

Catalytic and inhibitory effects of β -cyclodextrin on the hydrolysis of benzoic anhydride

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ABSTRACT: The hydrolysis of benzoic anhydride (Bz_2O) in the presence of β -cyclodextrin (β -CD) was studied in aqueous solution as a function of pH, temperature and ionic strength, at 25 °C. The experimental rate constant versus pH profiles show that, in the region of spontaneous water reaction (pH 3.0–6.5), β -CD inhibits the reaction and the isotope effect ($k_{H_2O}/k_{D_2O} = 4.7$) indicates that the rate determining step of the reaction corresponds to the water-catalyzed nucleophilic attack of water on the carbonyl group of Bz_2O . Conversely, whereas inhibition is observed at pH 6.0, catalysis of the hydroxide ion reaction is observed at pH 8.0 and it is found that the activation entropy is responsible for the catalytic phenomena in the basic hydrolysis of benzoic anhydride. Copyright © 2004 John Wiley & Sons, Ltd.

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KEYWORDS: cyclodextrin; benzoic anhydride; hydrolysis; kinetics; salt effect; thermodynamic parameters; isotope effect; ¹H NMR

INTRODUCTION

Cyclodextrins (CDs) have been widely used as models of artificial enzymes for a variety of reactions^{1,2} and as stabilizing agents and drug carriers.³ These typical applications require contrasting behavior, since an enzyme model needs to show catalytic efficiency and, in the latter case, stability requirements demand an inhibitory effect of cyclodextrins on the reactions involving inclusions.

Among the reactions affected by native α - and β -CD, cleavage of carboxylic esters,^{4–6} amides⁷ and phosphoric esters^{8,9} in basic media have been studied the most. In general, the mechanism involves nucleophilic attack of the ionized secondary hydroxyl group of CD on the carbonyl or phosphoryl group, but in some cases it also involves general base catalysis.^{10,11} Because of this particular mechanistic behavior, they have been highlighted as models for serine proteases, owing to the nucleophilic attack of the ionized secondary hydroxyl group, which is similar to the first step in the deacylation of esters in this type of enzyme.^{1,2}

CDs show many features characteristic of enzymatic reactions such as saturation, stereospecificity and formation

of an inclusion complex with the substrate, and a variety of intermolecular interactions are responsible for the stability of CD inclusion complexes in aqueous solution.^{12,13}

As far as we know, there have been no studies on the effect of native β -CD on the hydrolysis of anhydrides. The present work was undertaken to study the effect of native β -CD on the hydrolysis of benzoic anhydride (Bz_2O).

RESULTS

Benzoic anhydride is hydrolyzed in moderately acidic, neutral and basic aqueous solutions to form two molecules of benzoic acid (or benzoate). Figure 1 shows the pH dependence of the first-order rate constants for the hydrolysis reaction of Bz_2O with and without 0.01 M β -CD. The reaction carried out in water between pH 3.0 and 10.0 shows a pH-independent region typical of the spontaneous water reaction between pH 3.0 and 6.5 and a first-order dependence on hydroxide ion concentration, which denotes specific base catalysis, between pH 6.5 and 10.0. In the presence of β -CD, there is a decrease in the rate constant of the reaction in the region of spontaneous reaction and an increase in the rate in the region of the hydroxide ion-catalyzed reaction.

The effect of β -CD concentration on the observed rate constant for the hydrolysis of Bz_2O can be seen in Fig. 2.

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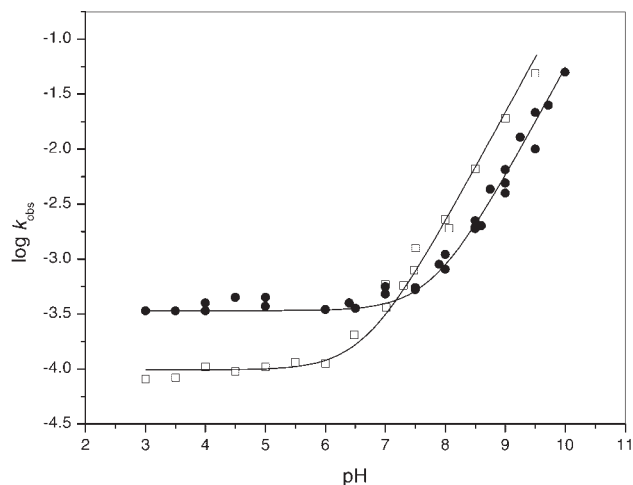


Figure 1. pH dependence of the logarithm of the first-order rate constant for the hydrolysis reaction of Bz_2O in water (●) and in the presence of 0.01 M β -CD (□) at 25 °C and ionic strength 0.5 (NaCl). The lines represent theoretical agreement with Eqn. (4) and the parameters are shown in Table 1

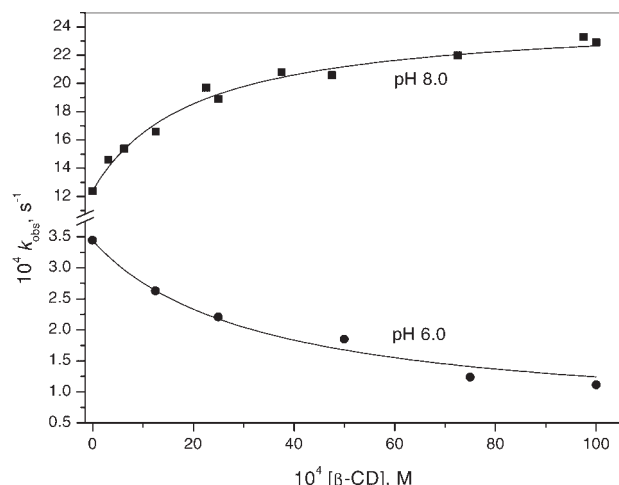
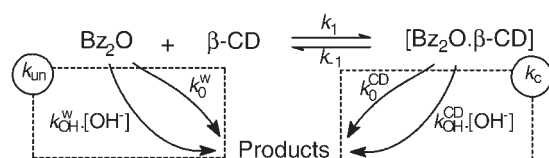


Figure 2. Effect of β -CD on the hydrolysis rate of Bz_2O at pH 6.0 (●) and 8.0 (■) at 25 °C and ionic strength 0.5 (NaCl). The lines were calculated using Eqn. (4) and the parameters are shown in Table 1

Whereas the hydrolysis reaction is inhibited at pH 6.0, it is slightly catalyzed at pH 8.0.

Our results are fully consistent with Scheme 1. In the presence of β -CD, the reaction goes through an anhydride-CD complex ($Bz_2O \cdot \beta$ -CD), which corresponds to a 1:1 inclusion complex between β -CD and Bz_2O .



Scheme 1

In Scheme 1, k_0 is the first-order rate constant for the spontaneous hydrolysis and k_{OH} is the second-order rate constant for the hydroxide ion-catalyzed hydrolysis of Bz_2O in the aqueous phase. The rate constants k_0^{CD} and k_{OH}^{CD} correspond to the reaction of water and HO^- with the fraction of Bz_2O incorporated into the CD cavity. The dissociation constant (K_{diss}) between Bz_2O and β -CD is given by k_{-1}/k_1 . At any given pH value, the observed rate constant corresponds to contributions from the reaction in water (k_{un}) and in β -CD (k_c).

The reaction in the aqueous phase, in the absence of β -CD, is given by

$$k_{un} = k_0^w + k_{OH}^w[OH^-] \quad (1)$$

and the rate constant k_c for the reaction of Bz_2O , fully incorporated into β -CD, is given by

$$k_c = k_0^{CD} + k_{OH}^{CD}[OH^-] \quad (2)$$

It follows that, under conditions where the substrate distributes rapidly between the free and complexed forms, the hydrolysis of Bz_2O according to Scheme 1 is described by means of

$$k_{obs} = k_{un}\chi_{Bz_2O}^w + k_c\chi_{Bz_2O}^{\beta-CD} \quad (3)$$

Expressing the mole fractions of substrate in water and in β -CD in terms of K_{diss} and the corresponding mass balance equations, it is possible to derive the equation

$$k_{obs} = k_{un} + (k_c - k_{un}) \frac{[CD]}{K_{diss} + [CD]} \quad (4)$$

which can be used in the Lineweaver-Burk form¹⁴ to calculate the dissociation constant between Bz_2O and β -CD (Table 1).

Thus, using the data in Figs 1 and 2 and Eqns (1)–(4), we can calculate the individual rate and equilibrium constants. At pH 6.0, in the absence and presence of β -CD, we can clearly see that the spontaneous water reaction is the only reaction in solution; therefore, $k_{un} = k_0^w$ in the absence of β -CD and $k_c = k_0^{CD}$ in the presence of high β -CD concentration. Thus, Eqn. (4) and the data in Fig. 2

Table 1. Calculated parameters for Bz_2O hydrolysis in the absence and presence of β -CD at 25 °C and ionic strength 0.5 (NaCl)

Rate constant	Value
k_0^w (s ⁻¹)	$(3.4 \pm 0.2) \times 10^{-4}$
k_{OH}^w (M ⁻¹ s ⁻¹)	515 ± 30
k_0^{CD} (s ⁻¹)	$(8.2 \pm 0.5) \times 10^{-5}$
k_{OH}^{CD} (M ⁻¹ s ⁻¹)	2530 ± 68
K_{diss} (M)	$(2.0 \pm 0.4) \times 10^{-3}$

permit the calculation of K_{diss} and k_0^{CD} (values are given in Table 1).

Using the experimental data at pH 8.0 (Fig. 2) and Eqns (2) and (4), it was possible to calculate values of $K_{\text{diss}} = (2.0 \pm 0.4) \times 10^{-3} \text{ M}$ and $k_c = (2.53 \pm 0.68) \times 10^{-3} \text{ s}^{-1}$ and thus the value of $k_{\text{OH}}^{\text{CD}}$ in Table 1. The value of k_{OH}^{w} , which was calculated from the slope of a plot of k_{obs} versus the hydroxide ion concentration, is also given in Table 1.

When the hydrolysis reaction of Bz_2O in the absence of $\beta\text{-CD}$ was carried out in dioxane–water mixtures, it was observed that the rate constant decreased as the concentration of dioxane increased. The observed rate constants for the reaction at pH 6.0 in dioxane–water mixtures with 10 and 20% (v/v) organic solvent content were 1.9×10^{-4} and $7.6 \times 10^{-5} \text{ s}^{-1}$, respectively.

All models in this work are consistent with a 1:1 stoichiometry between $\beta\text{-CD}$ and Bz_2O . In order to confirm the stoichiometry and the formation of the inclusion complex, the chemical shifts of H-3, H-5, and H-6 of $\beta\text{-CD}$ and H-*ortho*, -*meta* and -*para* of Bz_2O were measured by ^1H NMR spectroscopy (300 MHz). Upon inclusion complex formation, the chemical shift is the average of that for free (CD) and complexed (CDS) cyclodextrin (or substrate):

$$\delta_{\text{obs}} = \delta_{\text{CD}}\chi_{\text{CD}} + \delta_{\text{CDS}}\chi_{\text{CDS}} \quad (5)$$

Equation (5) can be used to derive Eqn. (6), where $\Delta\delta_{\text{obs}}$ represents the difference $\delta_{\text{obs}} - \delta_{\text{CD}}$ and $\Delta\delta_{\text{m}}$ the difference $\delta_{\text{CDS}} - \delta_{\text{CD}}$:

$$\Delta\delta_{\text{obs}} = \frac{[\text{CDS}]}{[\text{CD}]_0} \Delta\delta_{\text{m}} \quad (6)$$

A Job plot was used to obtain the stoichiometry of the inclusion complex between $\beta\text{-CD}$ and Bz_2O . We varied the amount of guest and CD, maintaining constant the total amount of CD plus guest ($[\text{CD}]_0 + [\text{S}]_0 = 8 \text{ mM}$). Figure 3 shows the results for H-5 of $\beta\text{-CD}$ and H_{meta} of the Bz_2O ; for all hydrogens analyzed, there was a maximum at 0.5 in the Job plot, indicating a 1:1 stoichiometry for the inclusion complex. The K_{ass} value calculated from ^1H NMR data in 1:1 $\text{D}_2\text{O}:\text{MeOD}$ was $317 \pm 17 \text{ M}^{-1}$ and

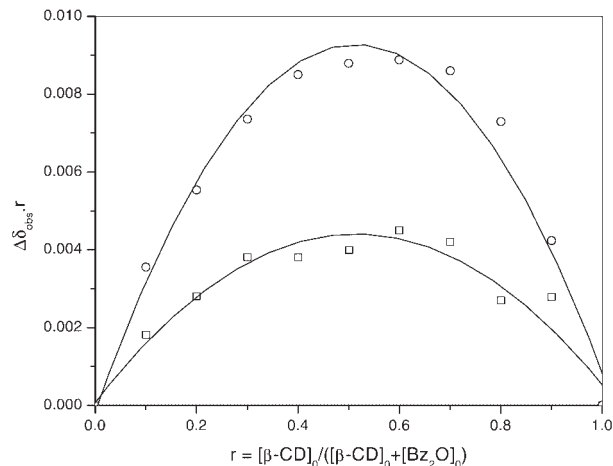


Figure 3. Job plot for ^1H NMR shifts of H-5 of $\beta\text{-CD}$ ($\square$) and H_{meta} of Bz_2O ($\circ$) at 300 MHz in $\text{D}_2\text{O}:\text{MeOD}$ (1:1)

as shown in Fig. 4 suggest that Bz_2O is deeply embedded in the CD cavity.

The effect of the ionic strength was studied by the addition of different NaCl concentrations and the results are shown in Fig. 5. Surprisingly, the increase in the ionic strength decreased the rate constants for Bz_2O hydrolysis with and without $\beta\text{-CD}$ at pH 6.0, but increased the rate constants in both cases at pH 8.0. The values of the activation parameters $\Delta H^\ddagger$ and $\Delta S^\ddagger$ for Bz_2O hydrolysis in the presence and absence of $\beta\text{-CD}$ at pH 6.0 and 8.0 were calculated following standard procedures¹⁵ and are given in Table 2. The results show that the values of $\Delta H^\ddagger$ and $\Delta S^\ddagger$, at pH 6.0 and 8.0, are greater in the presence of $\beta\text{-CD}$ than in aqueous solution.

The solvent isotope effect ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$) on Bz_2O hydrolysis in the presence of $\beta\text{-CD}$ at pH 6.0 and 8.0 gave values of 4.7 and 3.7, respectively. These values are similar to those determined by Butler and Gold¹⁶ for the hydrolysis of Bz_2O in the absence $\beta\text{-CD}$.

DISCUSSION

It is known that, in the case of enzyme catalysis the formation of a complex between substrate and catalyst brings the substrate into close contact with the active site of the enzyme and reactions can be favored owing to

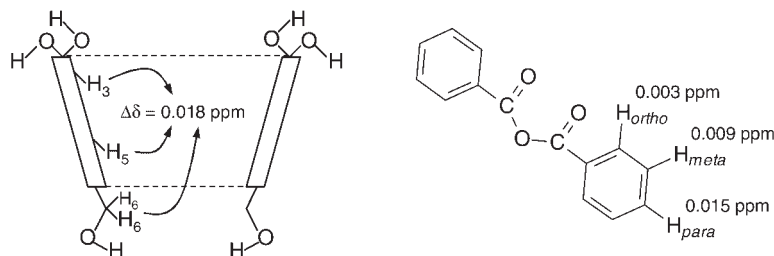


Figure 4. ^1H NMR shifts of H-3, H-5, and H-6 of $\beta\text{-CD}$ and H_{ortho} , H_{meta} and H_{para} of Bz_2O [300 MHz; $\beta\text{-CD}:\text{Bz}_2\text{O} = 1:1$; $\text{D}_2\text{O}:\text{MeOD}$ (1:1)]

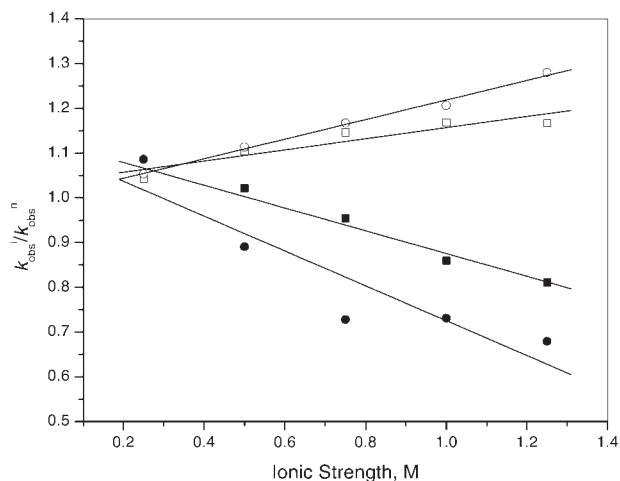


Figure 5. Effect of the concentration of NaCl in the absence (squares) and presence (circles) of β -CD at pH 6.0 (solid symbols) and 8.0 (open symbols) on the rate of Bz₂O hydrolysis at 25 °C. $k_{\text{obs}}^{\text{n}}$ and $k_{\text{obs}}^{\text{i}}$ are the observed constants in the absence and presence of NaCl

Table 2. Calculated thermodynamic parameters for the hydrolysis of Bz₂O in the absence and presence of 0.01 M β -CD, ionic strength 0.5 (NaCl)

Thermodynamic parameter	pH 6.0		pH 8.0	
	Bz ₂ O	Bz ₂ O · β -CD	Bz ₂ O	Bz ₂ O · β -CD
$\Delta H^\ddagger$ (kcal mol ⁻¹)	12.2 ± 0.7	16.6 ± 0.4	15.6 ± 0.4	17.2 ± 0.3
$\Delta S^\ddagger$ (e.u.)	-33.5 ± 2.2	-21.4 ± 1.4	-20.2 ± 1.5	-13.2 ± 1.1
$\Delta\Delta H^\ddagger$ (kcal mol ⁻¹)		4.4		1.6
$\Delta\Delta S^\ddagger$ (e.u.)		12.1		7.0

stereochemical effects.¹⁷ This has also been suggested in the case of catalysis by CDs and either general base or nucleophilic catalysis has been reported.¹⁸ Earlier studies showed that CDs caused either acceleration or inhibition of the alkaline hydrolysis of various alkyl benzoate esters, depending on the nature of the complexes formed. Lach and co-workers^{19,20} proposed that compounds which fit in close proximity to the hydroxyl groups of CD undergo an acceleration effect, whereas positioning of the carbonyl carbon atom further away from the secondary hydroxyl groups in CD results in a decrease in the rate constant. However, Bender and co-workers^{21,22} suggested that, in many cases, the unreactivity of the complexed esters could be attributed to an unfavorable partitioning of the tetrahedral intermediate. Attack of the alkoxide ion of CD on the carbonyl carbon atom of the substrate would lead to the formation of a tetrahedral intermediate having both the CD alkoxide ion and the alkoxide ion derived from the alkyl benzoate ester as potential leaving groups. The tetrahedral intermediate would

preferentially revert to reactants as the CD alkoxide ion is a better leaving group than the alkoxide ion derived from the ester.

Two mechanisms have been proposed to explain the base catalysis of anhydride hydrolysis. The first is nucleophilic catalysis, which involves the direct attack of the catalyst on the carbonyl group of the anhydride, forming an intermediate that decomposes faster than the substrate in the presence of water. The second mechanism involves a concerted attack on the carbonyl group of the anhydride by a water molecule, assisted by the catalyst. In this second case, the catalyst facilitates the reaction, accelerating the rate-limiting step through hydrogen bonding to the uncharged water molecule (general base catalysis).²³ In the hydrolysis of benzoic anhydride in the presence of carboxylate-type surfactants, it has been well characterized that the reaction proceeds via nucleophilic catalysis.²⁴

The experimental rate constant versus pH profiles in Fig. 1 show that, in the region of spontaneous water reaction (pH 3.0–6.5), the hydroxyl group of β -CD is not functioning as a general base type catalyst, a result which is fully consistent with the fact that, in aqueous solution, Bz₂O hydrolysis is not subject to efficient general base-type catalysis. Indeed, the mechanism of the Bz₂O hydrolysis reaction at neutral pH without β -CD shows a considerable isotope effect,¹⁶ which indicates that the rate-determining step of the reaction corresponds to the water-catalyzed nucleophilic attack of water on the carbonyl group of Bz₂O.²⁵ The large isotope effect observed in the presence of β -CD at pH 6.0 ($k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}} = 4.7$) is also consistent with this mechanism.

The NMR results are clear, showing that incorporation of the benzoate moiety into the CD cavity affects the chemical shifts of H-3, H-5, and H-6 of β -CD similarly. The shifts observed for Bz₂O follow the order $H_{\text{ortho}} > H_{\text{meta}} > H_{\text{para}}$, indicating as expected that H_{para} is located deeper into the cavity and H_{ortho} is in a more aqueous environment. The positioning of one of the carbonyl groups close to the upper ring of the CD cavity is strongly suggestive of a decrease in the microscopic polarity of the reaction site, a situation that is unfavorable in the light of the observed effect of added dioxane, where addition of 10 and 20% (v/v) of dioxane resulted in inhibition of the spontaneous reaction by 44 and 76%, respectively. Hence it could well be that the observed inhibition of the spontaneous reaction is mostly due to a decrease in microscopic polarity, a result which is consistent with previous observations in micellar solutions.²⁶ All the evidence indicates that the reaction mechanism in the presence of β -CD can, between pH 3 and pH 6, be considered to be similar to that postulated by Engbersen and Engberts,²⁵ that is, a classical 'water reaction' that involves a water-catalyzed nucleophilic attack of water on the carbonyl group of Bz₂O. This implies the contribution of at least two (or more) water molecules in the ground state and several water molecules in the transition state. Indeed, the

strongly negative $\Delta S^\ddagger$ value (-33.5 e.u.) for the hydrolysis in pure water in the absence of β -CD indicates that the transition state is highly hydrated relative to the ground state. This value is considerably more negative than that observed in the presence of the CD catalyst (-21.4 e.u.). Thus, the reaction slows owing to the increase in $\Delta H^\ddagger$ by $4.4 \text{ kcal mol}^{-1}$ ($1 \text{ kcal} = 4.184 \text{ kJ}$), which more than compensates the increase in activation entropy. It is important to consider that, in the β -CD cavity, there are considerable water-structure effects, which will appear strongly in the more hydrated structure of the transition state. Hence the loss of hydration of the transition state, relative to the ground state, is most probably promoted by a solvent organization effect in the CD cavity, which in turn produces a less negative activation entropy (Table 2).

The increase in ionic strength at pH 6.0, in the absence of β -CD, caused a small decrease in the rate constant for the hydrolysis reaction, which could be attributed to unspecific effects and changes in the structure of the solvent. In the presence of β -CD, the ionic strength effect at pH 6.0 is more pronounced, but the observed effect is complex since it could be the result of the sum of solvent-structure effects and an important salting-out effect favoring the equilibrium towards the inclusion complex (Fig. 5).

In order to understand the mechanism of Bz_2O hydrolysis in the region where the hydroxide ion reaction predominates in aqueous solutions, we examined the reaction in the presence of β -CD at pH 8.0, where a catalytic effect is observed (Figs 1 and 2). In this respect, the Bz_2O hydrolysis reaction is similar to the hydrolysis of trifluoroacetate and acetate esters, which show inhibition of the reaction at pH 6 and catalysis at pH 9. The authors suggested that this fact is related to, and depends on, the binding strength between the substrate and the CD relative to the transition state. When the binding of the substrate was stronger than that of the transition state, inhibition was observed.^{27,28} Clearly, this is not the case in the hydrolysis of Bz_2O . In fact, we have two different reactions under the selected pH conditions. Whereas inhibition of the water reaction is observed at pH 6.0, catalysis of the hydroxide ion reaction is observed at pH 8.0. Since the reactions are completely different, we contend that the reasons for the observed catalysis and/or inhibition are mechanistic in nature.

The cleavage of esters has been studied extensively and, in general, the hydrolysis of aryl esters follows a mechanism of acyl transfer to an ionized secondary hydroxyl group of the CD.^{1,2,4-6} In the reaction of benzoic anhydride, the ionized secondary hydroxyl group of β -CD could be reacting as a mechanistic general base-type catalyst or through nucleophilic catalysis. Nucleophilic catalysis can be ruled out because the final absorbance is consistent with the formation of 2 mol of benzoate. The benzoylated CD shows a slower rate of hydrolysis and a much higher molar absorptivity compared with the

benzoate ion. Hence it is possible that the reaction proceeds via general base catalysis promoted by the ionized secondary hydroxyl group of β -CD (the $\text{p}K_a$ of this OH group is ~ 12). Alternatively, the attack of the hydroxide ion on the substrate encapsulated in the CD could be a competitive route and this particular pathway has been confirmed by Breslow *et al.*²⁹ The value of the solvent isotope effect of 3.7 is fully consistent with the proposed mechanism since the final approximation of the hydroxide ion to the reacting carbonyl carbon will be assisted by at least two water molecules. Hence the observed isotope effect is consistent with the participation of several water molecules, which stabilize the transition state of the reaction through hydrogen bonding; various models which account for this participation have been proposed.^{30,31} The contribution of several water molecules in the ground state and in the transition state is consistent with the negative $\Delta S^\ddagger$ value (-20.2 e.u.) for the hydrolysis in pure water in the absence of β -CD, which again indicates higher hydration of the transition state relative to the ground state. In the presence of the CD catalyst, the activation entropy is -13.2 e.u.; therefore, the catalytic effect is the result of a large increase in activation entropy, which compensates the increase in $\Delta H^\ddagger$ of $1.6 \text{ kcal mol}^{-1}$. As discussed above, the water-structure effects in the β -CD cavity appear strongly in the transition state. It is interesting that in the water reaction, the activation enthalpy dominates the overall effect, whereas in the region of pH 8.0, it is the activation entropy that is responsible for the catalytic phenomenon (Table 2).

In conclusion, it is important to emphasize that the observed effects of β -CD on the hydrolysis of Bz_2O , i.e. inhibition of the water reaction and catalysis in the basic region, are related to the fact that different mechanisms are operative in these regions. At pH 6.0 an increase in activation enthalpy is responsible for the observed inhibition of the water reaction, whereas at pH 8.0 the activation entropy is responsible for the observed catalysis of the basic hydrolysis of benzoic anhydride.

EXPERIMENTAL

Materials. Bz_2O was prepared and purified as indicated in the literature.³² The β -CD (Lot C6003-1, MW 1135) was obtained from Cerestar (USA) with $>99\%$ purity and was dehydrated under vacuum at 100°C for 3 h before use. All other reagents and solvents were of analytical grade and freshly prepared distilled water was used throughout.

Kinetic procedure. The kinetic determinations were carried out in aqueous solution and the temperature and ionic strength used were varied. Rates of hydrolysis of benzoic anhydride were followed spectrophotometrically by monitoring the disappearance of Bz_2O at 245 nm by using a Hitachi U-2000 UV-visible spectrophotometer fitted with a thermostated water-jacketed cell holder.

Reaction was initiated by injection of 10 μ l of ~ 10 mM stock solutions of Bz₂O in dioxane (stored in a freezer) into 3 ml of aqueous solutions equilibrated at 25.0 °C (the initial concentration of Bz₂O was 3.33×10^{-5} M and the solutions contained 0.33% dioxane). Absorbance versus time data were stored directly on a microcomputer using a Microquimica 12-bit A/D interface board. First-order rate constants, k_{obs} , were estimated from linear plots of $\ln(A_{\infty} - A_t)$ against time for at least 90% reaction using an iterative least-squares program; correlation coefficients, ρ , were >0.999 for all kinetic runs and, between replicates, the standard deviation for the first-order rate constants was always $<2\%$. The pH during the reaction was maintained with the following buffers (0.01 M in all cases): HCOOH (pH 3–4.5); CH₃COOH (pH 4–5.5); NaH₂PO₄ (pH 5.5–7.8); H₃BO₃ (pH 8–9.5); NaHCO₃ (pH 9.5–11). The solutions were prepared immediately before use, except in the study of the solvent isotope effect, where the solutions were left to stand for 15 h at room temperature in order to allow the H/D balances to be reached. The value of pD was corrected considering that $\text{pD} = \text{pH} + 0.4$,³³ and the pH measurements were carried out with a Micronal Model B 374 digital pH-meter, previously calibrated with standard solutions of pH 4.00, 7.00 and 10.00 (Carlo Erba).

The values of the activation enthalpy and entropy were calculated from the experimental results (Supplementary Material, Tables S1 and S2) in the temperature range 20–40 °C using the equation¹⁵

$$\ln\left(\frac{k}{T}\right) = \left[\ln\left(\frac{k_B}{h}\right) + \frac{\Delta S^\ddagger}{R}\right] - \frac{\Delta H^\ddagger}{R} \left(\frac{1}{T}\right) \quad (7)$$

¹H NMR procedure. ¹H NMR spectra were recorded with a Bruker AC spectrometer at 300 MHz, using H₂O as an internal reference. The Bz₂O to β -CD ratio was continually varied, maintaining the total concentration at 8 mM in a 1:1 D₂O–MeOD mixture.

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Supplementary information

Job plots for ¹H NMR shifts of H-3 and H-6 of β -CD and of H_{ortho} and H_{para} of Bz₂O at 300 MHz in a 1:1 D₂O–MeOD mixture and rate constants as a function of

temperature in the presence and absence of β -CD at pH values of 6.0 and 8.0 are available at <http://www.interscience.wiley.com/jpages/0894-3230/suppmat/>.

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