JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Complexation of Several Benzimidazole-Type Fungicides with α- and β-Cyclodextrins

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The potential increase in water solubility of three benzimidazole-type fungicides (thiabendazole, carbendazim, and fuberidazole) due to complexation with α- and β-cyclodextrins was investigated. Fluorescence emission spectra of the fungicides in the presence of different concentrations of the cyclodextrins were measured. Analysis of these spectra by the method of principal components global analysis (PCGA) yielded precise values for the association constants and the emission spectra of the fungicide−cyclodextrin inclusion complexes. Phase-solubility diagrams confirmed the formation of inclusion complexes between each of the fungicides and β-cyclodextrin and showed significant increases of their solubilities due to complexation.

KEYWORDS: Cyclodextrins; fungicides; inclusion complexes; principal components analysis

INTRODUCTION

Cyclodextrins (CDs) are toroidally shaped polysaccharides with highly hydrophobic central cavities. CDs have the ability to form inclusion complexes with a variety of organic and inorganic substrates. As a result of complex formation, the physicochemical properties of the guest molecule can change significantly, and that is the basis of the widespread industrial use of cyclodextrins. In the pharmaceutical industry, cyclodextrins are frequently used to enhance the bioavailability of poorly soluble drugs, because complexation causes, in most cases, an increase of the water solubility of the guest molecule (*1*). The same effect has been demonstrated with other drugs of interest in the agriculture industry (*2*).

In this work three widely used fungicides derived from benzimidazole are investigated: thiabendazole, carbendazim, and fuberidazole (Figure 1). Their low water solubility is the main limit for their fungicide action, and it could be increased by complexation with cyclodextrins. Biocide−cyclodextrin interaction has been demonstrated for some benzimidazole-like fungicides as benomyl (*3*), carbendazim (*4*), and thiabendazole (*5*). The interaction leads in all cases to an increase in fungicide solubility (*3*, *4*). With the exception of carbendazim, for which a value for the equilibrium constant of the complex formation with β-CD has recently been obtained (*6*), the stability and structure of these fungicide−cyclodextrin complexes is still unknown. The stability can be measured through the dissociation constant, which is the inverse of the formation equilibrium constant. The knowledge of these constants is important as it allows calculation of the fungicide solubility at any given concentrations of guest and host, and that helps in designing a drug formulation.

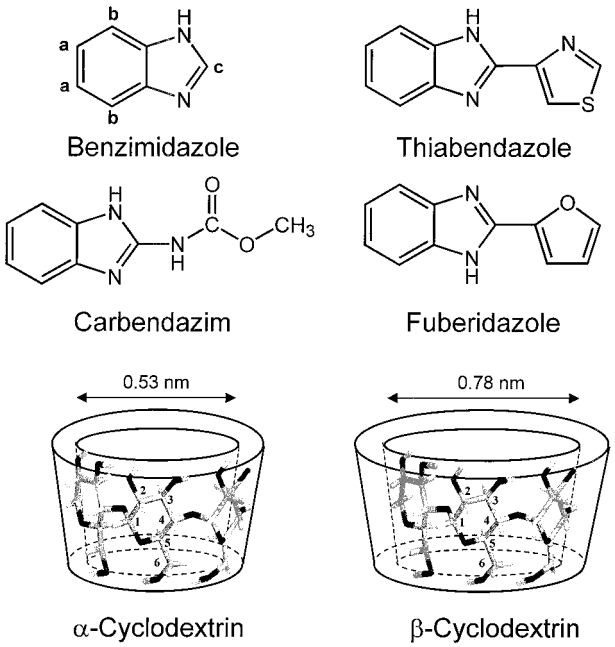

Figure 1. Structures of guest and host molecules used in this study.

In this work steady-state fluorescence spectroscopy was used because of its high sensitivity, and because all three fungicides under study show significant fluorescence. Solubility isotherms were also measured. With the aim of comparison, the complexation of benzimidazole by cyclodextrins was also studied by NMR techniques, which were feasible in this case because of the higher solubility of benzimidazole. The naturally occurring α- and β-cyclodextrins (α-CD and β-CD, respectively) were

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10.1021/jf010927y CCC: $22.00
Published on Web 12/04/2001

used in this work because the sizes of their cavities fit the molecules used as guests (Figure 1).

A powerful method, principal components global analysis (PCGA) (*7*), was used for the analysis of the fluorescence experimental data. This method allows us to obtain the emission spectra of the fungicide−cyclodextrin complexes, as well as more accurate values of the association constants than with conventional analysis, even if the spectral variations are small.

MATERIALS AND METHODS

Materials. Fuberidazole (Riedel-de Haën) was used without further purification. Thiabendazole and benzimidazole were purchased from Sigma and purified by recrystallization from ethanol and water, respectively. Carbendazim (Aldrich) was recrystallized three times from ethanol with active carbon. The β-CD used in this work was kindly supplied by Roquette, and the α-CD was purchased from Wacker. To be used in fluorescence measurements, both cyclodextrins were recrystallized several times from ethanol and acetone in order to remove fluorescent impurities which were still present after recrystallization from water. Purity was checked by measuring the fluorescence emission spectra of aqueous solutions of these cyclodextrins.

Fluorescence Measurements. Stock solutions of the fungicides in the range from 10^{-4} mol dm^{-3} to 10^{-5} mol dm^{-3} were prepared and kept protected from light. Because of its low water solubility, stock solutions of β-CD had a maximal concentration of 0.0120 mol dm^{-3}, whereas a 10× higher concentration was used for α-CD. All stock solutions were used within one week after their preparation. From these stock solutions samples were prepared just before the measurement of fluorescence. The fungicide concentration in these solutions was 6.0×10^{-6} mol dm^{-3} for thiabendazol, 1.1×10^{-5} mol dm^{-3} for carbendazim, and 1.9×10^{-7} mol dm^{-3} for fuberidazole. Cyclodextrin concentration was varied in the range from 4.0 to 92 mM in the case of α-CD, and from 0.6 to 9.6 mM in the case of β-CD. The pH of these solutions was adjusted to a constant value of 7.0 ± 0.1 by adding a phosphate buffer (total phosphate concentration = 0.020 mol dm^{-3}).

Steady-state fluorescence measurements were performed with an Edinburgh-Instruments F900 spectrofluorimeter, equipped with a Xenon lamp of 450 W as excitation source, and with a SPEX Fluoromax spectrofluorimeter. Series of emission spectra of samples with the same fungicide concentration and varying concentrations of CD were measured using suitable excitation wavelengths at a constant temperature of 25 °C. All emission spectra were corrected for the wavelength dependence of the detection system.

Phase-Solubility Diagrams. Samples were prepared by adding an excess of the solid fungicide and the same volumes of aqueous solutions of β-CD of different concentrations. The samples were kept with constant stirring at 25 °C for one week in order to achieve solubility equilibrium. The supernatants were then separated and, after 10× dilution, their absorption spectra were measured. Using a previously measured calibration curve, the concentration of fungicide−CD complex was determined for each sample. For each guest a calibration curve was determined by measuring the absorption spectra of aqueous solutions of different fungicide concentrations. The use of such a calibration curve to determine the concentration of complex involves the assumption that the absorption spectrum of the fungicide does not change by complexation. This was checked by comparing the absorption spectra of each pure fungicide and the fungicide in the presence of 0.010 M β-CD.

NMR Measurements. ^{13}C NMR spectra were recorded in a Bruker AC spectrometer at 300 MHz for mixtures of benzimidazole and β-CD prepared after the continuous variation method, i.e., constant sum of host and guest concentrations. Rotating-frame Overhauser effect spectroscopy (ROESY) experiments were recorded in a Bruker AMX spectrometer at 500 MHz. Samples for ROESY measurements had equal concentrations of both host and guest (10 mM) and were protected from light. The mixing time for the ROESY experiments was 300 ms. All NMR experiments were carried out in D_2O at 25 °C.

Data Analysis. Fluorescence data were analyzed by the principal components global analysis (PCGA) method (*7*). This method combines the determination of the minimal number of spectral components necessary to explain the variations of the emission spectra with CD concentration (principal components analysis) with the determination of the component-associated spectra and the parameters of the proposed model by nonlinear global analysis. Linear fits for the solubility experimental data were performed with the commercial program Origin 6.0 Professional (*8*).

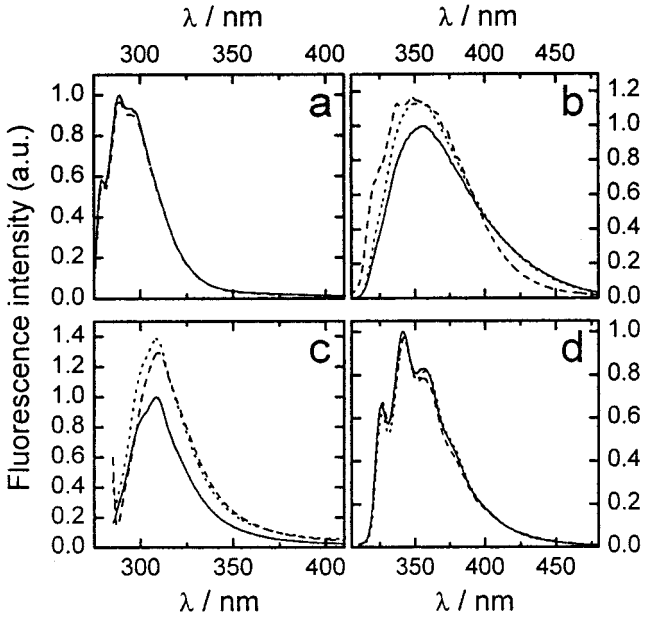

Figure 2. Emission fluorescence spectra of the guest molecules under study in water (–), in 9.6 mM β-CD aqueous solutions (- - -), and in 24 mM α-CD aqueous solutions (···), at 25 °C. (a) Benzimidazole (λ_{exc} = 270 nm); (b) thiabendazole (λ_{exc} = 290 nm); (c) carbendazim (λ_{exc} = 280 nm); and (d) fuberidazole (λ_{exc} = 305 nm). Spectra in pure water were normalized at the maximum to facilitate comparison among the different guest molecules.

RESULTS

Figure 2 shows the variation of the fluorescence emission spectra of benzimidazole and the three fungicides by addition of α-CD and β-CD. The spectrum of benzimidazole does not change at all by addition of 28 mM α-CD, and changes only slightly by addition of 9.6 mM β-CD (Figure 2a). On the contrary, addition of similar CD concentrations causes a significant increase of the fluorescence intensity for thiabendazole and carbendazim (Figures 2b and 2c). A blue shift of the carbendazim emission spectrum is also observed. In the case of fuberidazole, the fluorescence intensity decreases slightly by addition of both CDs and the spectrum shifts to longer wavelengths with α-CD (Figure 2d).

The dependency of fluorescence intensity on CD concentration at certain selected wavelengths is shown in Figure 3 for the three fungicides under study. To facilitate comparison, fluorescence intensities were normalized to unity at zero CD concentration. Similar dependencies are observed for thiabendazole and carbendazim with β-CD, whereas the increase of fluorescence intensity due to α-CD is greater in carbendazim. In the case of fuberidazole, the fluorescence intensity decreases with increasing CD concentration and the effect of the CDs is much smaller.

Phase-solubility diagrams, i.e., plots of total concentration of dissolved fungicide (total fungicide solubility, S_t) versus total CD concentration, were determined for the three fungicides with β-CD (Figure 4). All three diagrams show linear increase of the fungicide solubility with increasing CD concentration,

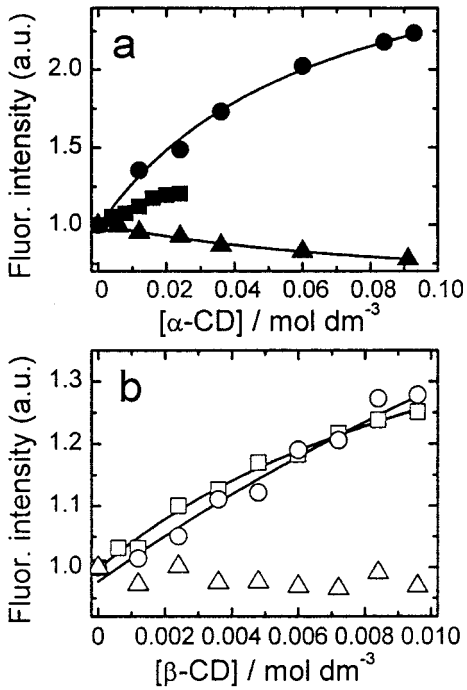

Figure 3. Fluorescence intensities of the three fungicides under study versus CD concentration at 25 °C. (a) Experimental data for α-CD: ■, thiabendazole (λ_{em} = 340 nm); ●, carbendazim (λ_{em} = 320 nm); and ▲, fuberidazole (λ_{em} = 340 nm). (b) Experimental data for β-CD: □, thiabendazole (λ_{em} = 337 nm); ○, carbendazim (λ_{em} = 310 nm); and △, fuberidazole (λ_{em} = 355 nm). Lines are the curves fitted to the experimental data (see text).

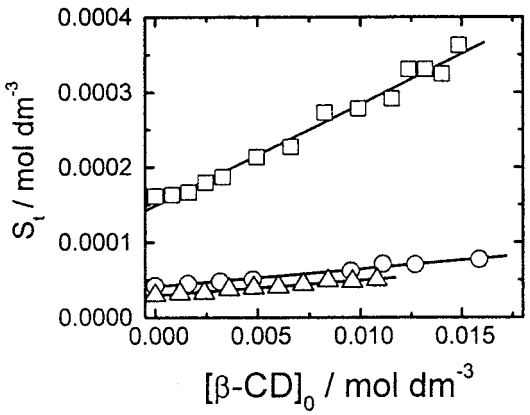

Figure 4. Phase-solubility diagrams for thiabendazole (□), carbendazim (○), and fuberidazole (△) with β-CD at 25 °C. Lines are the results of the linear regressions to the experimental data.

corresponding to type A_L diagram following the classification of Higuchi and Connors (*9*). For one and the same CD concentration the increase in solubility is greater for thiabendazole than for carbendazim and fuberidazole.

NMR measurements were only practicable with benzimidazole, as the fungicides are not soluble enough for NMR experiments. ROESY experiments showed no interactions between benzimidazole and α-CD protons. On the contrary, significant interactions of the aromatic benzimidazole protons with some of the β-CD protons were observed (Table 1). The increasing displacement of the peaks in the ^{13}C NMR spectra (chemical shift displacement, $\Delta\delta_C$) as β-CD concentration is increased also demonstrates the existence of interactions between benzimidazole and β-CD. Figure 5a shows the chemical shift displacements of the three cyclodextrin carbon atoms with the

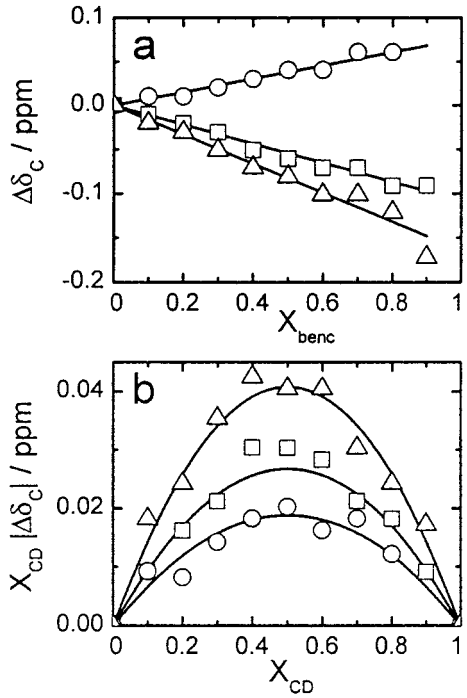

Figure 5. (a) Variations of the chemical shift displacements of carbons C4 (□), C5 (○), and C6 (△) of β-CD with molar fraction of benzimidazole. (b) Job plots corresponding to the data in figure (a). Lines are the curves fitted to the experimental data (see text).

Table 1. Cross Peaks Observed between the β-CD Protons Facing the Inner Cavity and Benzimidazole Protons, as Obtained in ROESY Experiments. Strength of Interaction Is Represented as Follows: ×, Weak; ××, Medium; ×××, Strong

	β-CD		
benzimidazole	H-3	H-5	H-6
H-a	×××	××	×
H-b	×××	××	×

largest displacements (see Figure 1 for location of these carbon atoms). The corresponding Job plots (*10*) are shown in Figure 5b.

DISCUSSION

Except for benzimidazole with α-CD, the emission spectra of all four compounds under study show more or less significant variations when α-CD or β-CD are added (Figure 2), which may be due to the formation of inclusion complexes. In the case of benzimidazole with β-CD, the change in the emission spectrum is too small to be quantified as a function of CD concentration. Therefore their possible complex formation was studied by NMR techniques and will be discussed later. The variation of the emission spectrum of fuberidazole by addition of α-CD or β-CD is also very small, but it is large enough to be detected and analyzed with PCGA. This method is specially suitable to analyze such small variations as it provides objective criteria to decide whether the spectral changes are due to complexation or to other effects, such as variation of the refraction index or other properties of the solvent.

In a first step of PCGA analysis the minimal number of spectral components (i.e, number of spectra whose linear combinations reproduce the systematic change in the experimental spectra) were determined for each series of data. Two spectral components were found to explain satisfactorily each series of experimental spectra except in the case of fuberidazole

Table 2. Values of the Association Constants of the Three Fungicides under Study with α-CD and β-CD Obtained by PCGA Analysis of Fluorescence Data and from Phase-Solubility Diagrams

	fluorescence (PCGA)		solubility
K_1/mol^{-1} dm^3	α-CD	β-CD	β-CD
carbendazim	14.2 ± 0.2	22.7 ± 2.6	57 ± 4
thiabendazole	14.7 ± 1.3	94.3 ± 2.5	92 ± 4
fuberidazole	16.3 ± 0.8	- -	70 ± 4

with β-CD, where a second component is not necessary and the variations observed are within the experimental error. The two spectral components found can be assigned to two fluorescent species present in each system, free and complexed fungicide, whose emission spectra differ to some extent. In the case of fuberidazole with β-CD two interpretations are possible: (i) no complexation takes place and the emission is only due to free fuberidazole, and (ii) the emission spectrum of fuberidazole is not sensitive to the change in environment due to inclusion into a β-CD molecule. The second hypothesis seems plausible taking into account the lack of sensitivity of fuberidazole to solvent polarity (*11*), and is supported by the results of the phase-solubility diagrams, as discussed later.

The second step in PCGA analysis is to determine the spectra of the components on the basis of a theoretical model which must describe the dependence of the fluorescence intensity on CD concentration. Assuming that the two spectral components correspond to free (FU) and complexed fungicide (C11), the following equilibrium must be considered:

$$\mathrm{FU} + \mathrm{CD} \overset{K_1}{\rightleftharpoons} \mathrm{C11} \tag{1}$$

where K_1 is the association equilibrium constant, which is defined as a function of the equilibrium concentrations of the three species:

$$K_1 = \frac{[\mathrm{C11}]}{[\mathrm{FU}][\mathrm{CD}]} \tag{2}$$

In the absence of association or dissociation processes in the singlet excited state, the measured fluorescence intensity at any wavelength (F^λ) is the sum of the contributions of the fluorescent species FU and C11, which are proportional to the ground-state equilibrium concentrations of these species. Under conditions of excess CD concentration ([CD] ≃ $[CD]_0$), the following equation is obtained which relates F^λ with the initial CD concentration $[CD]_0$:

$$F^\lambda = \frac{F^\lambda_{\mathrm{FU}} + F^\lambda_{\mathrm{C11}} K_1 [\mathrm{CD}]_0}{1 + K_1 [\mathrm{CD}]_0} \tag{3}$$

where F^λ_{FU} and F^λ_{C11} are the fluorescence intensities at any wavelength for free and complexed fungicide, respectively.

On the basis of this model, represented by eq 3, PCGA analysis was performed for each series of emission spectra, except that of fuberidazole with β-CD. Good fits were obtained in all cases, proving that the proposed model of 1:1 fungicide−cyclodextrin complexation can satisfactorily explain the experimental data. Precise values were obtained for the association equilibrium constants (Table 2), that for carbendazim with β-CD being in good agreement with the value reported in the literature (*6*). Very low values of K_1 are obtained for the complexes of all three fungicides with α-CD, indicating that host−guest interactions are weak in these complexes. For the complexes

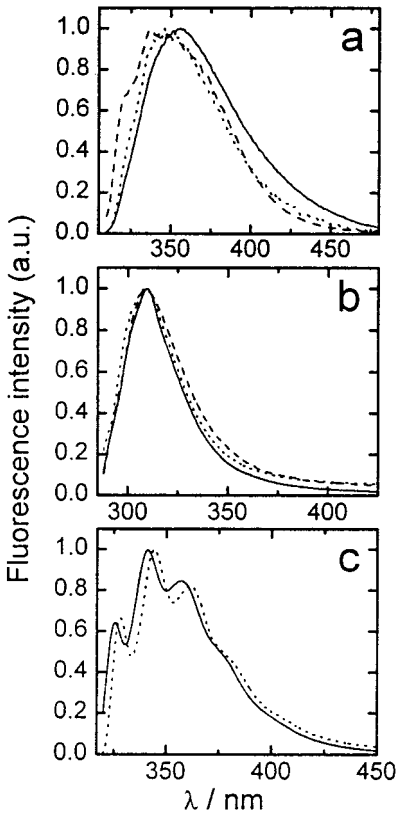

Figure 6. Emission fluorescence spectra determined by PCGA for free fungicides (–), fungicide–β-CD complexes (- - -), and fungicide–α-CD complexes (•••). (a) Thiabendazole, (b) carbendazim, and (c) fuberidazole. All spectra were normalized at the maximum to facilitate comparison (see relative fluorescence quantum yields in text).

with β-CD greater values of the association constants are obtained, but the strength of the host−guest interactions is still small. Only in the case of thiabendazole with β-CD does complexation occur in a greater extent.

PCGA analysis also yields the emission spectra of the *pure* fluorescent species, free fungicide and fungicide−CD complex (Figure 6). The emission spectra obtained for the free fungicides coincide perfectly with those measured experimentally, proving the validity of the method. The emission spectra of the thiabendazole−CD complexes are blue-shifted with respect to free thiabendazole, whereas no shift is observed for carbendazim complexes. For these two fungicides there is a significant increase of the fluorescence quantum yield due to complexation, which can be quantified by the ratio of areas under the emission spectra of complexed and free fungicide. In the case of thiabendazole, this ratio is 1.4 for the complex with α-CD and 1.3 for the complex with β-CD, indicating a significant enhancement of thiabendazole fluorescence by complexation. The effect is much more important for carbendazim, whose fluorescence quantum yield increases 3.3 and 3.5 times by complexation with α-CD and β-CD, respectively. On the contrary, the fluorescence quantum yield of the fuberidazole−α-CD complex is 0.7 times lower than that of free fuberidazole. Although enhancement of fluorescence is the general feature for most CD-complexed fluorophores, quenching of fluorescence has also been observed for some molecules in which specific interactions with the glycosidic oxygens of the cavity take place (*12*).

Further information about the potential increase of water solubility by complexation of the fungicides with β-CD was obtained from the corresponding phase-solubility diagrams. For

all three fungicides type A_L diagrams were obtained that correspond to the formation of soluble complexes of first order in CD (*9*). If the complexes are also of first order in fungicide, as derived from fluorescence studies, then the total solubility (S_t) varies linearly with the total CD concentration following the equation:

$$S_t = S_0 + \frac{K_1 S_0}{1 + K_1 S_0}[CD]_0 \quad (4)$$

where S_0 is the equilibrium fungicide solubility in the absence of CD (*9*). From the values of intercept and slope of the straight lines fitted to the experimental data (Figure 4), the association constant, K_1 can be estimated. For the three fungicides under study with β-CD, the values of K_1 obtained in this way are listed in Table 2. The most interesting result is the evidence that complexation of fuberidazole with β-CD takes place, with an association constant which is of the same order of magnitude as for the other two fungicides. Therefore, the fact that the emission spectrum of fuberidazole does not change in the presence of β-CD must be attributed to the lack of sensitivity of fuberidazole fluorescence to different environments. In the case of thiabendazole the value of K_1 is in very good agreement with that obtained from fluorescence measurements, whereas for carbendazim the two values agree only in order of magnitude. It must be noted that, due to the approximations involved, the values derived for the association constants from phase-solubility diagrams must be taken as rough estimations (*9*).

From the phase-solubility diagrams the increase in water solubility of the fungicides can also be estimated. For example, in the presence of a solution 0.010 mol dm^{-3} of β-CD, the relative increase of water solubility is 91%, 57%, and 69% for thiabendazole, carbendazim, and fuberidazole, respectively. The corresponding increase of the biological effect of these fungicides may be important for some applications. Moreover, since synergistic activity of the cyclodextrins could take place, as shown for the precursor of carbendazim called benomyl (*3*), the enhancement of biological activity may be even greater.

Finally, to give a tentative interpretation for the site of complexation of the fungicides by the CDs, the NMR data for benzimidazole will be considered. No interactions are observed between benzimidazole protons and α-CD protons, indicating that no complexation takes place. On the contrary, ROESY experiments for benzimidazole with β-CD show significant interactions among the protons of both molecules. These interactions are specially important between the benzimidazole protons H-a and H-b and protons H-3 and H-5 of β-CD (see Table 1 and Figure 1 for atom numeration), indicating that the benzene ring of benzimidazole penetrates into the wider part of the CD cavity. Furthermore, the chemical shift displacements measured for the β-CD carbons as a function of β-CD concentration (Figure 5) can be explained satisfactorily by the formation of a 1:1 (guest−host) inclusion complex. The global nonlinear least-squares fit of the corresponding equation (*13*) to these experimental data yields a value of about 16 mol^{-1} dm^3 for the association constant of the complex benzimidazole−β-CD. (Note that this value has a very large error due to the strong correlation among the fit parameters−association constant and maximal chemical shift displacements or displacements of the NMR peak when all the guest molecules are complexed.) This is a small value in comparison with those obtained for the fungicides with β-CD. This, together with the slight variation of benzimidazole emission spectrum by complexation, suggest that the complexation of the fungicides involves the moeity in position 2 of benzimidazole and not the benzimidazole ring. Also, the complexes with α-CD are probably formed by inclusion of the different substituents moieties, because the benzimidazole moiety does not penetrate into the α-CD cavity.

ABBREVIATIONS USED

CD, cyclodextrin; α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; ROESY, rotating-frame Overhauser effect spectroscopy; PCGA, principal components global analysis.

ACKNOWLEDGMENT

The authors thank Prof. M. Mosquera for making it possible to perform some of the fluorescence measurements at his laboratory.

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Received for review July 17, 2001. Revised manuscript received October 22, 2001. Accepted October 22, 2001. This work was supported by the Xunta de Galicia (Project XUGA PGIDT99PXI26201B) and by CYTED (Project VIII.3).

JF010927Y