The Binding Behavior of Cyclodextrins toward a Nitroxide Spin Probe in the Presence of Different Alcohols As Studied by EPR

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Stability constants, rates of association and dissociation, and thermodynamic and activation parameters for the formation of inclusion complexes between the radical guest, *N*-benzyl-*tert*-butyl-d_9-nitroxide and β- or 2,6-*O*-dimethyl-β-cyclodextrin (CDs), have been determined by EPR spectroscopy in water in the presence of 14 different alcohols, differing in size and lipophilicity. In all cases, it was found that addition of alcohol, depending on its structure and concentration, causes a reduction of the stability of the paramagnetic complex. Global analysis of EPR data allowed us to explain the CDs binding behavior: we discarded the formation of a ternary complex, where alcohol and radical guest are coincluded into CD cavity, while data were found more consistent with the formation of a binary complex alcohol:CD competing with the monitored complex nitroxide:CD. Both kinetic and thermodynamic analysis of the experimental results have revealed that the presence of alcohols affects to a larger extent the dissociation rather then the association of radical probe and CD and that the former process is of greater importance in determining the stability of the complex, this confirming the reliability of the competition model proposed. This competition has been used for the indirect determination of the stability constants of complexes between CD and examined alcohols. By using a similar approach, we showed EPR spectroscopy can be considered a rapid and accurate technique to investigate the CDs binding behavior toward different nonradical guest.

Introduction

The ability of cyclodextrins to form inclusion complexes with a variety of organic molecules in aqueous solution has been largely investigated and exploited.[1−5] Among the different factors affecting the formation of these complexes in water, the more important are the hydrophobic effect, according to which an apolar or weakly polar molecule is included inside the lipophilic cavity of a cyclodextrin to avoid contact with the aqueous medium, and the correspondence between shape and size of the guest molecule and those of the cyclodextrin cavity.[1] Because of the interest in modifying the inclusion properties of cyclodextrins in several fields of science such as organic and analytical chemistry,[6−8] enzymatic studies,[9] and controlled delivery of drugs,[10−14] attention has been paid to investigate the effect of adding a third component, especially alcohols, to binary systems consisting of a guest and a cyclodextrin. Guest molecules mostly submitted to this kind of studies are polycyclic aromatic hydrocarbons, especially polyaromatic derivatives, which are strongly fluorescent and are easily complexed by cyclodextrins due to their hydrophobicity.[15−24] Contradictory results have been reported on the possibility of forming ternary complex between the cyclodextrin, the probe, and the alcohol. For instance, Muñoz de la Peña et al.[16] found that addition of alcohols in the presence of complexes β-cyclodextrin:pyrene leads to the formation of a ternary complex β-cyclodextrin: pyrene:alcohol more stable than the binary one, while the opposite is true in the presence of the fluorescent probe acridine.[21] In 1996, Evans et al.[23] rejected the formation of ternary complexes between short-chain linear alcohols, 2-naphthol, and β-cyclodextrin, and they observed at the same time a decrease of the association strength of the binary complex 2-naphthol:β-cyclodextrin by increasing the number of carbon atoms in the aliphatic chain. Later, the same authors[24] reported that a ternary complex weaker than the binary one is formed when studying the complexation of naphthalene by β-cyclodextrin in the presence of linear and branched alcohols.

In more recent years, the effect of alcohols as the third component of systems cyclodextrin:drug has also been investigated. For instance, the addition of 1% linear alcohols reduces systematically the apparent equilibrium constant of association (K_{app}) of the complex between an anti-inflammatory drug, such as naproxene, and β-cyclodextrin;[25] analysis of the K_{app} variations with the alcohol concentration indicates the formation of a weak ternary complex. Also, in the case of the complex between the antimalaric dapsone and β-cyclodextrin,[26] the addition of linear alcohols decreases the value of K_{app} with increasing number of carbons along the alcohol chain. In this case, the variations of K_{app} with alcohol concentration do not reveal the formation of ternary complexes.

With the aim to broaden the range of observations reported in the literature on the behavior of binary complexes between cyclodextrins and organic substrates in the presence of alcohols of different size and lipophilicity and in particular to determine their influence, barely investigated so far, on the dynamics of the inclusion process, we have undertaken a systematic study on the effects of 14 different alcohols linear, cyclic, and branched (Chart 1) on the inclusion in aqueous solutions of the radical guest *N*-benzyl-*tert*-butyl-d_9-nitroxide (**1**) in β- and 2,6-*O*-dimethyl-β-cyclodextrin (β-CD and DM-β-CD), and in γ-cyclodextrin (γ-CD), by using electron spin resonance spectroscopy (EPR).

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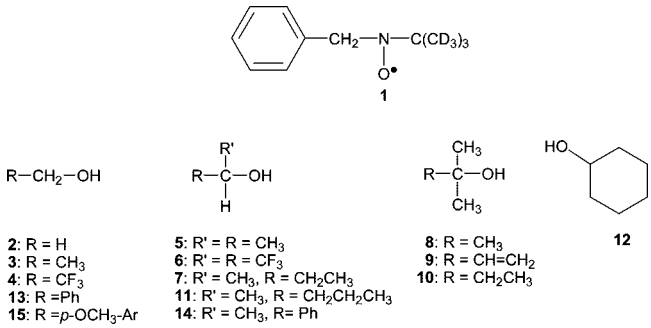
CHART 1: Structures of Radical Guest and Investigated Alcohols



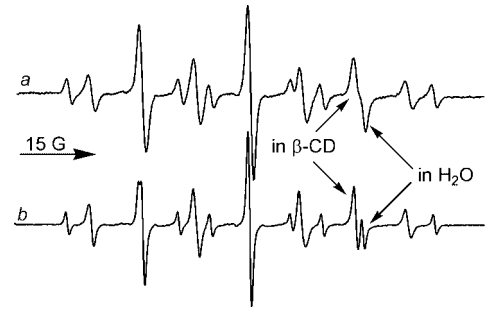


Figure 1. EPR spectra of *N*-benzyl-*tert*-butyl nitroxide (a) and *N*-benzyl-*tert*-butyl-d_9-nitroxide, **1** (b), both recorded in water at 298 K in the presence of β-CD (8.2 mM) and cyclohexanol, **12** (14 mM).

Experimental Section

Materials. All alcohols, **2**−**15**, γ-, β-, and 2,6-*O*-dimethyl-β-cyclodextrin were commercially available (Aldrich-Sigma) and were used as received. *N*-Benzyl-*tert*-butyl-d_9-amine, **1a**, the precursor of radical guest **1**, has been synthesized as described below.

***N*-Benzyl-*tert*-butyl-d_9-amine (1a).** A toluene solution (150 mL) of *tert*-butyl-d_9-amine (25 mmol) and benzaldehyde (25 mmol) was stirred and heated under reflux for 10 h, and the produced water was removed via a Dean−Stark apparatus. The solution was cooled and evaporated under reduced pressure to afford the imine (yield 76%). The imine (19 mmol) was then dissolved in a 1:1 solution of THF/methanol (150 mL), $NaBH_4$ was added (19 mmol), and the mixture was stirred at room temperature for 2 h. Another portion of $NaBH_4$ (19 mmol) was added to the reaction mixture and stirred for 16 h further. The solution was treated with HCl 6 N (150 mL), and the solvent was removed by evaporation under reduced pressure. The residue was treated with water NaOH 5 N (100 mL) and extracted with CH_2Cl_2 (100 mL × 3). The organic phase was washed with water (100 mL), before being dried (Na_2SO_4) and evaporated under reduced pressure to give the amine (yield 70%) [GC−MS (*m*/*z*): 172 (M^+), 154 (M^+ − 18, 100), 91 (95). ^1H NMR ($CDCl_3$): δ 3.73 (s, 2H), 7.25−7.37 (m, 5H)].

EPR Measurements. Radical **1** has been generated by mixing, in an aqueous solution (100 μL), 1 μL of a 5.0×10^{-2} M solution of **1a** in methanol with 1 μL of a 5.0×10^{-2} M aqueous solution of the magnesium salt of monoperoxyphthalic acid in the presence of variable amounts of CD and alcohol. To get higher radical concentrations, (occasionally) the solution was heated at 70 °C for 1 min. Twenty milliliters of these solutions were transferred in capillary tubes (internal diameter 1 mm), and the EPR spectra were recorded using a Bruker ELEXYS E500 spectrometer equipped with a NMR gaussmeter for field calibration and a microwave frequency counter for *g* factor determination. These have been corrected with respect to that of the perylene radical cation in concentrated H_2SO_4 (g = 2.00258). Temperature has been controlled with a standard variable-temperature accessory and has been monitored before and after each run with a copper-constantan thermocouple. Spectrometer settings were as follows: microwave power 5.0 mW; modulation amplitude 0.5 Gauss; frequency modulation 100 kHz; sweep time 180 s. Digitized EPR spectra were transferred to a PC and analyzed by comparison with simulated spectra obtained with a program developed by us and based on the density matrix theory and assuming a two-jump model.[27] The program requires as input data: values of hyperfine splitting constants, number and spin of the equivalent nuclei for both free and complexed radical, intrinsic line width in the absence of exchange, and rate constants of host−guest association and dissociation.

Results

N-Benzyl-*tert*-butyl nitroxide has been largely used in our previous work as paramagnetic guest species for studying different host systems,[27−34] because, due to the sensitivity of its spectroscopic parameters (in water: a(N) = 16.69 G, $a(2H_\beta)$ = 10.57 G, g = 2.0056), to the polarity of the environment, and to conformational changes, after inclusion it shows large variations of the hyperfine splitting constants at both nitrogen, a(N), and benzylic protons, a(N) = 15.74 G, $a(2H_\beta)$ = 7.88 G, g = 2.0058. Thus, the EPR spectra of this radical in the presence of a suitable host system show signals clearly different for the free and included species, and their ratio provides the value of equilibrium constant for the formation of the inclusion complex. In addition, because the lifetimes of the two species are comparable to the time scale of EPR spectroscopy, as suggested by the strong dependence on the temperature of the spectral line width, this technique permits one to obtain information on the kinetics of association and dissociation of the inclusion complex.

For the present study, the *tert*-butyl hydrogens of *N*-benzyl-*tert*-butyl nitroxide have been deuterated (**1**) to improve the spectral resolution and, thus, to get more accurate values of the kinetic rate constants. Figure 1 shows the increaseing resolution of the EPR lines of the free and included species observed upon deuteration.

As host systems, we have investigated β- and DM-β-CD, to check whether the three-component systems (probe−alcohol−CD) behave differently when increasing the internal length of the macrocyle cavity. The spectra of nitroxide **1** were recorded in the presence of CD concentrations ranging from 1 to 8 mM, while the alcohol concentration was always kept larger than that of CD although lower than 0.5 M so as to not significantly modify the solvent polarity.[35] From 5 to 15 measurements have been carried out within each concentration interval by analyzing mixtures containing different amounts of alcohol and CD. The effect of the added alcohol was evaluated at two temperatures: 298 K, to compare the present results with the few reported in the literature, and at 318 K, to guarantee that the exchange rate was high enough to permit a correct kinetic analysis. To evaluate the influence of the CD internal width on the behavior of a three-component system, the effect of 1,1,1,3,3,3-hexafluoro-2-propanol (**6**) on the stability of the complex between **1** and γ-CD has also been investigated at 318 K.

The apparent equilibrium constants (K_{EPR}) for the complex formation between nitroxide **1** and the investigated CD were

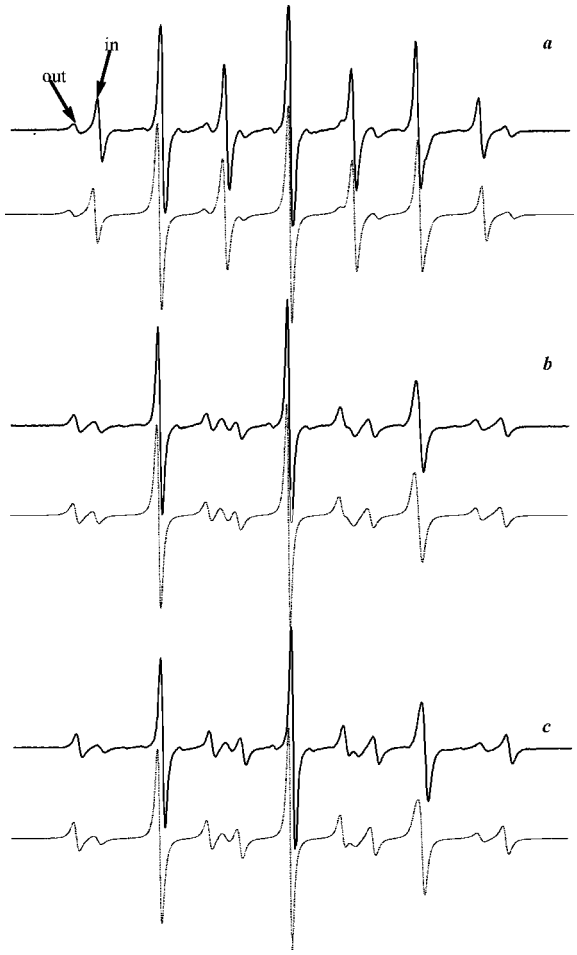


Figure 2. EPR spectra of radical **1** recorded in water at 318 K in the presence of β-CD (8.2 mM): (a) without a third component; (b) in the presence of 2-butanol (0.22 M); (c) in the presence of cyclohexanol (0.028 M). Below each experimental spectrum are shown the corresponding simulations computed by using the following rate constants: (a) $k_{ass} = 1.1 \times 10^9$ M^{-1} s^{-1}, $k_{diss} = 1.6 \times 10^6$ s^{-1}; (b) $k_{ass} = 7.6 \times 10^8$ M^{-1} s^{-1}, $k_{diss} = 7.6 \times 10^6$ s^{-1}; (c) $k_{ass} = 6.5 \times 10^8$ M^{-1} s^{-1}, $k_{diss} = 9.0 \times 10^6$ s^{-1}.

determined from the values of the rate constants for association (k_{ass}) and dissociation (k_{diss}) of the complex (see Scheme 1), obtained from the simulation of the exchange-broadened EPR spectra, by using well-established procedures based on the density matrix theory[27] and assuming a two-jump model as illustrated in Scheme 1. As an example, Figure 2 shows the EPR spectra at 318 K of **1** in the presence of β-CD 0.0082 M in water without the third component (Figure 2a), after the addition of 0.22 M of a moderately destabilizing alcohol such as 2-butanol, **7** (Figure 2b), and after the addition of just 0.028 M of a strongly destabilizing alcohol such as cyclohexanol, **12** (Figure 2c).

Discussion

Competitive Inclusion or Coinclusion of Alcohols. The data in Table 1 show that the addition to the complex of each of the investigated alcohols produces a reduction of the affinity constant in water of the guest radical toward the various cyclodextrins. The entity of effect depends on the alcohol structure and concentration and is less apparent with γ-CD. The changes induced by alcohol addition on the K_{EPR} values of inclusion complexes between **1** and cyclodextrins may be explained in three different ways.

1. Hydrophobic Effect. This is particularly important for alcohol concentrations larger than 5–10% v/v.[35] Under these conditions, the solvent polarity undergoes significant variations, and this may modify the affinity of the guest for the cyclodextrin cavity.

2. Competitive Effect. The alcohol molecule occupies the hydrophobic cavity of the CD, expelling the paramagnetic guest from it.

3. Coinclusion. Both guest molecule and alcohol are included within the cyclodextrin cavity, forming a ternary complex whose apparent association constant is larger or smaller than that of the binary complex guest:CD.

When the percent of alcohol is lower than ca. 2.0% v/v, the hydrophobicity of the medium is not substantially changed. Moreover, alcohol concentrations larger than 0.05% v/v ensure a favorable interaction between alcohol and cyclodextrin because the latter one is normally present at much lower concentration. Within this range of alcohol concentrations, the observed variations of K_{EPR} can essentially be attributed to the competitive effect or to the formation of a ternary complex. These conditions were found to be fulfilled with all of the investigated alcohols, with the only exception being methanol, ethanol, and isopropanol, for which the change in the medium polarity cannot be ignored at the concentration employed.

To decide which one of the three proposed hypotheses is the most likely, we should examine the variations of K_{EPR} as a function of the alcohol concentration on the basis of a model widely accepted in the literature,[22–26,36] which describes the complexation 1:1 of a guest in the presence of alcohol, ROH, in terms of three equilibria. In the present case, we can write

$$\mathbf{1} + \mathrm{CD} \overset{K_1}{\rightleftharpoons} \mathbf{1}\text{:CD} \tag{1}$$

$$\mathrm{ROH} + \mathrm{CD} \overset{K_2}{\rightleftharpoons} \mathrm{ROH:CD} \tag{2}$$

$$\mathbf{1} + \mathrm{ROH:CD} \overset{K_3}{\rightleftharpoons} \mathrm{ROH:CD:}\mathbf{1} \tag{3}$$

By assuming that the EPR spectrum due to the nitroxide complexed by CD is the same in the absence and in the presence of the alcohol, we can write a total equilibrium, (4):

$$\mathbf{1} + \mathrm{CD} + \mathrm{ROH} \overset{K}{\rightleftharpoons} (\mathbf{1})_{\mathrm{bound}} \tag{4}$$

where $(\mathbf{1})_{\mathrm{bound}}$ corresponds to the sum of the concentrations of **1**:CD and **1**:CD:ROH. When the alcohol concentration is constant and larger than those of CD and substrate, the apparent equilibrium constant is given by

$$K[\mathrm{ROH}] = K_{EPR} = [\mathbf{1}]_{\mathrm{bound}}/[\mathbf{1}][\mathrm{CD}] \tag{5}$$

The variation of K_{EPR} with alcohol concentration ($K_{EPR} = f([\mathrm{ROH}])$) is related to equilibria 1−3 by the following equation:

$$K_{EPR} = K_1 + K_2K_3[\mathrm{ROH}]/(1 + K_2[\mathrm{ROH}]) \tag{6}$$

If the formation of a ternary complex is considered negligible, equilibrium 3 can be neglected and eq 6 can be simplified to

$$K_{EPR} = K_1/(1 + K_2[\mathrm{ROH}]) \tag{7}$$

Experimentally, we checked if the variations of the K_{EPR} at 298 and 318 K of the complexes **1**:CD observed by changing alcohol concentration could be better fitted by the ternary model (eq 6) or by the competition model (eq 7). Although in both

TABLE 1: Variations of Thermodynamic (K_{EPR}, K_1, K_2) and Kinetic (k_{ass}, k_{diss}) Constants in the Presence of Different Alcohols

T (K)	host	alcohol	$k_{ass}/10^9 M^{-1}$ s^{-1} [a]	$k_{diss}/10^6 s^{-1}$ [a]	K_{EPR} (M^{-1})[a]	K_1 (M^{-1})[b]	K_2 (M^{-1})[b]	r^2
298	DM-β-CD	none	0.65	0.61	1079			
		2 (7.41 M)	0.75	2.6	288			
		3 (0.17–1.0 M)	0.76–1.0	1.1–1.8	913–471			
		4 (0.066–0.66 M)	0.84–1.1	1.4–5.0	794–168	1098 ± 28.0	6.6 ± 0.5	0.993
		5 (0.13–0.76 M)	0.71–0.88	1.3–3.0	677–248			
		6 (0.011–0.071 M)	0.31–0.58	1.8–6.9	322–45	1080 ± 15.7	230.3 ± 12.8	0.998
		7 (0.026–0.26 M)	0.84–1.1	1.4–3.3	741–254	1051 ± 37.9	13.8 ± 1.4	0.985
		8 (0.1 – 0.6 M)	0.54–0.68	2.9–8.7	235–62	1078 ± 14.6	31.9 ± 1.8	0.999
		9 (0.024–0.14 M)	0.78–0.96	1.7–3.7	566–211	1071 ± 20.3	32.7 ± 1.6	0.996
		10 (0.045–0.22 M)	0.58–0.73	2.7–6.6	277–92	1078 ± 16.0	58.9 ± 3.2	0.998
		11 (0.022–0.18 M)	0.77–1.0	1.6–5.0	625–154	1075 ± 15.3	31.6 ± 1.31	0.998
		12 (0.0047–0.047 M)	0.49–0.58	2.3–5.5	244–65	1076 ± 37.5	559.5 ± 57.5	0.992
		13 (0.024–0.14 M)	0.78–0.89	2.7–7.0	329–111	1075 ± 28.4	77.8 ± 6.5	0.995
		14 (0.0032–0.097 M)	0.58–1.4	2.1–7.5	644–89	1051 ± 20.6	138.5 ± 6.6	0.994
		15 (0.007–0.046 M)	0.56–0.63	2.0–4.0	317–140	1069 ± 51.3	228.7 ± 22.7	0.980
318		none	1.4	2.1	646			
		2 (7.41 M)	1.4	7.4	182			
		3 (0.16–0.82 M)	1.4–1.5	2.2–3.6	609–380			
		4 (0.066–0.66 M)	1.6–2.2	3.2–15	481–140	656 ± 9.4	5.6 ± 0.2	0.999
		5 (0.12–0.63 M)	1.4–1.5	2.9–8.1	467–191			
		6 (0.023–0.23 M)	1.0–1.3	5.9–49	207–18	653 ± 12.7	111.3 ± 5.6	0.995
		7 (0.05–0.22 M)	1.6–1.9	3.8–9.5	409–201	645 ± 14.0	9.8 ± 0.6	0.994
		8 (0.05–0.5 M)	1.5–2.0	5.8–40	241–47	649 ± 12.8	30.2 ± 1.9	0.995
		9 (0.024–0.14 M)	1.6–1.8	3.5–12	444–138	655 ± 16.0	23.1 ± 1.5	0.993
		10 (0.024–0.11 M)	1.4–1.6	5.3–15	294–98	646 ± 7.0	49.2 ± 1.4	0.999
		11 (0.022–0.18 M)	1.5–2.0	3.2–16	463–116	697 ± 22.5	31.0 ± 2.9	0.992
		12 (0.014–0.091 M)	1.1–1.4	6.1–50	182–29	652 ± 14.5	233.8 ± 17.0	0.994
		13 (0.012–0.09 M)	1.5–2.3	4.5–17	329–136	633 ± 31.0	56.7 ± 6.0	0.980
		14 (0.008–0.057 M)	1.4–1.6	3.5–9.3	404–156	629 ± 12.4	59.6 ± 3.0	0.995
		15 (0.007–0.046 M)	1.1–1.1	3.3–8.8	337–122	627 ± 19.7	100.7 ± 8.4	0.990
298	β-CD	none	0.68	0.53	1281			
		3 (0.17–1.0 M)	0.59–0.64	0.8–1.7	825–353			
		4 (0.13–0.8 M)	0.44–0.64	1.2–3.5	535–125	1276 ± 29.2	9.30 ± 0.6	0.996
		5 (0.13–0.76 M)	0.40–0.82	1.1–2.1	741–189			
		6 (0.019–0.094 M)	0.24–0.60	2.2–5.2	268–42	1281 ± 21.1	204.4 ± 14.9	0.998
		7 (0.054–0.32 M)	0.47–0.80	1.6–3.8	502–124	1280 ± 12.8	28.12 ± 0.83	0.999
		8 (0.025–0.3 M)	0.40–0.56	1.1 –5.1	510–79	1281 ± 11.5	50.3 ± 1.7	0.999
		9 (0.024–0.24 M)	0.46–0.62	1.7–5.4	330–84	1276 ± 59.1	97.7 ± 10.0	0.985
		10 (0.045–0.22 M)	0.37–0.56	2.3–6.5	245–60	1281 ± 12.1	103.2 ± 4.0	0.999
		11 (0.022–0.18 M)	0.55–0.89	1.4–3.9	635–141	1279 ± 10.7	45.3 ± 1.13	0.999
		12 (0.0033–0.043 M)	0.31–0.53	1.3–4.8	406–78	1274 ± 36.5	515.6 ± 49.4	0.992
		13 (0.028–0.14 M)	0.55–0.70	1.9–5.2	368–122	1279 ± 29.4	77.0 ± 6.1	0.997
		14 (0.008–0.081 M)	0.56–0.81	1.5–4.7	537–119	1267 ± 37.7	133.9 ± 10.5	0.991
		15 (0.007–0.046 M)	0.59–0.78	1.5–4.0	488–159	1273 ± 35.2	191.6 ± 19.5	0.994
318		none	1.1	1.6	688			
		3 (0.33–1.0 M)	0.82 – 1.00	2.3–3.2	451–256			
		4 (0.066–0.53 M)	0.9 –1.0	2.7–9.0	346–106	679 ± 23.0	11.7 ± 1.2	0.991
		5 (0.13–0.77 M)	1.0–1.1	2.4–8.6	432–123			
		6 (0.007–0.056 M)	0.71–0.92	2.0–6.9	461–102	697 ± 20.0	86.2 ± 7.1	0.993
		7 (0.026–0.22 M)	0.71–0.78	2.3–7.6	339–100	680 ± 18.6	32.2 ± 2.5	0.993
		8 (0.015–0.25 M)	0.70–0.85	2.3–14	371–52	669 ± 33.0	60.7 ± 6.0	0.980
		9 (0.014–0.19 M)	0.85–0.98	2.1–12	406–75	679 ± 18.0	44.4 ± 3.1	0.992
		10 (0.018–0.18 M)	0.87 –1.0	2.6–16	319–61	690 ± 31.3	52.8 ± 5.0	0.985
		11 (0.022–0.18 M)	1.1–1.2	2.4–12	465–89	697 ± 22.5	31.0 ± 2.9	0.992
		12 (0.0023–0.035 M)	0.65–0.83	2.3–10	340–60	676 ± 42.4	263.9 ± 30.8	0.989
		13 (0.028–0.14 M)	1.0–1.1	3.8–13	268–80	688 ± 6.5	55.7 ± 1.6	0.999
		14 (0.008–0.09 M)	1.0–1.1	2.0–10	482–106	694 ± 11.6	64.9 ± 2.7	0.996
		15 (0.007–0.046 M)	0.69–0.99	2.2–5.0	370–138	678 ± 20.5	93.6 ± 7.5	0.990
318	γ-CD	none	0.43	12	35.5			
		6(0.056–0.23 M)	0.53–0.87	16–48	30–18	35.7 ± 0.61	4.2 ± 0.26	0.991

[a] The values on the left and on the right correspond to the lower and to the upper concentrations of alcohol, respectively. [b] K_1 and K_2 are obtained by applying the competitive model, $K_{EPR} = K_1/(1 + K_2[ROH])$, to the plot $K_{EPR} = f([ROH])$.

cases the association constant (K_1) of the complexes **1**:CD in the absence of alcohol can be considered constant, we preferred to use it as an independent variable to control the reliability of the examined model. (We found in each case that K_1 calculated by using eq 6 or eq 7 was very close to the same equilibrium constants determined experimentally.)[27] Actually, the experimental points obtained by plotting the K_{EPR} values against the added alcohol could be satisfactorily interpolated for all of the investigated alcohols by using both models. Nevertheless, in the majority of cases, the coinclusion assumption could be discarded because the K_3 values for the formation of the ternary complex **1**:CD:ROH obtained by interpolation of the plot of

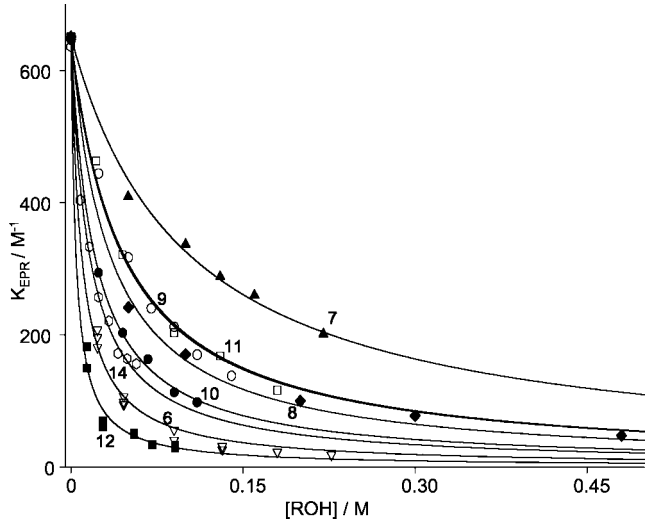
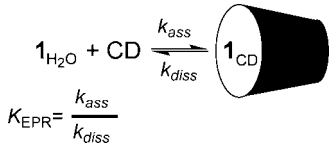


Figure 3. Variation of K_{EPR} at 318 K as a function of the concentration of different alcohols (referred to by a number) in the presence of DM-β-CD. Curves have been obtained by interpolating the experimental points, according to the competitive model (eq 7).

SCHEME 1



$K_{EPR} = f([ROH])$ according to eq 6 were found to be (i) negative (i.e., a solution without physical meaning), or (ii) negligibly small, or (iii) with an error greater than 25%, and thus completely unreliable.

The negligible presence of ternary complex in the investigated cases was confirmed by the values of hyperfine splitting constants (hfsc) of the complex, which, irrespective of the alcohol nature, are in all cases very similar to those of the complex in the absence of the alcohol (a(N) = 15.74 G, $a(2H_\beta)$ = 7.88 G, g = 2.0058). This observation does not agree with the formation of a ternary complexes in which the nitroxide probe is expected to show different spectroscopic parameters, depending on the nature of the coincluded alcohol.

Moreover, in favor of the competitive model, all of the values obtained for the formation constants of the binary complex ROH:CD (K_2), by applying eq 7 to the experimental points, were both reasonable and in good agreement with the few literature values of the same constants measured experimentally in water with other techniques[37−40] (see Supporting Information). In Table 1 are collected the values of K_1 and K_2 determined by using the competitive model. In all cases, good fitting was obtained with very good correlation coefficients ($r^2 \geq 0.98$) and experimental errors lower than 5% for K_1, and lower than 10% for K_2. For instance, in Figure 3 are shown the curves obtained by applying eq 7 to the experimental points obtained by plotting the K_{EPR} values at 318 K of the complex **1**:DM-βCD as a function of the concentration of different examined alcohols.

If the competitive model is assumed to hold and if the experimental conditions requested for its application are fullfilled ((i) the EPR spectrum of the CD complexed substrate is invariant to the presence of alcohol, and (ii) alcohol concentration is assumed to be constant and much larger than CD concentration), a very simple way to evaluate the effect of a particular alcohol on the complex cyclodextrin:substrate is to express the total amount of cyclodextrin available for substrate complexation through the following expression:[23]

$$[CD]_{free} = [1/(1 + K_2[ROH])]/[CD]_{tot} \quad (8)$$

For instance, at 298 K in the presence of ethanol, **3**, 0.05 M, 94% of DM-β-CD is available for nitroxide complexation, while in the presence of the same concentration of cyclohexanol, **12**, only 3.5% of CD is free, this emphasizing the hugely different behavior found between the various alcohols.

The competitive technique developed here can also be applied to the indirect determination of the affinity constants for CDs by any diamagnetic compounds. As examples, we choose two drugs: the anti-inflammatory ibuprofen and antiasmatic terbutalin; the stability constants at 37 °C of their complexes with β-CD, obtained by applying the competitive model to the plot $K_{EPR}^{310K} = f([drug])$, are $K_2 = 925 \pm 54$ M^{-1} ($r^2 = 0.995$) and $K_2 = 77 \pm 4$ M^{-1} ($r^2 = 0.995$), respectively.

By applying the competitive model to the plot $K_{EPR}^{298K} = f([chiral guest])$, we also evaluated, at room temperature, the ability of chiral recognition of DM-β-CD toward (*R*)- and (*S*)-1-phenylethanol ($K_2^R = 128 \pm 9$ M^{-1}, $r = 0.985$; $K_2^S = 153 \pm 12$ M^{-1}, $r^2 = 0.987$, ES = 8.9%), and of β-CD by determining its affinity for two enantiomeric couples: (D) and (L) *O*,*O*′-di-*p*-toluoyltartaric acid ($K_2^D = 266 \pm 19$ M^{-1}, $r = 0.998$; $K_2^L = 233 \pm 15$ M^{-1}, $r^2 = 0.998$, ES = 6.6%), and (D) and (L) *O*,*O*′-dibenzoyltartaric acid ($K_2^D = 68 \pm 4$ M^{-1}, $r^2 = 0.996$; $K_2^L = 44 \pm 2$ M^{-1}, $r^2 = 0.996$, ES = 21.4%). The enantioselectivities[41] of β-CD toward the examined enantiomeric couples found in the present work are in very good agreement with those reported by Inoue et al.,[42] who determined directly the stability constants of the complexes with β-CD of these couples by microcalorimetric analysis. This confirms the potential of the present methodology (the experimental data concerning the reported examples are given as Supporting Information).

Correlation between log K_2 and log P_0. The investigated alcohols (**2**−**15**) show very different efficiency in destabilizing the nitroxide:CD complexes. This behavior can be understood on the basis of the different tendency of these alcohols to be included in the CD cavity.

It is well-known that hydrophobic interactions are mostly considered responsible for the inclusion process of an organic substrate in a host system in water. Because the partition coefficient of a given organic compound is considered a practical and reliable index of the hydrophobicity of that compound, we plotted the value of log K_2 (we selected the data set reported in Table 1 relative to β-CD at 298 K) against the log P_0[43] (partition coefficient 1-octanol/water) of the given alcohol (see Figure 4). Actually, when taking into account only primary (**2**, **3**, **4**, **13**, **15**) or secondary (**5**, **6**, **7**, **11**), with R′ = CH_3, aliphatic alcohols, it is possible to fit the data with a straight line having a slope very close to 1 (log $K_2 = 0.40 + 1.17(\log P_0)$, $n = 9$, $r^2 = 0.972$).[44] The great increase of affinity for β-CD found going on from ethanol (**3**) to 1,1,1-trifluoroethanol (**4**) and even more from 2-propanol (**5**) to 1,1,1,3,3,3-hexafluoro-2-propanol (**6**) is proportional to the hydrophobicity increase of the fluorinated with respect to the nonfluorinated alcohol. Similarly, the affinity increase observed by increasing the number of carbon atoms of the linear chain (see alcohols **2**, **3**, **5**, **7**, **11**) seems directly correlated to the hydrophobicity of the substrate. A similar behavior is instead absent with branched (**8**, **9**, **10**, **15**) or cyclic (**12**) alcohols. In these cases, the alcohol affinity for β-CD cannot be explained only in terms of hydrophobic interactions, but also a strong contribution from van der Waals interactions that increase with the alcohol molecular volume must be considered.

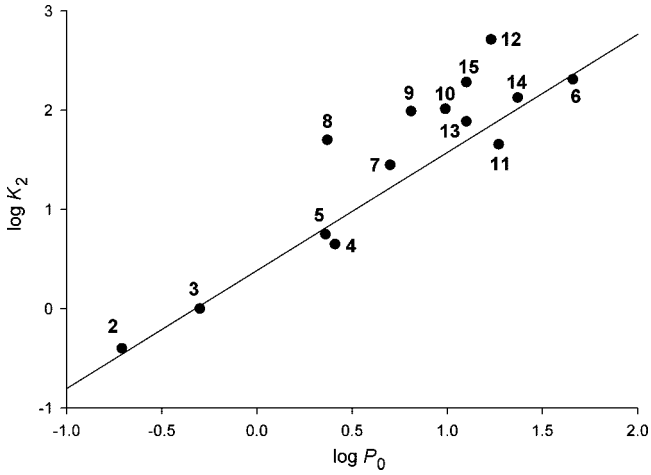


Figure 4. Plot reporting the log K_2 at 298 K value vs log P_0, where the association constant K_2 of the complex ROH:β-CD has been defined above and P_0 is the 1-octanol/water partition coefficient of the investigated alcohols. Data for **2**, **3**, and **5** are from ref 37.

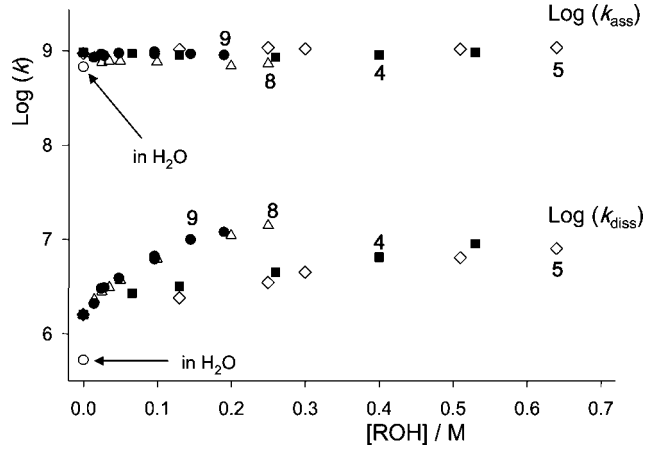


Figure 5. Variation of the log k_{ass} (association rate constants) and log k_{diss} (dissociation rate constants) values for the complex **1**:DM-β-CD at 318 K as a function of the concentration of some of the investigated alcohols. Numbers of the referred alcohols are reported close to the symbols.

For instance, it is noteworthy that structural isomers such as 2-pentanol (**11**) and *tert*-amylalcohol (**10**) or 2-butanol (**7**) and *tert*-butanol (**8**) show a great affinity difference for β-CD that can be justified on the basis of a different spatial interaction between substrate and host cavity.

Alcohol Effects on the Kinetics of Complexation. Previous literature studies on the effects induced by alcohol addition on the interactions between cyclodextrins and guest molecules deal mostly with the stability and stoichiometry of the formed complexes, while the kinetics of the association and dissociation of these complexes has been rarely investigated[22,23] despite the importance of knowing how to change the strength of association of the complex and how to modulate the complexation dynamics, to utilize CD:guest complexes to affect chemical processes, mimic enzymatic systems, and develop controlled release drugs.

By using EPR spectroscopy, which, as previously mentioned, is characterized by a time scale (microseconds or less) sufficiently short to study relatively fast processes such as the formation of complex between the radical probe and CD, we have been able to investigate the mechanism of the destabilization of the complexes **1**:CD, induced by alcohol addition, by studying the kinetics of complexation. Table 1 reports the values of the association (k_{ass}) and dissociation (k_{diss}) rates constants

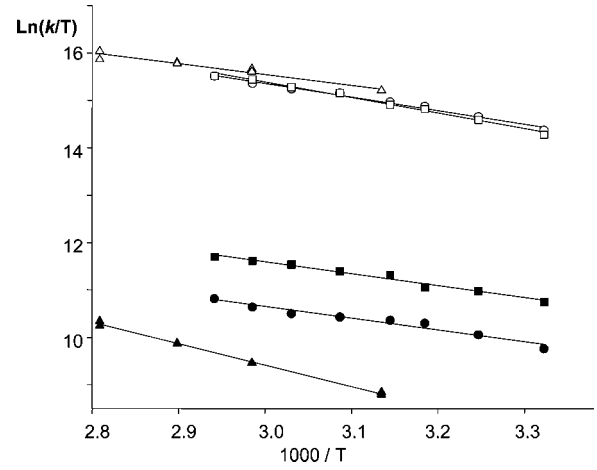


Figure 6. Plots of ln(k/T) against T^{-1} for the rate constants of association (k_{ass}, empty symbols) and dissociation (k_{diss}, full symbols) between radical **1** and DM-β-CD in the absence (triangle) and in the presence of **6** 0.046 M (circle) and 0.09 M (square).

of these complexes in the presence of different amounts of the investigated alcohols. Analysis of the data obtained by simulating the experimental spectra recorded at 318 K shows that the process of formation of the complexes is only slightly affected by the addition of alcohols. Particularly, the rate of inclusion of the nitroxide **1** in the β-CD decreases slightly, while a small increase of this rate is observed with DM-β-CD. In line with what was reported in the literature,[45,46] this result can be interpreted by admitting that alcohol association with cyclodextrins takes place only at the entry surface because the more favorable interaction geometry is that where the nonpolar tail of carbon atoms is localized inside the host cavity and the hydroxyl group sits on the border between water and cyclodextrin. This location hinders the entry of the nitroxide into the narrower rim of β-CD, while it does not preclude the entry into the larger rim of DM-β-CD, which, instead, is made easier by the elimination of water molecules and consequent increased hydrophobicity of the cavity. The effect is stronger with the larger cavity γ-CD where, in the presence of 1,1,1,3,3,3-hexafluoro-2-propanol (**6**), faster inclusion of the nitroxide takes place.

Stronger variations have been, instead, observed for the rate of complexes dissociation. All of the examined alcohols induce a significant increase of the rate of exclusion of nitroxide **1** from the various cyclodextrins depending on the concentration and structure of the given alcohol. The plots of Figure 5 show, for instance, the variation of the logarithm of the rate constants of association and dissociation of the complex **1**:DM-β-CD at 318 K as a function of the concentration of some of the investigated alcohols. The fact that the destabilization of the complexes **1**:CD in the presence of alcohols is essentially caused by the increased dissociation rate of the complexes is in agreement with the series of papers by Nishikawa et al.[47−49]on binary systems alcohol-β-CD (studied by means of ultrasonic relaxation)[50] where it is reported that the stability of alcohol:CD complexes is substantially independent of the rate of association, k_{ass}, which is more or less the same for the various alcohols, and quite similar to that determined by us for the nitroxide **1**, but depends on the rate of dissociation that decreases by increasing the lipophilicity and the spatial interactions with the internal β-CD cavity of the alcohol.

If the principal mechanism of action of alcohols is the competition with the nitroxide for the cyclodextrin cavity, it seems reasonable to admit that the ease of expulsion of the

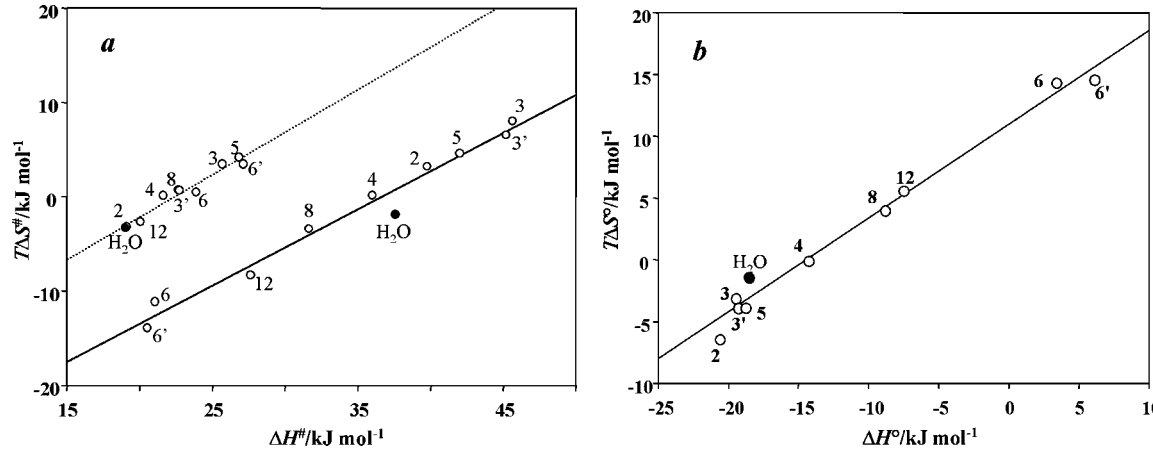


Figure 7. $T\Delta S$ against ΔH for the complexation of nitroxide **1** with DM-β-CD in water (full symbol) and in the presence of alcohols: **2** (methanol 7.41 M), **3** (ethanol 0.86 M), **3′** (ethanol 1.72 M), **4** (1,1,1-trifluoroethanol 0.33 M), **5** (isopropanol 0.44 M), **6** (1,1,1-3,3,3-hexafluoro-2-propanol 0.046 M), **6′** (1,1,1-3,3,3-hexafluoro-2-propanol 0.09 M), **8** (*tert*-butanol 0.15 M), **12** (cycloexanol 0.021 M). Thermodynamic activation parameters are reported on the left (upper and lower lines for the association and dissociation process, respectively), and equilibration parameters are on the right.

TABLE 2: Thermodynamic Parameters of Activation and of Reaction for the Complex 1:DM-β-CD in the Presence of Several Alcohols at 294 K

alcohol	$\Delta H_{ass}^{\ddagger}$/ kJ mol^{-1}	$\Delta S_{ass}^{\ddagger}$/ kJ mol^{-1} K^{-1}	$\Delta H_{diss}^{\ddagger}$/ kJ mol^{-1}	$\Delta S_{diss}^{\ddagger}$/ kJ mol^{-1} K^{-1}	K^{294K}/M^{-1}	$\Delta G°_{294K}$/ kJ mol^{-1}	$\Delta H°$/ kJ mol^{-1}	$\Delta S°$/ kJ mol^{-1} K^{-1}
none	19.02	−11.12	37.58	−6.40	1064	−17.01	−18.56	−4.72
2 (7.41 M)	19.10	−10.87	39.75	10.87	342.8	−14.25	−20.65	−21.74
3 (0.86 M)	25.67	11.79	45.19	22.28	835.0	−16.43	−19.52	−10.49
3′ (1.72 M)	22.66	2.55	42.00	15.68	573.0	−15.51	−19.35	−13.13
4 (0.33 M)	21.61	0.42	35.99	0.46	344.0	−14.30	−14.34	−0.042
5 (0.44 M)	26.84	14.21	45.64	27.25	458.6	−14.96	−18.81	−13.04
6 (0.046 M)	23.87	1.76	20.53	−47.23	95.3	−11.03	3.34	48.91
6′ (0.09 M)	27.13	11.79	21.06	−37.87	33.0	−8.53	6.06	49.74
8 (0.15 M)	22.74	2.26	31.64	−11.54	236.3	−12.96	−8.90	13.80
12 (0.02 M)	20.06	−9.07	27.63	−28.26	227.3	−13.25	−7.67	19.19

nitroxide from cyclodextrin will be greater the larger is the residence time of alcohol in the CD cavity. The study of the kinetics of complexation is therefore consistent with the formation of binary complexes ROH:CD of different stability, depending on the alcohol structure, which compete with the complexes **1**:CD, while the formation of ternary complexes ROH:CD:**1** can be rejected.

Thermodynamic Parameters for the Equilibrium Reaction and for the Activated Complex. We have also measured, for the first time, the effects of alcohol addition on the thermodynamic parameters of equilibration and of activation of inclusion complexes of cyclodextrins. This was done by recording the EPR spectra of the complex **1**:DM-β-CD at various temperatures in the presence of constant concentrations of the examined alcohols.

The rate constants of association and dissociation of the complex at the given temperatures, obtained from spectral simulation (see Supporting Information), have been analyzed to get the activation parameters $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (an example is shown in Figure 6). From these values, the thermodynamic parameters of reaction have been obtained by difference. Table 2 collects the experimental data.

To check whether the experimental results are consistent with the Inoue et al.[51] theory stating that, in the presence of supramolecular complexes, enthalpy variations ($\Delta\Delta H$) induced by any change in the host system, in the guest, or in the solvent are compensated by the corresponding entropy variation ($\Delta\Delta S$), we plotted the $T\Delta S$ against the ΔH values of activation and of reaction for the complex **1**:DM-β-CD, in the presence of the investigated alcohols. For the association (upper line in Figure 7a) and dissociation (lower line in Figure 7a) processes of the complex in the transition state, nice linear regressions ($r^2 = 0.96$, $\alpha = 0.90$, $T\Delta S = -20.21$ for association and $r^2 = 0.97$, $\alpha = 0.81$, $T\Delta S = -29.27$ for dissociation) have been obtained.

The comparison of the activation parameters for the dissociation process measured in the presence and in the absence[27] of alcohols shows two different behaviors. One, observed with methanol (**2**), ethanol (**3**, **3′**), and isopropanol (**5**), is characterized by an entropy increase compensated by an enthalpy increase: these alcohols, without being bounded to the CD, favor the dissociation of the nitroxide, thus increasing the system disorder. The second, observed with more lipophilic and sterically crowded alcohols such as *tert*-butanol (**8**), 1,1,1,3,3,3-hexafluoro-2-propanol (**6**, **6′**), and cycloexanol (**12**), is characterized by an enthalpy decrease compensated by an entropy decrease giving rise to a sort of competition effect.

In the case of the association process, on the other hand, we observed only a single behavior: the addition of alcohols to the water system produces positive variations of both enthalpy and entropy, similarly to processes dominated by the classical hydrophobic interaction.[52]

The enthalpy−entropy compensation theory can be successfully applied also to the equilibrium thermodynamic parameters because a good straight line ($r^2 = 0.98$) is obtained by linear regression of the data (see Figure 7b). Evidence that the final stability of the complex is determined by dissociation process is provided by the comparison of the thermodynamic parameters for the complex formation in the presence and in the absence[27]

of alcohols. The alcohols can be arranged in two groups on the basis of their behavior: those lying on the regression line before the point referring to pure water (full symbols in Figure 7b), such as methanol (**2**), ethanol (**3** and **3′**), and isopropanol (**5**), and those lying on that line after water, such as 1,1,1-trifluoroetanol (**4**), *tert*-butanol (**8**), 1,1,1,3,3,3-hexafluoro-2-propanol (**6** and **6′**), and cyclohexanol (**12**). The first group includes alcohols destabilizing the complex essentially by decreasing the hydrophobic driving to complexation (an entropy decrease is observed). The second group contains alcohols having a greater affinity to the CD cavity that destabilize the complex by competition with the nitroxide for the occupancy of the host as indicated by the enthalpy increase, partially counterbalanced by the entropy increase that is greater the more lipophilic is a given alcohol.

If considering the slope α of the straight line $T\Delta S^\circ = \alpha\Delta H^\circ + T\Delta S$ as an index of the conformational variation (freedom) of the host system, the rather high value ($\alpha = 0.76$) obtained might mean that the apparently rigid structure of the cyclodextrin undergoes conformational variations of the molecular skeleton, presumably due to a rearrangement occurring in the portion of the peripheral hydrogen bonds. If additionally considering that, according to the compensation theory, $\Delta\Delta G^\circ = (1 - \alpha)\Delta\Delta H^\circ$, which means that only the fraction $(1 - \alpha)$ of the enthalpy contribution affects the final stability of the complex, it is found that only 20% of the enthalpy gain or loss ($\Delta\Delta H^\circ$) induced on the system by the variations resulting from the addition of the investigated alcohols reflects on the change of the whole complex stability ($\Delta\Delta G^\circ$).

If, on the other hand, the value obtained for the intercept ($T\Delta S^\circ = 11$ kJ/mol) is attributed to desolvation of the guest resulting from the inclusion process, it can be concluded that this desolvation is quite remarkable. However, a similar entropic gain originates even from the release of water molecules originally localized inside the CD cavity and from the induced dehydration of the peripheral hydroxyl groups of the cyclodextrin. Finally, it should be emphasized that the values obtained for the slope, α, and intercept $T\Delta S^\circ$ are remarkably similar to those found by Inoue et al.,[38] $\alpha = 0.80$ and $T\Delta S^\circ = 11.0$ kJ/mol, by evaluating the complexations enthalpy and entropy variations observed in the presence of hundreds of inclusion complexes with β-CD.

This result provides additional support to the reliability of the method developed here and demonstrates the usefulness of EPR spectroscopy to investigate supramolecular species poorly stable thermodynamically and kinetically labile, resulting from weak intermolecular interactions.

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Supporting Information Available: Tables containing spectroscopic parameters, and K_{EPR}, k_{ass}, k_{diss} values for the complexes formation between **1** and CDs; plots reporting $K_{EPR} = f$([third component]) and relative curves obtained by fitting the experimental data to eq 7; plots reporting $\log k = f$([third component]) and reporting $\ln k/T = f(1/T)$, for both association and dissociation processes; table containing some literature values relative to direct measure of equilibrium constant for CD:ROH complex formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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(52) In the absence of alcohols, the various factors governing the magnitude of the entropy of activation for guest complexation by CD are rather difficult to visualize, because $\Delta S_{ass}^{\ddagger}$ and $\Delta S_{diss}^{\ddagger}$ are related to the change in randomness on passing from reactants or complex, respectively, to the transition state. In general, the following four terms should contribute to the activation entropy: (a) the freezing of motional degrees of freedom for the guest molecule, (b) the desolvation around the guest molecule and the reorganization of solvent water, (c) the release of water molecules from the CD cavity, and (d) any conformational changes of the CD torus. While the first term will be negative, the other ones are expected to contribute positively to the activation entropy. In the presence of alcohol, the picture is even more complicated, because the inclusion and the release of alcohol molecules by CD can significantly modify this picture. For this reason, we preferred to omit any detailed discussion on the activation entropies.

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