γ-Cyclodextrin Inclusion Complex of a New Organophosphorus Insecticide. Determination of Stability Constant with HPLC

Yannis L. Loukas,† Ekaterini Antoniadou-Vyza,*,† Aspasia Papadaki-Valiraki,† and Kiriaki G. Machera‡

Department of Pharmacy, Division of Pharmaceutical Chemistry, University of Athens, Panepistimioupoli Zografou 15710, P.O. Box 64064, Athens, Greece, and Laboratory of Pesticide Toxicology, Benaki Phytopathological Institute, 8 Stephanou Delta Street, Kifissia 14561, Athens, Greece

The effect of γ-cyclodextrin (γCD) as a complexing agent with a new insecticide (DCPE) has been examined. The liquid DCPE was transformed to a white powder form, which gives the opportunity to formulate the complex as a stabilized dust. In γCD complex form, DCPE is "packed" into a hydrophilic cover, so that its affinity to hydrophobic surfaces is decreased and therefore its dermal absorption during application reduced. The stoichiometry coefficient of the inclusion complex is found to be 1:1. Differential scanning calorimetry and X-ray powder diffraction analysis have been used to confirm the inclusion in solid state. ^{1}H NMR has been used for proving the complexation in aqueous solution. γCD has a stabilizing effect on DCPE in solutions. In the presence of 2.8×10^{-3} M γCD there is a 2-fold increase in the stability. The association constant of the DCPE/γCD complex was determined kinetically.

INTRODUCTION

The applications of cyclodextrins (CDs) are based on their ability to form inclusion complexes with a variety of compounds. The new low-cost production of γCD (Duchene, 1991a) comparable to that of βCD encourages the use of this molecule as a complexing agent. The properties of γCD are superior to those of α- and β-cyclodextrins: it has the highest water solubility (23.5% at 25 °C), and its cavity is larger than that of α- and β-cyclodextrins, allowing the complexation of molecules which are not—or poorly—complexed by α- and β-cyclodextrins. Pesticides can be complexed with CDs as well as drug molecules or food flavors (Szejtli, 1988).

The complexation of pesticides may result in considerable improvement of their properties (Szejtli, 1988; Jones et al., 1984), namely enhancement of solubility and bioavailability, increase in stability of volatile and/or unstable pesticides, diminution of unpleasant taste and odor, and reduction of contact toxicity to humans.

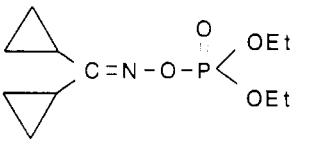
α-(Diethoxyphosphinoximino)dicyclopropylmethane (DCPE) (Chart 1) is a very active organophosphorus insecticide, prepared in our laboratory (Kostakis and Rouman, 1984). It is a yellow oily liquid that is unstable in aqueous media and poorly soluble in water, which could be used as an "emulsifiable concentrate". In this study an attempt to develop better formulation and application methods of this insecticide has been made by complexation with γCD. Better handling, better storage stability, and less contact toxicity to humans were also aims of this complexation.

EXPERIMENTAL PROCEDURES

Materials. DCPE was synthesized in our laboratory; γCD was obtained from Sigma Chemical Co. (St. Louis, MO). Acetonitrile was of HPLC grade, and water was deionized and double distilled by a Millipore MilliQ Plus system.

Preparation of Complex. The inclusion complex was prepared (Camileri and Boocock, 1985) by the coprecipitation method. Three grams of γCD (2.3 mmol, MW γCD = 1297) was dissolved in 15 mL of water. The solution was slowly treated by

Chart 1



stirring with an excess of DCPE (3 g or 11.5 mmol, MW DCPE = 262), which was added dropwise. The mixture was stirred for 1 h in ambient temperature, cooled in an ice bath, and stirred for a further 3 h. The white precipitate formed was filtered off and washed with ethyl ether (3 × 5 mL) to remove the uncomplexed DCPE and the decomposition product of DCPE [*N*-cyclopropyl-cyclopropylcarboxamide (**3**), Scheme 1]. Finally, the complex was dried in a desiccator in vacuum in the presence of P_2O_5 to give a yield of 2.4 g of DCPE/γCD complex.

Nuclear Magnetic Resonance Spectroscopy. In aqueous solution, the structure of the complex was investigated by ^{1}H NMR spectroscopy. NMR spectra were recorded on a Bruker AC 200 spectrometer in 99.9% D_2O (Sigma). The probe temperature was regulated to 293 K, and the concentrations were 4 mM. All chemical shifts were related to external standard TMS peak at 0 ppm, which was used as a separate sample. Typical conditions were as follows: 16K data points with zero filling; sweep width, 1.4 kHz, giving a digital resolution of 0.17 Hz/point; pulse width, 2 μs (90° pulse, 5.5 μs); acquisition time, 2.9 s. Gaussian enhancement was used for the displayed spectra (GB = 0.2, LB = −2).

X-ray Powder Diffraction. The identification (evidence) of the complex in the solid state was first confirmed with X-ray powder diffraction. X-ray diffraction photographs of powdered γCD and DCPE/γCD complex were taken on a Debye Sherrer camera with Cu Kα radiation. DCPE by itself is a liquid guest and does not produce any diffraction patterns.

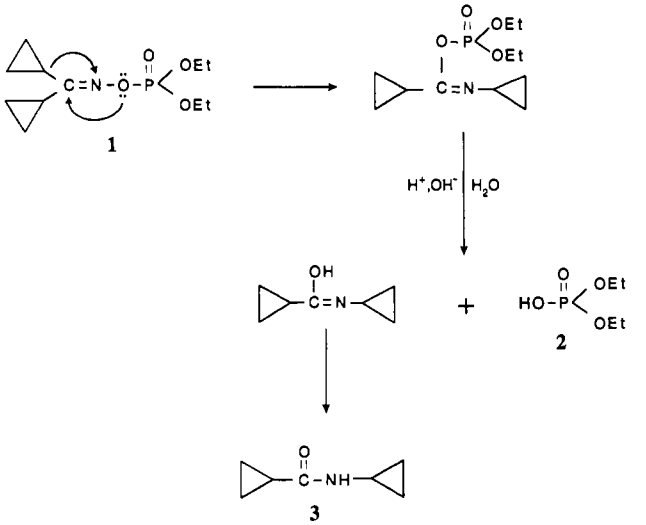
Differential Scanning Calorimetry (DSC). Thermograms were obtained using a Perkin-Elmer DSC 7 differential scanning calorimeter, using vented aluminum pans. Typical conditions were as follows: temperature range, 50–250 °C; scanning rate, 10 °C/min; sample weight, 3–10 mg. Baseline optimization was performed before each run.

High-Performance Liquid Chromatography. All HPLC assays for DCPE were performed on a Waters chromatographic system (Waters Associates, Milford, MA), consisting of a pump (Model 590) and a multiwavelength UV detector (Lamda-Max, Model 481). The separation of DCPE from its degradation products was performed on a reversed-phase, 10-μm μ-Bondapak C_{18} column, 300 × 3.9 mm (Waters). The mobile phase consisted

† University of Athens.
‡ Benaki Phytopathological Institute.

0021-8561/94/1442-0944$04.50/0

Scheme 1



of acetonitrile–water (55:45) vacuum filtered through a 0.45-μm-pore nylon membrane filter (Sartolon, GmbH, Germany) prior to use. A flow rate of 1.5 mL/min was maintained, and the effluent was monitored at 215 nm with a detector sensitivity of 0.05 AUFS. The injection volume was 20 μL (Rheodyne 717S fitted with a 20-μL loop). Quantitation was performed by peak height integration (Waters, Baseline 810 integration system for personal computer).

Kinetics. All kinetics studies were performed on DCPE in aqueous solutions containing various concentrations of γCD. The reactions were initiated by the addition of 1 mL of aqueous solution of DCPE into 10-mL volumetric flasks. The flasks were brought to 10 mL with an aqueous solution of γCD to give an initial DCPE concentration of 7×10^{-2} mM. At appropriate intervals, samples were taken and analyzed for remaining DCPE by the HPLC assay described above. First-order rate constants for the disappearance of DCPE (k_{obs}) were determined from the slopes of linear plots of the logarithm of the percentage of remaining DCPE against time. The degradation pathway of DCPE in aqueous media is shown in Scheme 1. The conversion to the corresponding amide **3** was due to a Beckmann rearrangement (Donamura and Heldt, 1960).

RESULTS AND DISCUSSION

Characterization of the Inclusion Complex in the Solid State. Differential scanning calorimetry (DSC) indicated true complexation (Ford and Timmins, 1989). Thus, in all cases the DSC thermogram of the complex was different in the number and/or position of peaks, compared to the DSC curves of both a mechanical mixture of DCPE/γCD and pure γCD. Figure 1 illustrates the DSC curves of the solid complex, pure γCD, and mechanical mixture of DCPE and γCD.

A more reliable and widely used method for the characterization of complexes in the solid state is the method of X-ray diffractometry of powdered samples (Szejtli, 1982a). The diffraction diagrams showed that the reflection pattern of DCPE/γCD complex was dramatically different from that of the parent γCD. The fact that the guest was liquid simplified the qualitative characterization of the complex by this method. Figure 2 illustrates the diagrams of γCD and DCPE/γCD complex.

Characterization of the Complex in Solution. Proton NMR spectroscopy is commonly used for the investigation of the nature of the complexes in solution (Duchene, 1991). For our soluble complex the observed resonances were the time-averaged peaks of pure and complexed compounds (fast exchange regime on the NMR time scale at 293 °C). The inclusion of the DCPE in aqueous solutions is evidenced by the modification of the

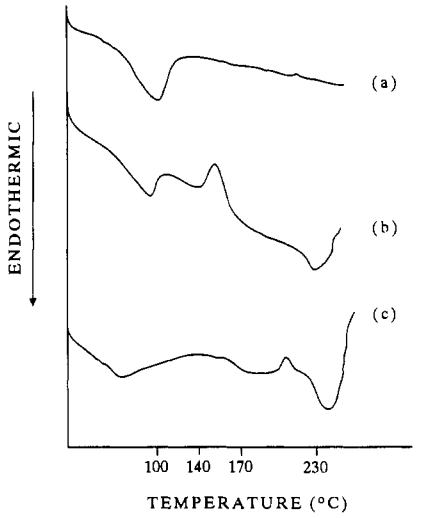


Figure 1. DSC curves of (a) γCD, (b) mechanical mixture, and (c) DCPE/γCD complex.

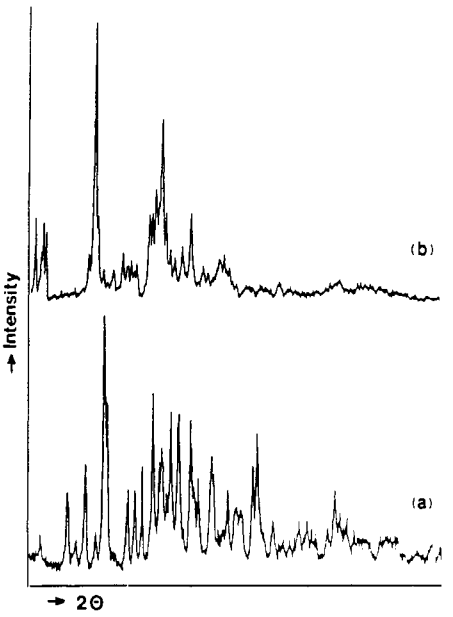


Figure 2. X-ray powder diffraction diagrams of (a) γCD and (b) DCPE/γCD complex.

NMR spectra of both the guest and host molecules. The partial 200-MHz NMR spectra of the pure compounds and of the complex are displayed in Figure 3. Under the present conditions, only shift changes of the signals were observed. No new peaks appeared that could be assigned to the pure complex. This observation implies that the complexation is a dynamic process, the included guest being in fast exchange (relative to the NMR time scale) between the free and bonded states.

γCD has a torus shape formed by eight glucose units, with a cavity having a diameter of about 8 Å, which can accommodate a guest molecule without covalent bonding. Protons located inside the cavity (H-3 and H-5) are expected to experience large chemical shift variations upon inclusion of the guest, whereas the ones residing outside (H-1, H-2, and H-4) should undergo minimal changes (Figure 3). Specifically, the ^{1}H NMR spectrum of the complex γCD/DCPE in D_2O showed upfield shifts of H-3 and H-5 (Table 1), whereas the frequencies of H-1, H-2, and H-4 were not altered. Meanwhile, the induced chemical shifts of the peaks due to methylene and methyl groups of DCPE, which are centered at 4.23 and 1.36 ppm, respectively, undergo 0.08 and 0.13 ppm downfield shifts,

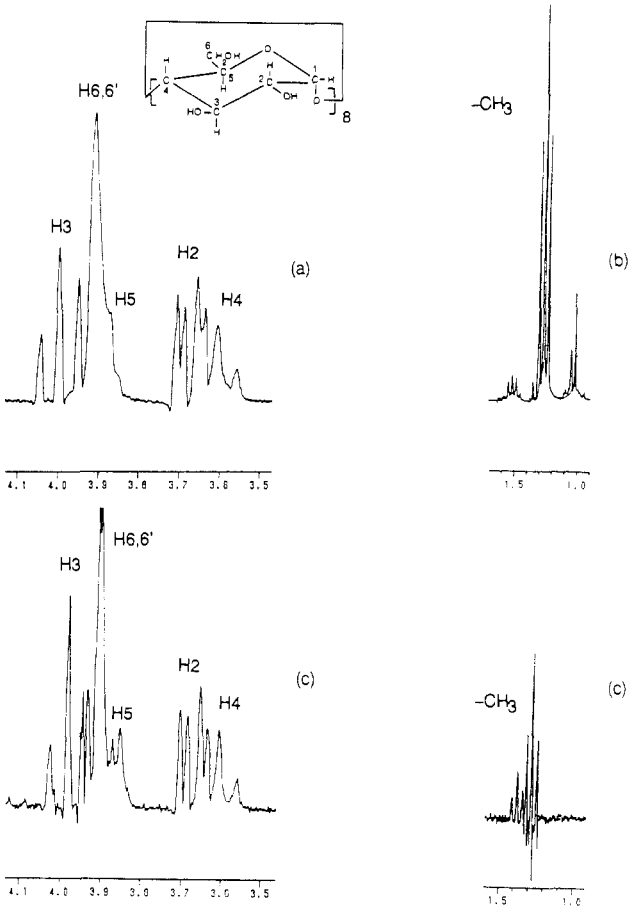


Figure 3. Partial 200-MHz ^{1}H NMR spectra of 4 mM solutions of (a) γCD, (b) DCPE, and (c) DCPE/γCD complex in D_2O at 293 K. The chemical shift at 1.05 ppm corresponds to the cyclopropyl protons of amide **3** (Scheme 1).

Table 1. Chemical Shifts of the Protons of γCD and DCPE in the Free and Complex States

proton	δ_0 (free)	δ_c (complex)	$\Delta\delta$ ($\delta_c - \delta_0$)
γCD			
H1	5.125	5.116	−0.009
H2	3.665	3.657	−0.008
H3	3.953	3.931	−0.022
H4	3.603	3.595	−0.008
H5	3.846	3.825	−0.021
H6	3.890	3.883	−0.007
DCPE			
CH_2	4.23	4.31	0.08
CH_3	1.36	1.49	0.13
	0.827	0.838	0.005

respectively. It is also evident that in the case of pure guest the triplets at 1.36 ppm corresponding to the methyl group of DCPE and the same group of its degradation product, diethylphosphoric acid (**2**) (Scheme 1), are overlapped. In the case of the complex these two triplets are separated due to the large downfield shift of the triplet which corresponds to DCPE (Figure 3). The triplet that corresponds to diethylphosphoric acid (**2**) (Scheme 1) did not show any chemical shift change. Also, the protons of cyclopropyl groups of the DCPE and the protons of the second product of degradation in D_2O, amide **3** (Scheme 1), were not altered.

In an attempt to observe probable intermolecular interaction between DCPE and γCD, NOE difference experiments were performed. Cyclodextrin complexes are molecules in the range of $\omega\tau_c = 1$, and so at room temperature (298 K) zero NOEs were observed.

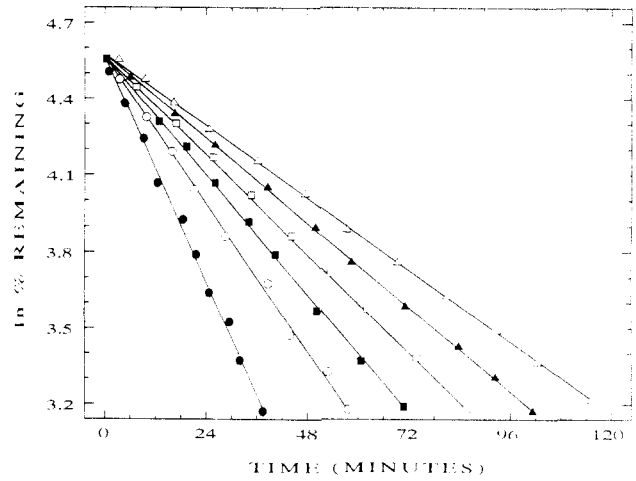


Figure 4. Degradation of 0.07×10^{-3} M DCPE in the presence of various concentrations of γCD. Key: [γCD] = (●) 0.0 M; (○) 0.7×10^{-3} M; (■) 1×10^{-3} M; (□) 1.4×10^{-3} M; (▲) 2.1×10^{-3} M; (△) 2.8×10^{-3} M.

Stoichiometry of the Inclusion Complex. The stoichiometry was measured by ^{1}H NMR, HPLC, and elemental analysis. Digital integration of ^{1}H NMR relevant lines from DCPE (methyl groups) and from γCD (H-1) provided direct access to the stoichiometry coefficient (Duchene, 1991). In the case of HPLC a known amount of complex in aqueous solution was injected to HPLC and the concentration of the guest (DCPE) in the chromatogram was measured by calibration curve. Finally, independent confirmation of the stoichiometry of the complex was obtained from elemental analysis, which was carried out at the Central Department of Microanalysis (CNRS, Vernaison, France). The results agreed with those calculated by the other two methods. In all cases the calculated stoichiometry coefficient was found to be 1:1.

Stability Studies. The DCPE/γCD complex was stored as a solid and assayed in solution. The stability of the complex was measured by HPLC. Solutions of known concentration of the complex were analyzed by HPLC, and the DCPE content was calculated by calibration curve. This experiment was repeated many times for a period of 6 months, and the DCPE content of the complex did not change.

Determination of Stability Constant. The degradation of DCPE in aqueous solution follows pseudo-first-order kinetics in the absence and presence of γCD, as is evident from the linear first-order plots (Figure 4). It is clear that γCD has a pronounced stabilizing effect on DCPE. The rate constant for the degradation reaction of 7×10^{-2} mM DCPE decreased from 30.3×10^{-3} to 13.1×10^{-3} min^{-1} in the presence of 2.8 mM γCD. Figure 5 shows the effect of γCD concentration on the observed pseudo-first-order rate constant for the degradation of 7×10^{-2} mM DCPE. It is evident that the k_{obs} is not a linear function of the concentration of added CD but rather asymptotically approaches a minimum value with increasing CD concentration (Szejtli, 1982a,b). This so-called "saturation behavior" is characteristic of the reaction through complex formation occurring prior to the rate-determining step and may be accommodated by the reaction mechanism illustrated in Scheme 2, where k_0 is the rate constant for decomposition of free DCPE, k_c is the rate constant for totally complexed DCPE, and K_{st} is the apparent stability constant for complex formation (Duchene, 1987).

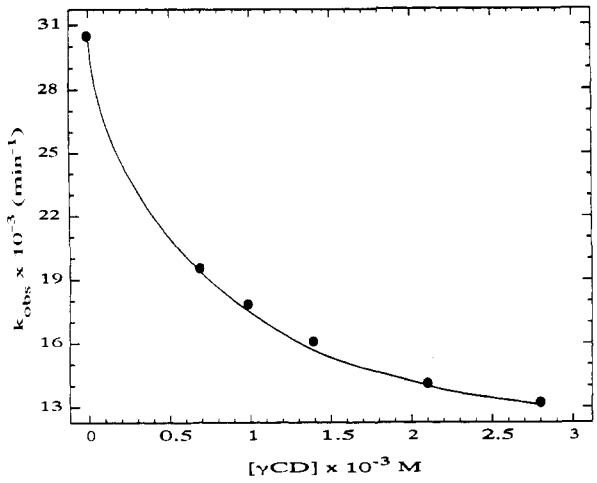
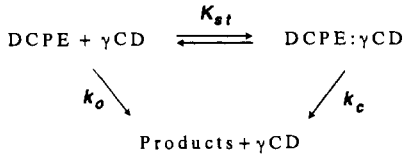


Figure 5. Effect of γCD on the pseudo-first-order constant for the degradation of 7 × 10^{-2} mM DCPE.

Scheme 2



Usually a 10-fold excess of cyclodextrin should be present to ensure the conditions of a first-order reaction. From Scheme 2 the following rate expression can be derived:

$$\frac{-d[DCPE]}{dt} = k_0[DCPE] + k_c[DCPE/\gamma CD] \quad (1)$$

The observed reaction rate for DCPE degradation in the presence of γCD is a weighted average of the rate of reaction of free DCPE and the rate of reaction of DCPE included in γCD, and, therefore, K_{st} can be determined from the dependence of the observed rate constant on the concentration of added γCD. The actual measurable rate constant is

$$k_{obs} = k_0 + \frac{(k_c - k_0)[CD]}{1/K_{st} + [CD]} \quad (2)$$

This equation can be solved graphically according to the procedure of Eadie (1942) and on rearrangement gives

$$k_0 - k_{obs} = \frac{-[1/K_{st}](k_0 - k_{obs})}{[CD]} + (k_0 - k_c) \quad (3)$$

The correlation of $(k_0 - k_{obs})$ vs $(k_0 - k_{obs})/[CD]$ gives $-[1/K_{st}]$ as the slope and $(k_0 - k_c)$ as the intercept. In Figure 6 the rate data of Figure 5 are plotted according to eq 3. From the slope and intercept of the plot, the values of K_{st} and k_c were obtained. The data are shown in Table 2.

To calculate the activation energy for the degradation of DCPE in the absence and presence of γCD, the rate of degradation for DCPE was measured at several temperatures. Activation energy (E_a) was obtained graphically from the temperature dependence of the rate constants for free (k_0) and complexed (k_c) DCPE, according to the Arrhenius equation (eq 4). The Arrhenius plot in the presence of γCD was parallel to that in its absence (Figure 7), suggesting that the mechanism of degradation was unchanged.

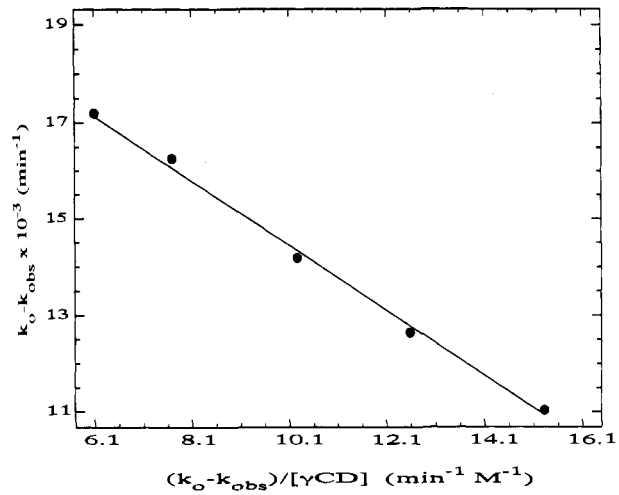


Figure 6. Plot of the rate data in Figure 5 according to eq 3.

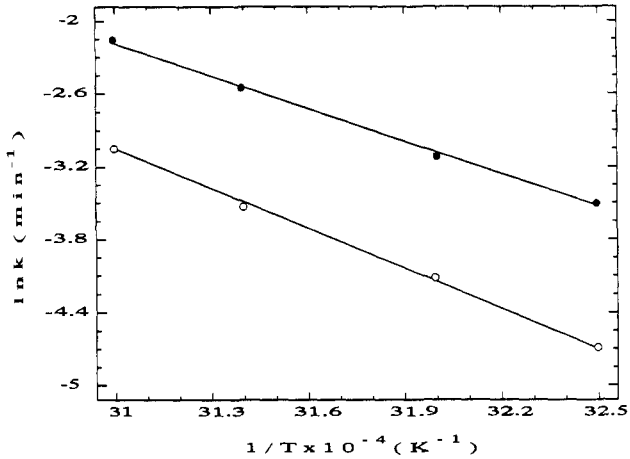


Figure 7. Arrhenius diagrams for the degradation of DCPE alone and DCPE/γCD complex. Key: (●) DCPE alone; (○) DCPE/γCD complex.

Table 2. Kinetic Parameters for the Degradation of DCPE and DCPE/γCD Complex in Various Temperatures

θ (°C)	$k_0 \times 10^{-3}$ (min^{-1})	$k_c \times 10^{-3}$ (min^{-1})	K_{st} (M^{-1})
35	30.3	9.1	1488
40	44.5	16	1262
45	77	29.6	877
50	115	48.3	714

$$\ln k = -\frac{E_a}{R}\frac{1}{T} + \ln A \quad (4)$$

The activation energy for DCPE degradation was determined to be 17.75 kcal/mol. When DCPE is totally in complexed form, E_a is increased to 21.6 kcal/mol. The increase in activation energy may be due to steric hindrance for the formation of the intermediate compound, as is shown in the degradation mechanism in Scheme 1, perhaps because the inclusion phenomenon creates a shield to the susceptible moiety of DCPE from attack by aqueous media.

The enthalpy of complexation of DCPE and γCD was evaluated from the temperature dependence of the stability constant K_{st}. The enthalpy (ΔH_0) of DCPE/γCD complex formation, determined using the van't Hoff relationship (eq 5), is exothermic and equal to −9.99 kcal/mol (Figure 8). Negative enthalpies of CD complex formation may be due to enhanced van der Waals interactions between the guest and CD, hydrogen bonding

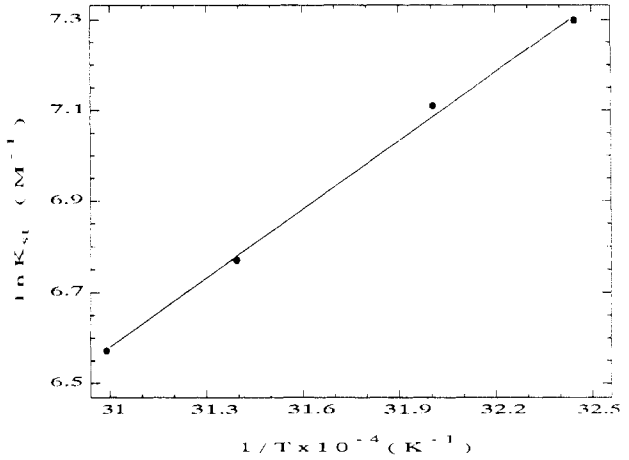


Figure 8. Van't Hoff plot for the stability constant of DCPE/γCD complex.

$$\ln K_{st} = -\frac{\Delta H_0}{R}\frac{1}{T} + \frac{\Delta S_0}{R} \quad (5)$$

between the guest and the hydroxyl groups of CD, or changes in the degree of aggregation of water associated with the molecules on complex formation (Tabushi et al., 1978). Water molecules inside the CD cavity are in an energetically unfavorable position due to polar–nonpolar interactions and therefore cannot form as many hydrogen bonds as in the bulk. These high-enthalpy water molecules are readily displaced by a less polar guest (Saenger, 1980) and combined with the bulk solvent to increase the number of solvent–solvent hydrogen bonds.

A negative entropy change (ΔS_0) of −9 cal mol^{-1} K^{-1} is observed for complex formation between DCPE and γCD, showing the increased order in the complex vs the unassociated free molecules.

Determination of Acute Oral Toxicity. The acute oral toxicity of the DCPE/γCD complex was determined on Wistar rats. Four female rats of the same age, weighing about 200 g ± 10%, were used at each dose level. The test complex was administered to the experimental animals with water as carrier, at a standard volume of 10 mg/kg of body weight. The tested doses were chosen on the basis of the acute oral toxicity of DCPE and the DCPE content of the complex. Table 3 shows the respective LD_{50} for DCPE that has been determined in a previous study and was found to be 10.86 mg/kg for male and 7.85 mg/kg for female Wistar rats. The toxic symptoms were typically cholinergic and appeared almost immediately after the administration of lethal doses (Machera and Kostakis, 1991, 1992).

The experimental animals were observed for toxic symptoms, general behavior, and deaths for 14 days after the administration of the test compound. The respective LD_{50} values were determined graphically. The doses of DCPE/γCD complex administered to female Wistar rats and the respective percentages of mortality are shown in Table 3. The LD_{50} of the DCPE/γCD complex was found to be approximately 51 mg/kg of body weight. The toxic symptoms were typical cholinergic symptoms and appeared approximately 30–40 min after the administration of the test substance. The amount of DCPE contained at the determined median lethal dose of the DCPE/γCD complex is 8.61 mg/kg, as the stoichiometry coefficient for the complex is 1:1.

The results of this preliminary study indicate that the complexation of DCPE with γCD decreased the acute oral toxicity of the parent compound without a significant decrease of its anticholinergic activity. This fact in combination with the delay of the appearance of toxic symptoms is an indication that a safer product has been prepared, especially for the applicator.

Table 3. Toxicity of DCPE and DCPE/γCD Complex on Female Wistar Rats

DCPE		DCPE/γCD complex	
doses (mg/kg)	mortality (%)	doses (mg/kg)	mortality (%)
6.5	0	32.5	0
6.6	30	40.0	25
7.9	60	49.0	50
9.9	80	60.5	50
11.5	100	74.5	100
LD_{50} = 7.85 mg/kg of bw		LD_{50} = 51 mg/kg of bw	

ACKNOWLEDGMENT

We thank Dr. G. Bonas for the NOE difference experiments which were performed at the Institute of Organic and Pharmaceutical Chemistry of the National Hellenic Research Foundation.

LITERATURE CITED

Camilleri, P.; Boocock, G. β-Cyclodextrin Inclusion Complex of Mevinphos. *J. Agric. Food Chem.* **1985**, *33*, 1032–1034.

Donamura, G.; Heldt, W. The Beckmann Rearrangement. *Organic Reactions*; Wiley: New York, 1960; Vol. 11.

Duchene, D. *Cyclodextrins and their industrial uses*; Editions de Sante: Paris, 1987; pp 144–146.

Duchene, D. *New trends in cyclodextrins and derivatives*; Editions de Sante: Paris, 1991a; pp 25–55.

Duchene, D. *New trends in cyclodextrins and derivatives*; Editions de Sante: Paris, 1991b; pp 215–247.

Duchene, D. *New trends in cyclodextrins and derivatives*; Editions de Sante: Paris, 1991c; pp 223.

Eadie, G. S. *J. Biol. Chem.* **1942**, *146*, 85–94.

Ford, L.; Timmins, P. *Pharmaceutical Thermal Analysis*; Wiley: New York, 1989; pp 152, 164–165, 171–172.

Jones, S. P.; Grant, D. J.; Hadgraft, J.; Parr, G. Cyclodextrins in the Pharmaceutical Sciences. *Acta Pharm. Technol.* **1984**, *30*, 263–277.

Kostakis, E.; Rouman, A. Synthesis and pharmacological study of new anti-cholinesterasic compounds. Ph.D. Thesis, University of Athens, 1984.

Machera, K.; Kostakis, E. Acute oral toxicity of the organophosphorous compound diethylphosphoric ester of dicyclopropylketoxime and efficacy of antidotes on white rats. *Ann. Inst. Phytopathol. Benaki* **1991**, *16*, 75–87.

Machera, K.; Kostakis, E. Anti-Cholinesterase Action of a New Organophosphorous Ester, Diethylphosphoric Ester of Dicyclopropylketoxime, on Wistar Rats. *Bull. Environ. Contam. Toxicol.* **1992**, *48*, 108–114.

Saenger, W. Cyclodextrin inclusion compounds in research and industry. *Angew. Chem.* **1980**, *19*, 344–362.

Szejtli, J. *Cyclodextrins and their inclusion complexes*; Akademiai Kiado: Budapest, 1982a; pp 115–116.

Szejtli, J. *Cyclodextrins and their inclusion complexes*; Akademiai Kiado: Budapest, 1982b; pp 144–146.

Szejtli, J. *Cyclodextrin Technology*; Kluwer Academic Publishers: Dordrecht, 1988; pp 335–362.

Tabushi, I.; Kiyosuke, Y.; Sugimoto, T.; Yamamura, K. Approach to the Aspects of Driving Force of Inclusion by α-Cyclodextrin. *J. Am. Chem. Soc.* **1978**, *100*, 916–919.

Received for review May 13, 1993. Revised manuscript received October 14, 1993. Accepted November 29, 1993.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts*, February 1, 1994.