs}, s^{-1} ß-CD
0	0.0494	0.0494
1.0	0.0740	0.104
2.0	0.0897	0.128
4.0	0.104	0.147
6.0	0.113	0.153
8.0	0.121	0.160
10.0	0.125	0.164
	$k_c = 0.146\ s^{-1}$	$k_c = 0.179\ s^{-1}$
	$K_s = 2.88$ mM	$K_s = 1.34$ mM

b) Obtained by Bozzi:

[CD], mM	k_{obs}, s^{-1} α-CD	[CD], mM	k_{obs}, s^{-1} ß-CD
0	0.0467	0	0.0451
4.0	0.0785	1.5	0.0897
8.0	0.0884	3.0	0.105
12.0	0.0945	6.0	0.117
16.0	0.0957	9.0	0.123
20.0	0.0975	12.0	0.126
		15.0	0.128
	$k_c = 0.107\ s^{-1}$		$k_c = 0.137\ s^{-1}$
	$K_s = 3.52$ mM		$K_s = 1.60$ mM

Experimental Data: ß-CD with pNPA and Various Inhibitors [q]

	[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
(1) Alkanols		**ß-CD / pNPA / n-propanol / exp #19**			
	0	0	0.334	0.403	0.660
	80	77.7	0.412	0.497	0.925
	160	156	0.481	0.580	1.209
	240	235	0.506	0.610	1.401
	320	314	0.529	0.638	1.601
	400	394	0.532	0.641	1.742
		ß-CD / pNPA / 2-propanol / exp #13			
	0	0	0.318	0.403	0.660
	60	58.2	0.385	0.488	0.884
	120	117	0.436	0.552	1.095
	180	176	0.448	0.567	1.215
	240	235	0.464	0.588	1.353
	300	295	0.491	0.622	1.536
		ß-CD / pNPA / n-butanol / exp #6			
	0	0	0.316	0.403	0.660
	30	26.9	0.371	0.473	0.926
	60	55.2	0.374	0.477	1.084
	120	113	0.380	0.484	1.416
	180	173	0.392	0.500	1.796
		ß-CD / pNPA / 2-butanol / exp #14			
	0	0	0.321	0.403	0.660
	40	36.4	0.362	0.454	0.920
	80	74.7	0.384	0.482	1.170
	120	113	0.395	0.496	1.406
	160	153	0.404	0.507	1.647
	200	193	0.398	0.499	1.823

[q] The concentrations of inhibitor in these tables are the nominal concentration [I] and $[I]_{corr}$, after correction for binding to the CD. Likewise, $k_{obs'}$ corresponds to the rate constants k_{obs} after scaling.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPA / iso-butanol / exp #15			
0	0	0.352	0.403	0.660
40	34.1	0.382	0.437	1.127
80	72.5	0.378	0.432	1.563
120	111	0.364	0.416	1.936
160	151	0.359	0.411	2.340
200	191	0.346	0.396	2.657
	β-CD / pNPA / t-butanol / exp #8			
0	0	0.308	0.403	0.660
20	15.7	0.319	0.417	0.888
40	33.8	0.317	0.415	1.112
60	52.8	0.315	0.412	1.343
80	72.3	0.326	0.426	1.653
100	91.9	0.314	0.411	1.829
	β-CD / pNPA / n-pentanol / exp #3			
0	0	0.362	0.403	0.660
20	15.1	0.357	0.397	0.890
40	33.2	0.365	0.406	1.207
60	52.3	0.356	0.396	1.474
80	71.8	0.347	0.386	1.728
100	91.5	0.344	0.383	2.007
	β-CD / pNPA / 2-pentanol / exp #27			
0	0	0.366	0.403	0.660
30	25.6	0.375	0.413	0.889
60	53.8	0.374	0.412	1.115
90	82.8	0.368	0.405	1.327
120	112	0.363	0.399	1.538
150	142	0.360	0.396	1.754

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPA / iso-pentanol / exp #4			
0	0	0.315	0.403	0.660
5	2.19	0.331	0.423	0.804
10	5.19	0.325	0.416	0.932
20	13.0	0.321	0.410	1.287
40	31.5	0.327	0.418	2.206
50	41.2	0.326	0.417	2.663
	β-CD / pNPA / neo-pentanol / exp #20			
0	0	0.346	0.403	0.660
15	6.99	0.273	0.318	1.273
30	20.8	0.248	0.289	2.454
45	35.5	0.241	0.281	3.722
60	50.3	0.234	0.272	4.895
75	65.3	0.229	0.267	6.037
	β-CD / pNPA / c-pentanol / exp #16			
0	0	0.344	0.403	0.660
30	22.7	0.367	0.430	1.471
60	51.4	0.349	0.409	2.295
90	80.9	0.330	0.386	3.017
120	111	0.320	0.375	3.750
150	141	0.306	0.358	4.348
	β-CD / pNPA / n-hexanol / exp #31			
0	0	0.436	0.403	0.660
4.0	1.52	0.421	0.389	0.717
8.0	3.61	0.401	0.370	0.784
12.0	6.24	0.391	0.361	0.892
16.0	9.31	0.382	0.353	1.013
20.0	12.7	0.366	0.338	1.113

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPA / 2-hexanol / exp #18			
0	0	0.342	0.403	0.660
7.5	4.50	0.337	0.397	0.758
15.0	10.1	0.332	0.391	0.878
22.5	16.4	0.327	0.385	1.009
30.0	23.1	0.327	0.385	1.165
37.5	30.1	0.325	0.383	1.317
	β-CD / pNPA / c-hexanol / exp #17			
0	0	0.323	0.403	0.660
7.5	2.23	0.320	0.399	0.938
15	7.18	0.280	0.349	1.337
21	12.4	0.269	0.335	1.806
30	20.9	0.232	0.289	2.209
60	50.4	0.225	0.281	4.496
90	80.2	0.229	0.286	7.066
120	110	0.230	0.287	9.591
150	140	0.232	0.289	12.22
	β-CD / pNPA / n-heptanol / exp #56			
0	0	0.344	0.403	0.660
0.8	0.11	0.338	0.396	0.667
1.6	0.22	0.331	0.388	0.672
4.0	0.70	0.310	0.363	0.701
	β-CD / pNPA / 1,6-hexanediol [r] / exp #79			
0	0	0.308	0.403	0.660
10	7.35	0.286	0.374	0.694
20	15.7	0.250	0.327	0.676
30	24.5	0.231	0.302	0.694
40	33.7	0.217	0.284	0.718
50	43.2	0.212	0.277	0.771

[r] In this experiment, the inhibitor appears to be inhibiting.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
(2) Alkanoates				
		β-CD / pNPA / butyrate / exp #38		
0	0	0.322	0.403	0.660
20	19.3	0.349	0.437	0.742
40	38.7	0.353	0.442	0.773
60	58.2	0.362	0.453	0.817
80	77.7	0.370	0.463	0.859
100	97.3	0.372	0.465	0.887
		β-CD / pNPA / pentanoate / exp #25		
0	0	0.356	0.403	0.660
20	18.0	0.392	0.443	0.804
40	36.7	0.403	0.456	0.904
60	55.7	0.407	0.465	1.003
80	75.0	0.412	0.466	1.086
100	94.4	0.409	0.466	1.167
		β-CD / pNPA / 4-methylpentanoate / exp #54		
0	0	0.333	0.403	0.660
2.5	1.55	0.336	0.406	0.694
5.0	3.21	0.344	0.416	0.743
7.5	4.97	0.348	0.421	0.785
10.0	6.83	0.351	0.425	0.827
12.5	8.76	0.356	0.431	0.877
		β-CD / pNPA / hexanoate / exp #26		
0	0	0.355	0.403	0.660
2.0	1.27	0.359	0.407	0.689
4.0	2.60	0.359	0.407	0.711
6.0	4.00	0.371	0.419	0.756
8.0	5.46	0.366	0.415	0.774
10.0	6.97	0.370	0.420	0.809

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPA / heptanoate / exp #33			
0	0	0.289	0.403	0.660
1.8	0.72	0.279	0.403	0.692
3.6	1.54	0.281	0.406	0.733
5.4	2.46	0.285	0.410	0.783
7.2	3.49	0.285	0.410	0.829
9.0	4.61	0.280	0.406	0.869
	β-CD / pNPA / octanoate / exp #58			
0	0	0.320	0.403	0.660
0.8	0.16	0.315	0.396	0.666
1.6	0.33	0.314	0.395	0.683
2.4	0.53	0.311	0.391	0.697
3.2	0.75	0.312	0.393	0.723
4.0	0.99	0.307	0.386	0.736
	β-CD / pNPA / cyclohexanecarboxylate / exp #39			
0	0	0.362	0.403	0.660
2.5	0.89	0.356	0.396	0.697
5.0	1.99	0.341	0.379	0.722
7.5	3.31	0.337	0.375	0.780
10.0	4.86	0.325	0.362	0.825
12.5	6.61	0.310	0.345	0.861
	β-CD / pNPA / suberate[a] / exp #68			
0	0	0.373	0.403	0.660
7.0	5.53	0.356	0.384	0.670
14.0	11.4	0.338	0.365	0.674
21.0	17.5	0.323	0.349	0.681
28.0	23.7	0.306	0.330	0.680
35.0	30.1	0.287	0.310	0.668

[a] Suberate behaved as an inhibitor proper. Experiment #71 was a repeat of expt. #68, albeit at a larger concentration of inhibitor, to ensure that results were not an artefact. Suberate inhibited the reaction in this experiment also.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPA / suberate / exp #71[t]			
0	0	0.337	0.403	0.660
20.0	16.6	0.289	0.345	0.668
40.0	34.8	0.250	0.299	0.665
60.0	53.7	0.217	0.259	0.646
80.0	73.0	0.191	0.228	0.621
100.0	92.6	0.169	0.202	0.586
3) Sulfonates				
	β-CD / pNPA / butanesulfonate / exp #40			
0	0	0.342	0.403	0.660
30	27.6	0.346	0.407	0.750
60	56.1	0.341	0.402	0.820
90	85.1	0.353	0.416	0.940
120	114	0.349	0.411	1.015
150	144	0.345	0.406	1.088
	β-CD / pNPA / pentanesulfonate / exp #48			
0	0	0.342	0.403	0.660
25	19.6	0.336	0.396	0.944
50	42.8	0.325	0.383	1.244
75	67.0	0.311	0.366	1.513
125	116	0.290	0.34[illegible]	2.007
	β-CD / pNPA / hexanesulfonate / exp #30			
0	0	0.375	0.403	0.660
10	5.19	0.346	0.372	0.821
20	13.0	0.334	0.359	1.100
30	22.0	0.325	0.349	1.411
40	31.5	0.320	0.344	1.741
50	41.2	0.309	0.332	2.016

[t] This experiment confirms inhibition. However, note that k_{corr} decreases at high [I], perhaps because of the onset of 2:1 binding.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPA / heptanesulfonate / exp #28			
0	0	0.362	0.403	0.660
10	3.78	0.289	0.322	0.833
20	11.6	0.262	0.292	1.320
30	21.0	0.247	0.275	1.858
40	30.7	0.246	0.274	2.505
50	40.5	0.238	0.265	3.030
	β-CD / pNPA / octanesulfonate / exp #29			
0	0	0.386	0.403	0.660
10	2.67	0.252	0.263	0.814
20	10.8	0.216	0.225	1.651
30	20.5	0.205	0.214	2.603
40	30.3	0.194	0.202	3.398
50	40.2	0.189	0.197	4.231
	β-CD / pNPA / decanesulfonate / exp #32			
0	0	0.336	0.403	0.660
3.0	0.10	0.301	0.361	0.677
6.0	0.32	0.244	0.292	0.687
9.0	0.98	0.180	0.216	0.771
12.0	2.79	0.156	0.187	1.284
15.0	5.42	0.148	0.177	2.049

ß-CD with pNPH and Various Inhibitors [u]

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	k^{cal} (s^{-1})	%diff [v]
(1) Alkanols				
	ß-CD / pNPH / n-propanol / exp #44			
0	0	0.149	0.149	0.00
80	77.8	0.226	0.229	-1.47
160	156.3	0.287	0.286	0.28
240	235.3	0.331	0.329	0.75
320	314.6	0.362	0.361	0.24
400	394.0	0.385	0.387	-0.52
	ß-CD / pNPH / isopropanol / exp #85*			
0	0	0.112	0.112	0.00
60	57.3	0.162	0.162	0.21
120	115.4	0.200	0.201	-0.53
180	174.0	0.234	0.233	0.46
240	233.0	0.259	0.259	-0.08
300	292.1	0.281	0.281	-0.04
	ß-CD / pNPH / n-butanol / exp #86*			
0	0	0.123	0.123	0.00
60	53.0	0.230	0.236	-2.51
120	110.3	0.293	0.289	1.35
180	168.9	0.324	0.319	1.64
240	228.1	0.338	0.337	0.18
300	287.6	0.345	0.350	-1.50

[u] The data from these experiments gave curved plots with the progam PIANALYSIS. Therefore, they were analyzed for saturation behaviour using CDFIT to fit to the model in equation 23, in the Results section, so that $K_t = K_d$ and $k_t = k_c$. The fitted values of k_t were then scaled to the appropriate run for pNPH on page 114. The experiments with an asterisk had [ß-CD] = 15mM.

[v] This column relates the amount of scatter affecting the points, by representing the difference between k^{obs} and k^{cal} values. where k^{cal} values correspond to the position of the points which fall on the best line through the points.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	k^{cal} (s^{-1})	%diff
	β-CD / pNPH / 2-butanol / exp #87*			
0	0	0.122	0.122	0.00
40	34.8	0.183	0.185	-1.16
80	72.1	0.229	0.227	0.72
120	110.6	0.256	0.255	0.23
160	149.5	0.276	0.276	0.03
200	188.8	0.291	0.291	-0.27
	β-CD / pNPH / iso-butanol / exp #59			
0	0	0.107	0.107	0.00
40	34.1	0.194	0.197	-1.37
80	72.5	0.236	0.235	0.54
120	111.7	0.258	0.254	1.45
160	151.4	0.267	0.266	0.37
200	191.1	0.270	0.274	-1.41
	β-CD / pNPH / t-butanol / exp #98*			
0	0	0.110	0.110	0.00
20	14.0	0.155	0.154	0.55
40	31.1	0.185	0.186	-0.50
60	49.5	0.206	0.207	-0.84
80	68.5	0.226	0.222	1.70
100	87.9	0.231	0.233	-0.81
	β-CD / pNPH / n-pentanol / exp #88*			
0	0	0.116	0.116	0.00
20	13.2	0.173	0.176	-1.61
40	30.2	0.217	0.219	-1.09
60	48.7	0.252	0.247	2.13
80	67.9	0.270	0.265	1.78
100	87.3	0.272	0.278	-2.28

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	k^{cal} (s^{-1})	%diff
	β-CD / pNPH / 2-pentanol / exp #43			
0	0	0.132	0.132	0.00
20	16.6	0.165	0.164	0.66
40	34.8	0.186	0.188	-1.27
60	53.7	0.207	0.207	0.11
80	73.1	0.224	0.221	1.39
100	92.6	0.230	0.232	-0.84
	β-CD / pNPH / neo-pentanol / exp #61			
0	0	0.120	0.120	0.00
15	6.99	0.171	0.172	-0.41
30	20.8	0.188	0.186	1.15
45	35.5	0.189	0.190	-0.45
60	50.3	0.191	0.192	-0.35
	β-CD / pNPH / c-pentanol / exp #89*			
0	0	0.126	0.126	0.00
20	11.4	0.223	0.225	-0.68
40	28.4	0.279	0.278	0.22
60	47.3	0.305	0.304	0.25
80	66.7	0.323	0.319	1.36
100	86.3	0.323	0.328	-1.42
	β-CD / pNPH / n-hexanol / exp #99*			
0	0	0.124	0.124	0.00
1.5	0.37	0.129	0.128	0.44
3.0	0.79	0.133	0.132	0.61
4.5	1.26	0.134	0.135	-1.04
6.0	1.79	0.138	0.138	0.10
7.5	2.37	0.141	0.141	0.37

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	k^{cal} (s^{-1})	%diff
	β-CD / pNPH / c-hexanol / exp #91*			
0	0	0.123	0.123	-0.00
12	3.00	0.170	0.171	-0.71
24	11.3	0.219	0.216	1.18
36	22.2	0.233	0.235	-0.97
48	33.8	0.244	0.244	0.07
60	45.6	0.249	0.249	0.16
	β-CD / pNPH / 1-6, hexanediol[w] / exp #80			
0	0	0.233[x]		
10	7.35	0.210		
20	15.7	0.209		
30	24.5	0.202		
40	33.8	0.195		
50	43.2	0.195		

[w] Inhibition was observed with this diol when it was analysed using PIANALYSIS. Because of the inhibition that was demonstrated by this diol, CDFIT analysis was not carried out on the data. Some evidence for the belief that a hydroxy group prefers to ride high in the pocket is provided by this experiment, as well as by experiments 68,69 and 70. The different nature of the interaction is not as efficient at providing catalysis.

[x] These values clearly indicate inhibition, not catalysis.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
(2) Alkanoates[y]				
	ß-CD / pNPH / butyrate / exp #73			
0	0	0.122	0.163	0.178
20	19.3	0.126	0.168	0.185
40	38.7	0.128	0.171	0.190
60	58.2	0.133	0.178	0.199
80	77.7	0.135	0.180	0.203
100	97.3	0.138	0.184	0.209
	ß-CD / pNPH / pentanoate / exp #74			
0	0	0.116	0.163	0.178
20	18.0	0.138	0.194	0.218
40	36.7	0.154	0.216	0.250
	ß-CD / pNPH / hexanoate / exp #75			
0	0	0.117	0.163	0.178
2	1.27	0.126	0.176	0.195
4	2.60	0.125	0.175	0.194
6	4.00	0.130	0.182	0.204
8	5.46	0.138	0.193	0.219
10	6.97	0.145	0.203	0.232
	ß-CD / pNPH / heptanoate / exp #53			
0	0	0.116	0.163	0.178
2	0.80	0.118	0.166	0.183
4	1.73	0.122	0.171	0.192
8	3.96	0.127	0.179	0.207
10	5.27	0.129	0.181	0.215

[y] These experiments were analysed with PIANALYSIS and scaled to Du's experiment for pNPH and ß-CD, on p 114.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	β-CD / pNPH / octanoate / exp #52			
0	0	0.118	0.163	0.178
2	0.43	0.122	0.169	0.187
4	0.99	0.130	0.180	0.204
6	1.73	0.137	0.189	0.222
8	2.65	0.142	0.196	0.238
10	3.79	0.147	0.203	0.257
	β-CD / pNPH / suberate[z] / exp #69			
0	0	0.116	0.163	0.178
5	3.91	0.118	0.166	0.183
10	8.00	0.113	0.159	0.177
15	12.2	0.113	0.159	0.179
20	16.6	0.110	0.155	0.176
25	21.0	0.113	0.159	0.183
	β-CD / pNPH / suberate / exp #70			
0	0	0.122	0.163	0.178
20	16.6	0.113	0.151	0.172
40	34.8	0.105	0.140	0.166
60	53.7	0.0955	0.128	0.156
80	73.0	0.0904	0.121	0.152
100	92.6	0.0798	0.107	0.136

[z] Suberate demonstrated straight inhibition under these conditions. Experiment #69 did not yield definitive results, so the concentration range of inhibitor was increased and the experiment was rerun (#70). Calculation by PIANALYSIS again confirmed inhibition.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
(3a) Sulfonates[aa]				
	β-CD / pNPH / pentanesulfonate / exp #46			
0	0	0.113	0.163	0.178
25	19.6	0.152	0.219	0.268
50	42.8	0.170	0.245	0.338
75	67.0	0.181	0.261	0.402
100	91.5	0.192	0.277	0.473
125	116.3	0.192	0.277	0.518
	β-CD / pNPH / hexanesulfonate / exp #111			
0	0	0.124	0.163	0.178
6	2.74	0.131	0.172	0.196
12	6.61	0.143	0.188	0.228
18	11.3	0.154	0.203	0.264
24	16.6	0.162	0.213	0.298
30	22.0	0.173	0.228	0.344
	β-CD / pNPH / heptanesulfonate / exp #50			
0	0	0.124	0.163	0.178
10	3.78	0.152	0.200	0.253
20	11.6	0.175	0.230	0.376
30	21.0	0.188	0.247	0.514
40	30.7	0.189	0.248	0.630
50	40.5	0.186	0.245	0.730
	β-CD / pNPH / octanesulfonate / exp #108			
0	0	0.099	0.124	0.137
3	0.35	0.111	0.139	0.160
9	2.13	0.126	0.158	0.215
12	3.97	0.138	0.172	0.275
15	6.33	0.151	0.189	0.362

[aa] See footnote cc.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	ß-CD / pNPH / octanesulfonate / exp #117 [bb]			
0	0	0.114	0.124	0.137
10	2.67	0.151	0.164	0.236
20	10.8	0.156	0.170	0.412
30	20.5	0.157	0.171	0.615
40	30.3	0.147	0.160	0.752
50	40.2	0.134	0.146	0.829
	ß-CD / pNPH / decanesulfonate / exp #49			
0	0	0.103	0.163	0.178
3	0.10	0.112	0.177	0.201
6	0.32	0.117	0.185	0.227
9	0.99	0.129	0.204	0.308
12	2.80	0.146	0.231	0.533
15	5.43	0.145	0.230	0.786

[bb] When this data is analyzed according to PIANALYSIS it exhibits marked saturation. If CDFIT is used, the program crashes. Due to the high concentration of inhibitor used in the experiment and the high affinity that octanesulfonate anion possesses for ß-CD, there is probably another process which is unaccounted for such as 2:1 binding or micellization, that is affecting the observed rate constant in this reaction.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	k^{cal} (s^{-1})	%diff
(3b) Sulfonates[cc]				
	β-CD / pNPH / pentanesulfonate / exp#46			
0	0	0.113	0.113	0.00
25	19.6	0.152	0.152	0.06
50	42.8	0.170	0.171	-0.37
75	67.0	0.181	0.182	-0.35
100	91.5	0.192	0.189	1.63
125	116.3	0.192	0.194	-1.04
	β-CD / pNPH / hexanesulfonate / exp #111			
0	0	0.124	0.124	0.00
6	2.74	0.131	0.132	-0.89
12	6.61	0.143	0.142	0.41
18	11.3	0.154	0.153	0.69
24	16.6	0.162	0.164	-0.93
30	22.0	0.173	0.173	0.32
	β-CD / pNPH / heptanesulfonate / exp #50			
0	0	0.124	0.124	0.00
10	3.78	0.152	0.154	-1.34
20	11.6	0.175	0.175	-0.14
30	21.0	0.188	0.184	2.39
40	30.7	0.189	0.188	0.61
50	40.5	0.186	0.190	-2.36

[cc] Because of the ambiguity of results obtained with sulfonates and pNPH, this set of data was analysed using both PIANALYSIS (section 3a) and CDFIT (section 3b). Depending on the concentration range of inhibitor used, one or the other method affords a better fit.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	k^{cal} (s^{-1})	%diff
	ß-CD / pNPH / octanesulfonate / exp #108 [dd]			
0	0	0.099	0.099	0.00
3	0.35	0.111	0.106	5.08
9	2.13	0.126	0.127	-0.94
12	3.97	0.138	0.140	-1.61
15	6.33	0.151	0.149	-1.14
	ß-CD / pNPH / decanesulfonate / exp #49 [ee]			
0	0	0.103	0.103	0.00
3	0.10	0.112	0.109	2.85
6	0.32	0.117	0.118	-0.68
9	0.99	0.129	0.131	-1.78
12	2.80	0.146	0.142	2.49
15	5.43	0.145	0.147	-1.33

[dd] In the experiments with this inhibitor, the problem of micellization was demonstrated. Experiment #77, (not listed) was carried out at a higher octanesulfonate ion concentration. Judging from the results, either the CMC for this inhibitor is 40mM in the presence of 10 mM ß-CD, or a 2:1 binding process involving 2 CD molecules with one octansulfonate molecule is occuring. From a k_{obs} of 0.120 s^{-1} at [I] = 40mM in the mixture, the rate constants dropped to 0.0362 s^{-1} when [I] was raised to 50mM. To avoid this effect, experiment #108 was rerun at a lower concentration of octansulfonate ion (0-15mM). Another experiment #117 (not listed) was conducted which duplicates the conditions of exp #77. Marked saturation was observed in exp #117, but the sudden drop in reaction rate did not occur again as in exp #77.

[ee] Note that although decanesulfonate is longer than octanesulfonate anion the 2:1 binding or micellization phenomenon was not observed in this experiment due to the low concentration of inhibitor used. Since decanesulfonate ion has a high affinity for ß-CD, there was very little free decanesulfonate left over after complexation with the 10mM ß-CD in solution. Presumably not enough to form micelles.

The Determination of K_i Values using mNPA as Substrate

Experiments with ß-CD

	[I] (mM)	k^{obs} (s^{-1})	k^{scaled} (s^{-1})	K_i (mM)
1) Alkanoates [ff]		**ß-CD / mNPA / butyrate / exp #34**		
	0	0.413	0.454	
	20	0.402	0.441	
	40	0.376	0.413	
	60	0.361	0.396	
	80	0.344	0.378	
	100	0.327	0.359	258
		ß-CD / mNPA / pentanoate / exp #35		
	0	0.410	0.454	
	20	0.366	0.405	
	40	0.324	0.358	
	60	0.284	0.314	
	80	0.263	0.291	
	100	0.231	0.256	74.4
		ß-CD / mNPA / 4-methylpentanoate / exp #55		
	0	0.360	0.454	
	2.5	0.331	0.417	
	5.0	0.288	0.363	
	7.5	0.270	0.340	
	10.0	0.247	0.311	
	12.5	0.234	0.295	14.7

[ff] Literature sources provided us with adequate values of K_i for alkanols, therefore mNPA experiments were not necessary for them, other than to validate the efficacy of our method.

[I] (mM)	k^{obs} (s^{-1})	k^{scaled} (s^{-1})	K_i mM)
	β-CD / mNPA / heptanoate / exp #36		
0	0.404	0.454	
1.8	0.344	0.386	
3.6	0.295	0.331	
5.4	0.258	0.290	
7.2	0.233	0.262	
9.0	0.215	0.241	5.86
	β-CD / mNPA / octanoate / exp #37		
0	0.423	0.454	
2	0.260	0 285	
4	0.210	0.225	
6	0.170	0.182	
8	0.146	0.157	
10	0.129	0.138	0.82
	β-CD / mNPA / adipate / exp #64		
0	0.401	0.454	
40	0.354	0.400	
80	0.323	0.365	
120	0.294	0.333	232
	β-CD / mNPA / suberate / exp #67-2		
0	0.399	0.454	
7	0.350	0.398	
14	0.314	0.357	
21	0.284	0.323	
28	0.256	0.291	
35	0.237	0.269	31.7

Experiments with α-CD

[I] (mM)	k^{obs} (s^{-1})	k^{scaled} (s^{-1})	K_i (mM)	
(1) Alkanoates[ss]	**α-CD / mNPA / propanoate / exp #101**			
0	5.34	7.08		
40	5.05	6.70	426.1	
80	4.69	6.22	374.4	
120	4.30	5.70	327.7	
160	3.81	5.05	265.6	
200	3.56	4.72	269.2	ave.=332.6
	α-CD / mNPA / butyrate / exp #102			
0	5.38	7.08		
25	4.41	5.80	69.3	
50	3.56	4.69	61.5	
75	3.08	4.05	64.3	
100	2.68	3.53	64.2	
125	2.33	3.07	62.1	ave.=64.3
	α-CD / mNPA / pentanoate / exp #103-1			
0	4.82	7.08		
8	4.01	5.89	18.9	
16	3.18	4.65	15.4	
24	2.61	3.83	15.0	
32	2.26	3.31	15.6	
40	1.65	2.86	15.4	ave. = 16.0

[ss] The analysis employed a different technique due to an error commited in the experimental procedure. Instead of using the customary [CD] of 1mM, 10mM was used. This inaccuracy invalidates the use of the INHIB program since the condition $[I]_o >$ [CD] no longer applies and the simplification of equation 6 to equation 8 in the Treatment of Results section would be innacurate. Instead, the following rearrangement of equation 4 was used: $[CD] = \{(k_{obs}-k_u)/(k_c-k_{obs})\}K_s$. Substitution from this into eq 6 yields K_i values for each $[I]_o$ used. This calculation was arranged in a Lotus 1-2-3 spreadsheet to give the average value of K_i from the different concentrations used in the experiment.[9]

	[I] (mM)	k^{obs} (s^{-1})	k^{scaled} (s^{-1})	K_i (mM)	
		α-CD / mNPA / hexanoate / exp #105			
	0	0.83	1.04		
	2	0.602	0.754	3.99	
	4	0.478	0.599	4.06	
	6	0.394	0.494	3.98	
	8	0.338	0.424	3.94	
	10	0.291	0.365	3.75	ave. = 3.94
(2) Sulfonates		**α-CD / mNPA / butanesulfonate / exp #106**			
	0	5.02	7.08		
	10	3.93	5.54	17.3	
	20	3.32	4.86	21.1	
	30	2.81	3.96	21.6	
	40	2.39	3.36	21.1	
	50	2.16	3.05	22.6	ave. = 20.7
		α-CD / mNPA / pentanesulfonate /exp#107			
	0	5.12	7.08		
	3.5	4.46	6.17	7.63	
	7.0	3.84	5.31	7.65	
	10.5	3.42	4.73	8.68	
	14.0	2.87	3.97	7.59	
	17.5	2.47	3.42	7.34	ave. = 7.78
		α-CD / mNPA / hexanesulfonate / exp #110-3 [hh]			
	0	0.85	1.04		
	2.0	0.52	0.64	2.13	
	6.0	0.29	0.35	2.01	
	8.0	0.24	0.29	1.96	
	10.0	0.21	0.26	2.02	ave. = 2.03

[hh] This and the following three experiments were analysed using the Lotus spreadsheet calculation (see footnote gg). The [CD] was maintained low, but so was $[I]_o$, due to the high affinity that long-chained sulfonates have for α-CD.

[I] (mM)	k^{obs} (s^{-1})	k^{scaled} (s^{-1})	K_i (mM)	
	α-CD / mNPA / heptanesulfonate / exp #113			
10	0.14	0.17	0.81	
15	0.11	0.13	0.70	
20	0.10	0.12	0.64	
25	0.09	0.11	0.55	ave. = 0.72
	α-CD / mNPA / octanesulfonate / exp #114			
1.0	0.38	0.49	0.31	
2.0	0.22	0.30	0.33	
3.0	0.17	0.23	0.36	
4.0	0.14	0.18	0.34	
5.0	0.13	0.16	0.36	ave. = 0.36
	α-CD / mNPA / decanesulfonate / exp #115-2			
1.0	0.22	0.28	0.05	
2.0	0.16	0.15	0.07	
3.0	0.09	0.12	0.06	
4.0	0.08	0.10	0.04	
5.0	0.07	0.09	0.02	ave. = 0.05

Experiments with α-CD and pNPH

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
1) Alkanols[ii]	α-CD / pNPH / ethanol / exp #92			
0	0	0.0917	0.0913	0.107
80	77.0	0.0889	0.0885	0.110
240	234	0.0896	0.0865	0.119
320	314	0.0875	0.0871	0.127
400	393	0.0892	0.0888	0.136
	α-CD / pNPH / n-propanol / exp #93*(15mM)			
0	0	0.0874	0.0951	0.107
60	51.8	0.0960	0.105	0.135
120	109	0.0972	0.106	0.155
180	168	0.0981	0.107	0.176
240	227	0.104	0.113	0.211
300	287	0.103	0.112	0.230
	α-CD / pNPH / n-butanol / exp #94*(20mM)			
0	0	0.0870	0.0975	0.107
20	10.4	0.0962	0.108	0.129
40	26.0	0.0981	0.110	0.147
60	44.0	0.101	0.113	0.171
80	63.0	0.104	0.117	0.198
100	82.4	0.108	0.121	0.231
	α-CD / pNPH / n-pentanol / exp #95-2			
0	0	0.0821	0.0913	0.107
2.4	0.654	0.0848	0.0943	0.115
4.8	1.51	0.0840	0.0934	0.118
7.2	2.62	0.0871	0.0969	0.130
9.6	3.97	0.0876	0.0974	0.138
12.0	5.57	0.0897	0.0998	0.152

[ii] The experiments in this section were all analysed using PIANALYSIS. Those marked with an * were conducted with a [α-CD] of 15mM or 20mM, as specified in parentheses.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
	α-CD / pNPH / n-hexanol / exp #96-2			
0	0	0.0833	0.0913	0.107
1.5	0.17	0.0794	0.0870	0.103
3.0	0.40	0.0808	0.0886	0.109
4.5	0.69	0.0772	0.0846	0.106
6.0	1.08	0.0772	0.0846	0.111
7.5	1.61	0.0739	0.0810	0.110
2) Alkanoates[ii]				
	α-CD / pNPH / butyrate / exp #102			
0	0	0.0890	0.0913	0.107
25	22.6	0.0889	0.0912	0.112
50	46.0	0.0885	0.0908	0.117
75	70.0	0.0852	0.0874	0.116
100	94.3	0.0825	0.0846	0.116
125	118	0.0809	0.0830	0.117
	α-CD / pNPH / hexanoate / exp #105			
0	0	0.0904	0.0913	0.107
2.0	1.03	0.0853	0.0861	0.102
4.0	2.16	0.0825	0.0833	0.0991
6.0	3.39	0.0933	0.0942	0.117
8.0	4.71	0.0866	0.0895	0.112
10.0	6.11	0.0822	0.0891	0.114

[ii] Based on the following experiments, there is no definitive evidence that cocatalysis of pNPH cleavage occurs with alkanoates (and most of the sulfonate ions) in -CD; they basically show inhibition.

[PI] (mM)	$[PI]_{corr}$ (mM)	k^{obs} (s^{-1})	$k^{obs'}$ (s^{-1})	k^{corr} (s^{-1})
3) Sulfonates				
	α-CD / pNPH / butanesulfonate / exp #106-3			
0	0	0.0840	0.0913	0.107
10	7.82	0.0866	0.0945	0.116
20	16.3	0.0845	0.0922	0.118
30	25.3	0.0842	0.0919	0.122
40	34.5	0.0803	0.0876	0.120
50	43.9	0.0814	0.0888	0.127
	α-CD / pNPH / pentanesulfonate / exp # 107			
0	0	0.0673	0.091	0.107
2.0	0.93	0.0671	0.091	0.109
4.0	1.98	0.0693	0.094	0.115
6.0	3.13	0.0769	0.104	0.133
8.0	4.39	0.0823	0.112	0.147
10.0	5.75	0.0844	0.115	0.156
	α-CD / pNPH / heptanesulfonate / exp #113			
0	0	0.0868	0.0913	0.107
1.0	0.13	0.0851	0.0895	0.106
2.0	0.28	0.0876	0.0921	0.112
3.0	0.46	0.0858	0.0902	0.111
4.0	0.67	0.0838	0.0881	0.110
	α-CD / pNPH / octanesulfonate / exp #114-3			
0	0	0.0985	0.0913	0.107
1.0	0.013	0.0975	0.0904	0.107
2.0	0.029	0.0939	0.0870	0.105
3.0	0.050	0.0888	0.0823	0.100
4.0	0.078	0.0889	0.0775	0.103
5.0	0.115	0.0878	0.0814	0.105
	α-CD / pNPH / decanesulfonate / exp #115-3			
0	0	0.0767	0.0913	0.107
1.0	0.005	0.0752	0.0895	0.106
2.0	0.012	0.0724	0.0862	0.104
3.0	0.021	0.0636	0.0757	0.090