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The β-Cyclodextrin-accelerated Hydrolysis of Aryl Sulfates: A Model for Enzymic Catalysis Through Binding

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The reversible binding and catalyzed hydrolysis of Aryl sulfates by β-cyclodextrin has been studied over the pH range 2.92–13.2 and at several temperatures. Analysis of the pH-rate curves, various thermodynamic and Arrhenius parameters, substituent effects and titration of the inorganic sulfate formed on hydrolysis leads to a catalytic mechanism which does not involve nucleophilic participation by β-cyclodextrin. A unimolecular mechanism analogous to that of the uncatalyzed process is proposed. The significance of these data is discussed especially in relation to the concept of strain and distortion in enzymatic catalysis.

INTRODUCTION

Cyclodextrins have been shown to form inclusion complexes with many organic compounds (*1–3*), and to exhibit many enzyme-like properties (*4*, *5*). The outstanding features of this model are that its structure, i.e., cyclic $\alpha - 1$, 4 linked D(+) glucose polymers containing six (α), seven (β), or eight (γ) residues per molecule (*6*), and usual mode of catalysis are relatively simple (*4*).

It was thought that β-cyclodextrin might be a useful model to examine the concept of strain and distortion in enzymatic catalysis (*7*). This concept has been described as a maximum affinity of an enzyme active site for the transition state as compared to its affinity for substrates or products. The optimal fit of the transition state has been proposed to be compensated for by a less favorable binding energy for the substrate due to its less than optimal fit. Further, this is said to lead to an increase in the ground state energy of the bound substrate and consequent lowering of the activation energy of the reaction.

To make meaningful comparisons between a catalyzed and an uncatalyzed process both processes must have the same mechanism. This requirement eliminates as substrates such compounds as esters (*4*), pyrophosphates (*8*), and phosphonates (*5*), which have been shown to be catalyzed by β-cyclodextrin via nucleophilic mechanisms. Aryl sulfates, however, of importance biologically (*9*), have been shown to hydrolyze via a unimolecular mechanism under most conditions (*10*) and have been used for this study.

EXPERIMENTAL

p-Nitrophenyl sulfate, potassium salt, was purchased from Calbiochem. Spectrophotometric assay (*11*) revealed 98% purity. 2,4-Dinitrophenyl sulfate, potassium salt,

was a gift from Dr. E. J. Fendler. Repeated purification gave a 90% pure product. *Ortho-* and *meta*-nitrophenyl sulfates were synthesized as previously described (*10*) and assayed greater than 98%. Salicylic sulfate was synthesized according to previously described procedures used for the *m*-carboxyphenyl sulfate, dipotassium salt (*12*), and assayed 95%. Barium chloranilate was purchased from Fisher Scientific.

Buffer solutions were prepared from reagent grade chemicals and doubly distilled water. The following buffers were used: formate, pH 2.92, 3.49; acetate, pH 4.37, 4.68; phosphate, pH 6.80, 7.29, 11.7; carbonate, pH 9.98. The alkali solutions were prepared from standard 1 *N* sodium hydroxide (British Drug House). pH values were measured on a Radiometer 26 expanded-scale pH meter with a radiometer type K4016 HF glass electrode versus S.C.E.

The spectrophotometric method of kinetic assay has been previously described (*10*, *13*). A Gilford 2000 with thermostated cell compartment (±0.1°C) was used for kinetics. Spectrophotometric titrations were carried out on a Cary Model 14 spectrophotometer at ambient temperature.

For the initial rates, *p*-nitrophenyl sulfate concentrations were on the order of 10^{-3} *M* and the first 0.04–0.10 half-life was monitored. For *ortho-* and *meta*-nitrophenyl sulfates, concentrations in the range 2–6 × 10^{-3} *M* were used. 2,4-Dinitrophenyl sulfate and salicylic sulfate hydrolyses were monitored under first-order conditions. First-order rate constants at aryl sulfate concentrations of 2–8 × 10^{-5} *M* were concentration independent. Infinity values were taken after 7–10 half-lives and duplicate kinetic runs normally agreed to within 4%.

Inorganic sulfate was determined with barium chloranilate using the chloranilic acid absorption at 530 nm (*14*). With *p*-nitrophenyl sulfate, 1.5 × 10^{-2} *M* β-cyclodextrin, $I = 0.2$, $T = 60.0$°C, and pH = 7.08, 97% inorganic sulfate was found and with salicylic sulfate, 1.5 × 10^{-2} *M* β-cyclodextrin, $I = 0.2$, $T = 60.0$°C, and pH = 4.0, 94% inorganic sulfate was found.

RESULTS

In Table 1, k_u the uncatalyzed rate, k_2 the calculated catalyzed rate, K_s the calculated dissociation constant, and several complex constants are listed. These values were calculated by a least-squares computerized Eadie method (*15*). The error limits quoted for k_2 and K_s represent a 90% confidence interval. In Table 2 are shown the Arrhenius parameters and in Table 3 the thermodynamic binding parameters for *p*-nitrophenyl and 2,4-dinitrophenyl sulfate. The error values quoted for these parameters represent the maximum possible error using the 90% confidence limits for each constant used to calculate each parameter.

Figure 1 shows a plot of the pseudo-first-order rate constants, k_ψ, versus the concentration of β-cyclodextrin for the hydrolysis of *p*-nitrophenyl sulfate. This plot appears to approach a limiting value, typical of Michaelis–Menten kinetics, and has been used with the Eadie correlation (*15*) to calculate rates and binding constants. Figure 2 shows plots of $\log k_2$ and $\log k_u$ versus pH for *p*-nitrophenyl sulfate hydrolysis. These, in conjunction with the complex constant (k_2/k_u) values listed in Table 1 may be interpreted mechanistically. In alkali, the uncatalyzed unimolecular mechanism is supplemented by an hydroxide ion-catalyzed nucleophilic mechanism and at pH = 12, for *p*-nitrophenyl sulfate, the mechanism has been shown to proceed by 33% nucleophilic aromatic substitution (*16*). Since k_2 does not increase with pH, one can conclude that the secondary hydroxyl groups on β-cyclodextrin, which are known to have $pK_a \cong 12$ (4),

TABLE 1

MAXIMAL RATE ACCELERATIONS AND DISSOCIATION CONSTANTS OF β-CYCLODEXTRIN-ARYL SULFATE COMPLEXES

Aryl sulfate	pH[a]	Temp. (°C)	k_u (min^{-1})	k_2 (min^{-1})	k_2/k_u	K_s (10^{-2} M)	k_2/K_s
p-Nitrophenyl	2.92[b]	60.3	1.14×10^{-4}	$1.99 \pm 0.04 \times 10^{-4}$	1.75	1.24 ± 0.19	1.6×10^{-2}
	7.08[e]	60.3	2.71×10^{-6}	$11.0 \pm 0.22 \times 10^{-6}$	4.06	0.93 ± 0.05	1.18×10^{-3}
	9.98[f]	50.3	6.81×10^{-7}	$2.60 \pm 0.19 \times 10^{-6}$	3.82	0.49 ± 0.12	5.31×10^{-4}
	9.98[f]	60.3	2.72×10^{-6}	$8.87 \pm 0.13 \times 10^{-6}$	3.26	0.86 ± 0.05	1.03×10^{-3}
	9.98[f,g]	60.3	2.70×10^{-6}				
	9.98[f]	70.6	8.73×10^{-6}	$3.01 \pm 0.48 \times 10^{-5}$	3.45	1.42 ± 0.50	2.12×10^{-3}
	11.58[e]	60.3	2.84×10^{-6}	$8.20 \pm 0.06 \times 10^{-6}$	2.89	0.76 ± 0.02	1.08×10^{-3}
	13.2[h]	60.3	7.79×10^{-6}	$9.13 \pm 0.12 \times 10^{-6}$	1.17	0.75 ± 0.14	1.22×10^{-3}
2,4-Dinitrophenyl	2.92[b]	37.3	7.96×10^{-3}				3.04
	3.49[c]	37.3	8.00×10^{-3}				3.94
	4.68[d]	37.3	7.42×10^{-3}	$78 \pm 8 \times 10^{-3}$	10.6	1.90 ± 0.30	4.13
	4.68[d]	50.3	2.70×10^{-2}	$23 \pm 1.9 \times 10^{-2}$	8.5	2.20 ± 0.50	10.3
	6.80[e]	37.3	7.16×10^{-3}	$134 \pm 8 \times 10^{-3}$	18.7	3.03 ± 0.40	4.4
	6.80[e]	50.3	2.57×10^{-2}	$37.6 \pm 1.8 \times 10^{-2}$	14.7	3.50 ± 0.30	10.7
	7.29[e]	37.3	7.09×10^{-3}				4.85
	9.98[f]	37.3	7.24×10^{-3}	$105 \pm 8 \times 10^{-3}$	14.6	2.40 ± 0.30	4.40
	9.98[f]	50.3	2.36×10^{-2}	$27 \pm 2 \times 10^{-2}$	7.9	2.40 ± 0.60	11.3
	11.7[e]	37.3	8.00×10^{-3}				3.08
m-Nitrophenyl	2.92[b]	60.3	4.48×10^{-5}				4.7×10^{-4}
o-Nitrophenyl	9.98[f,i]	60.3	6.90×10^{-6}				
Salicylic	2.92[b]	50.3	2.73×10^{-3}	$9.9 \pm 1.9 \times 10^{-3}$	3.6	4.00 ± 1.70	0.25
	3.49[c]	50.3	2.16×10^{-3}	$7.0 \pm 1.3 \times 10^{-3}$	3.2	3.20 ± 1.60	0.22
	4.37[d]	50.3	8.75×10^{-4}	$2.3 \pm 0.14 \times 10^{-3}$	2.6	1.80 ± 0.40	0.13

[a] Ionic strength of 0.2 used throughout.
[b,c] 0.2 M sodium formate buffer. pH adjusted by addition of formic acid.
[d] 0.2 M sodium acetate buffer. pH adjusted by addition of acetic acid.
[e] Phosphate buffers.
[f] Carbonate buffer of 0.1 M total concentration.
[g] 0.083 M methyl α-D + glucoside.
[h] 0.18 N NaOH.
[i] The following first-order rates were obtained: 7.8×10^{-3} M β-cyclodextrin, $k_\psi = 6.75 \times 10^{-6}$ min^{-1}, 1.06×10^{-2} M β-cyclodextrin, $k_\psi = 6.51 \times 10^{-6}$ min^{-1}, 1.64×10^{-2} M β-cyclodextrin, $k_\psi = 6.23 \times 10^{-6}$ min^{-1}.

TABLE 2

ARRHENIUS PARAMETERS FOR THE β-CYCLODEXTRIN-CATALYZED HYDROLYSIS OF *p*-NITROPHENYL AND 2,4-DINITROPHENYL SULFATES

Sulfate		$\Delta H^{\ddagger}$ (kcal/mole)	$\Delta S^{\ddagger}$ (e.u.)	$\Delta G^{\ddagger}$ (kcal/mole)
p-Nitrophenyl				
pH = 9.98[c]	k_u	27.0 ± 1.1[a]	−11.0 ± 2.4[a]	30.7
	k_2	25.9 ± 0.3[a]	−12.0 ± 1.1[a]	29.9
2,4-Dinitrophenyl[b,d]				
pH = 9.98	k_u	18.3 ± 1.1	−17.5 ± 2.5	24.0
	k_2	13.9 ± 2.3	−26.5 ± 4.5	22.5
pH = 6.80	k_u	19.0 ± 1.0	−15.3 ± 2.4	24.0
	k_2	15.3 ± 1.1	−21.7 ± 5.1	22.5
pH = 4.68	k_u	19.3 ± 0.9	−14.4 ± 2.1	24.0
	k_2	15.8 ± 2.8	−21.0 ± 8.6	22.6

[a] Calculated at 60.3°C.
[b] Calculated at 50.3°C.
[c] For the uncatalyzed reaction at $I = 1.0$, $T = 35$°C, pH = 9.96, $\Delta H^{\ddagger} = 24.6$ kcal/mole, $\Delta S^{\ddagger} = -18.5$ eu (*13*).
[d] For the uncatalyzed reaction at $I = 0.01$, $T = 75$°C, pH = 8.0, $\Delta H^{\ddagger} = 18.2$ kcal/mole, $\Delta S^{\ddagger} = -18.0$ eu (*10*).

TABLE 3

THERMODYNAMIC ASSOCIATION PARAMETERS FOR β-CYCLODEXTRIN ARYL SULFATE COMPLEXES

Aryl sulfate	ΔG^{a}_{Assn} (kcal/mole)	ΔH^{a}_{Assn} (kcal/mole)	ΔS^{a}_{Assn} (eu)	ΔG^{b}_{Assn} (kcal/mole)	K^{c}_{s} (*M*)
p-Nitrophenyl[d] (60.3°C)					
pH = 9.98	−3.1	−8.9 ± 0.2	−17.4 ± 0.9	−3.9	2.3×10^{-3}
2,4-Dinitrophenyl (50.3°C)					
pH = 9.98	−1.12			−2.6	1.7×10^{-2}
pH = 6.80	−1.61			−3.1	7.9×10^{-3}
pH = 4.68	−1.02			−2.4	2.3×10^{-2}

[a] Association constants for substrate binding to β-cyclodextrin.
[b] Association constant for transition state binding to β-cyclodextrin.
[c] Dissociation constant for the transition state β-cyclodextrin complex.
[d] From previous work (*1*) the following values have been calculated for *p*-nitrophenolate-α-cyclodextrin association: $\Delta G^{\circ 60.3^{\circ}C}_{Assn} = 1.35$ kcal/mole; $\Delta H^{\circ}_{Assn} = -7.2$ kcal/mole; $\Delta S^{\circ}_{Assn} = -30.8$ eu.

do not catalyze the reaction.[1] Further, one can rule out an hydroxide ion attack on the substrate at any position for the catalyzed process.[2] k_2/k_u decreases due to the increase in k_u only. In dilute acid solution, *p*-nitrophenyl sulfate hydrolyzes via an acid-catalyzed

[1] For a nucleophilic cyclodextrin catalyzed mechanism one would expect a linear dependence of $\log k_2$ on pH such as that for the cyclodextrin catalyzed hydrolysis of *m*-tolyl acetate (*4*). Since there is no participation by ionized hydroxyl groups ionization should affect only K_s. The fact that *p*-nitrophenyl sulfate shows no increase in K_s may be attributed to (*1*) the ionic strength of the medium (*2*) binding of the substrate preferentially in a position with anionic sulfate moiety removed from the anionic secondary hydroxyl groups and (3) an increase in the pK_a of the secondary hydroxyl groups in the anionic enzyme-substrate complex.

[2] At high pH values k_u but not k_2 increases with pH so one can conclude the complexed substrate does not show a term with respect to hydroxide. This is attributed to steric inhibition of hydroxide attack on the bound substrate and is consistent with Lach and Chin's study of the alkaline hydrolysis of ethyl *p*-aminobenzoate inhibited by β-cyclodextrin (*20*).

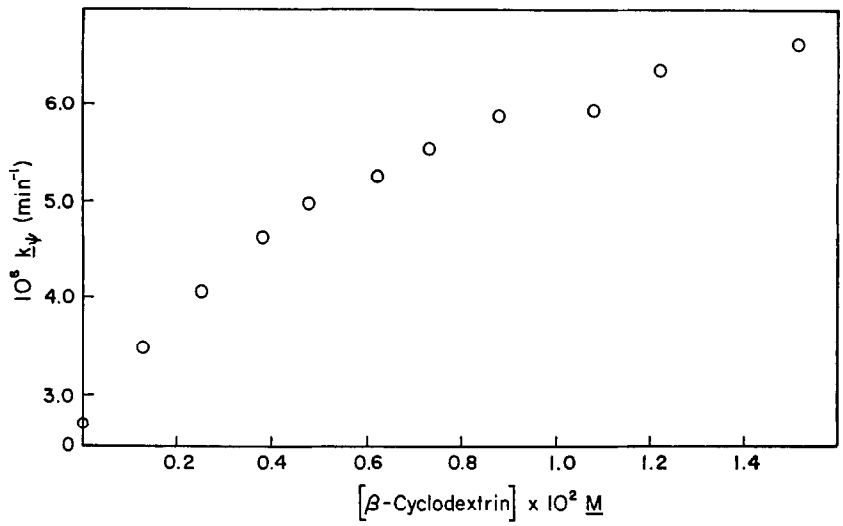

FIG. 1. Variation of the apparent first-order rate constant (k_ψ) for the hydrolysis of *p*-nitrophenyl sulfate as a function of the concentration of β-cyclodextrin at pH = 9.98, $T = 60.3°C$, $I = 0.2$.

path. From Fig. 3 one can adduce that this process is also catalyzed by β-cyclodextrin and, overall, both the catalyzed and the uncatalyzed processes are much more rapid than the neutral reaction, which can be essentially neglected in calculation. However, k_2/k_u decreases from that for the neutral reaction showing a lower catalytic efficiency for this process.

Figure 3 shows plots of $\log(k_2/K_s)$ versus pH for *p*-nitrophenyl and 2,4-dinitrophenyl sulfates. The insensitivity of this ratio to pH (although there is scatter due to large K_s values) shows that ionization is not important for the overall process. In acid solution, for the para derivative, k_2/K_s increases due to the acid-catalyzed mechanism.

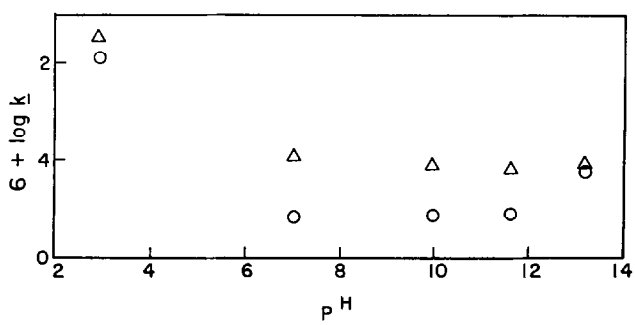

FIG. 2. pH-logk_x profile for *p*-nitrophenyl sulfate hydrolysis at $T = 60.3°C$, $I = 0.2$, $k_2 = \triangle$, $k_u = o$.

Another kind of evidence for a unimolecular mechanism comes from a comparison of k_2/K_s values (a measure of an enzyme's sensitivity toward a substrate) for *meta*- and *para*-nitrophenyl sulfates (0.03) with the corresponding ratio for *meta*- and *para*-nitrophenyl acetates (5.2). The meta isomer would be bound more poorly in each case but binding places the reacting group in each case very close to the reactive secondary hydroxyl groups of β-cyclodextrin. Phenyl acetates reacting by a nucleophilic mechanism react more rapidly. For aryl sulfates the meta derivative is not well catalyzed, since, just as for salicylic sulfate (*17*) and 2-(4(5)-imidazoyl)-phenyl sulfate (*18*), proximity of a nucleophile apparently does not lead to nucleophilic catalysis.

The preceding results preclude a nucleophilic mechanism by ionized β-cyclodextrin hydroxyl groups but do not rule out nucleophilic attack by the un-ionized hydroxyl

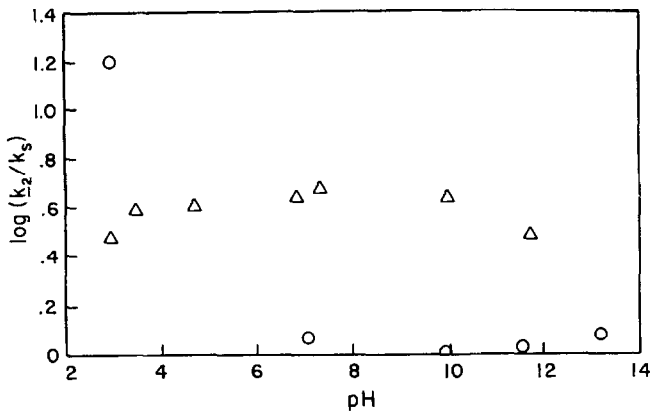

FIG. 3. pH versus $3 + \log(k_2/K_s)$ for *p*-nitrophenyl sulfate hydrolysis at 60.3°C, $I = 0.2$ (○) and pH versus $\log(k_2/K_s)$ for 2,4-dinitrophenyl sulfate at 37.3°C, $I = 0.2$ (△).

groups or a unimolecular mechanism which leads to transfer rather than hydrolysis. Near quantitative formation of inorganic sulfate, as determined spectrophotometrically, would not rule out a cyclodextrin sulfate covalent intermediate, but it does indicate that the intermediate would have to be labile under the reaction conditions.

If one can assume (as for the uncatalyzed reaction (*10*)) a relationship such as:

$$\Delta \log k_2 / \Delta \mathrm{p}K_a(\text{of phenol}) = \text{constant},$$

the reactivity of β-cyclodextrin sulfate can be estimated. Evaluated at pH = 9.98, $T = 50.3$°C, $I = 0.2$ considering 2,4-dinitrophenyl and *p*-nitrophenyl sulfates $\Delta \log k_2 / \Delta \mathrm{p}K_a = -1.5$. This is close to that for the uncatalyzed neutral process (*10*), and leads to a value for k_2 of 10^{-13} min^{-1} (corresponding to a half-life of 10^7 years) for β-cyclodextrin sulfate ($\mathrm{p}K_a = 12$ (*4*)), thus precluding unionized hydroxyl nucleophilic attack. Possible general acid catalysis, however, can not be ruled out, although, if true, it would be expected for methyl α-D(+) glucoside as well, which is not observed. Scheme I indicates a probable mechanism for the cyclodextrin-accelerated hydrolysis of aryl sulfates.

Scheme I

$$\mathrm{Ar{-}O{-}SO_3^- + \beta\text{-CD} \overset{K_s}{\rightleftharpoons} Ar{-}O{-}SO_3^- \cdot \beta\text{-CD (noncovalent)}}$$

$$\mathrm{Ar{-}O{-}SO_3^- \cdot \beta\text{-CD (noncovalent)} \xrightarrow{k_2} Ar{-}O^- \cdot \beta\text{-CD} + SO_3}$$

$$\mathrm{SO_3 + H_2O \xrightarrow{fast} H^+ + HSO_4^-}$$

For the acid-catalyzed process one must also include:

$$\mathrm{Ar{-}OSO_3^- \cdot \beta\text{-CD (noncovalent)} \overset{H^+}{\rightleftharpoons} Ar{-}\overset{H}{\overset{|}{O^+}}{-}SO_3^- \cdot \beta\text{-CD (noncovalent)}}$$

$$\mathrm{Ar{-}\overset{H}{\overset{|}{O^+}}{-}SO_3^- \cdot \beta\text{-CD (noncovalent)} \xrightarrow{k_2} ArOH \cdot \beta\text{-CD} + SO_3}$$

DISCUSSION

Acceleration of the magnitude observed in this study ($k_2/k_u \cong 18$ maximum) is difficult to ascribe to a specific substrate–catalyst interaction and might be considered a simple microsolvent effect.[3] However, since the catalyzed process occurs by an analogous mechanism to that for the uncatalyzed process, as shown by a similar dependence of k_2 and k_u on pH (below 12), a similar dependence of k_2 and k_u on phenol pK_a and by inorganic sulfate titration, a more detailed analysis of the data is thought warranted.

For slow reactions, which both the catalyzed and uncatalyzed processes surely are, the products could be reasonable models for the transition state after Hammond's postulate. *p*-Nitrophenolate and other weak bases exhibit on complexation with cyclodextrin, large red ultraviolet absorption shifts (*1*) and a decreased basicity (*21*). Complexation with cyclodextrin desolvates the charged bases and is manifested in a highly unfavorable binding entropy and a highly favorable binding enthalpy (See Table 3). It is thought that the changes in characteristics on binding are indicative of an increased delocalization of charge over the molecule. The transition state for the hydrolysis would, as shown by the logk_2-pK_a (phenol) correlation, have a great deal of charge forming on the phenol portion of the molecule. Since the transition state would be bound, the charge forming on the phenol could be more readily delocalized, essentially lowering the transition state energy. This can equivalently be described as catalysis resulting from a change in the leaving phenolate pK_a due to complexing with cyclodextrin. As might be expected for this type of catalysis (See Table 2) the rate enhancement results from a decrease in ΔH^* incompletely compensated for by a decrease in ΔS^*.[4] Using Eq. (1), one can calculate

$$\Delta G^{\ddagger}_{uncat} - \Delta G^{\circ}_{Assn\,(ground\ state)} + \Delta G^{\circ}_{Assn\,(transition\ state)} = \Delta G^{\ddagger}_{cat} \qquad (1)$$

values for the energy decrease of the bound transition state system (Eq. 1). This parameter can be calculated for any type of catalysis and may be interpreted as purely an association constant for the transition state complex or more correctly a sum of several factors leading to catalysis. It is a convenient measure of catalysis and literally shows that catalysis results from a lower free energy of association of the transition state than the substrate.

Strain may be defined as a change in the angle or bond order or length of the reactive bond, in this case the O–S ester bond, resulting from an external force (*22*). The bound transition state has a lower energy due to greater delocalization of the incipient charge. This would decrease the O–S bond separation necessary for dissociation, effectively inducing a change in form of the reactive bond. This then may constitute an example of catalysis due purely to complex formation, inducing strain and reducing the energy of the transition state.

Up to now catalysis has been ascribed to a lowering of the transition state energy but only energy differences can be measured and there is no *a priori* reason why bound ester (the ground state) could not be even more responsible for catalysis. However, the hydrolysis of *ortho*-nitrophenyl sulfate (See Table 1) is inhibited by cyclodextrin. Since the product is a reasonable model for the transition state one can infer that (1) the substrate must bind to cyclodextrin since there is an effect and (2) since the model for the

[3] The effect of added β-cyclodextrin is shown (see Table 1) not to be a macrosolvent effect by the lack of effect of 0.083 *M* methyl α(D)+-glucoside on the rate of hydrolysis of *p*-nitrophenyl sulfate.

[4] This result is opposite to that found for hydrolysis of aryl sulfates in mixed solvents (*10*) where rate increases are accompanied by large increases in ΔS^*. For the latter process the increase in ΔS^* has been attributed to desolvation of the transition state, but the result may as easily come about by a favorable entropy of solvation of the ground state (*19*).

transition state does not bind, β-cyclodextrin is not affecting the transition state. From these observations one can conclude that the bound substrate O–S bond is of lower energy than the unbound form, as pictured in Fig. 4, and that catalysis results only when the transition state is well bound.

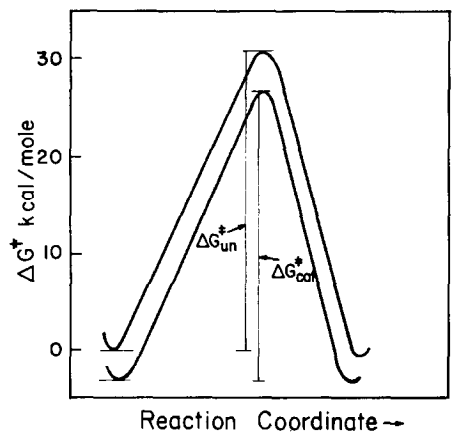

FIG. 4. Transition-state diagram based on the observed free-energy parameters for the uncatalyzed and catalyzed hydrolysis of *p*-nitrophenyl sulfate.

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