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PHYSICOCHEMICAL AND TOXICOLOGICAL STUDIES ON 4-CHLORO 3,5-DINITROBENZOIC ACID IN AQUEOUS SOLUTIONS

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Abstract—Physicochemical characterization of hazardous compounds often is required for the development of structure–reactivity correlations. Physical, chemical, and toxicological properties of target pollutants require determination for an efficient application of wastewater treatments. In the present work, we chose a chloro-nitro-aromatic derivative (4-chloro-3,5-dinitrobenzoic acid [CDNBA]), as a model compound on which to perform physicochemical and toxicological studies. Several properties of CDNBA are not available in the literature, although many aromatic nitro-compounds are considered hazardous materials. Measurements of solubility in water, acid dissociation constant, and kinetic parameters for the nucleophilic substitution of chlorine atom in alkaline media are reported. We also performed cytotoxicity studies of CDNBA and ultraviolet-irradiated CDNBA solutions. From the analysis of CDNBA solubility in water at different temperatures, an enthalpy of solution of 23.2 ± 2.5 kJ/mol was found. The study of the acid dissociation constant K_c by using conductivity measurements and the modified Gran's method yielded values for the equilibrium constant K_a of 2.36×10^{-3} and 2.26×10^{-3}, respectively. The bimolecular rate constant for the reaction of $CDNB^-$ and hydroxyl ion (HO^-) measured at room temperature and 0.1 M of ionic strength was 5.92/M/s, and the activation energy for this process was 70.7 ± 3.4 kJ/mol. Cytotoxicity assays with aqueous suspensions of *Tetrahymena pyriformis* showed lethal effects due to the pH change induced by CDNBA. On the other hand, in buffered solutions, a value of 104.47 μM was observed for the median effective concentration, that is, the concentration of CDNBA at which the proliferation was restricted to one half of the blank. Irradiation of CDNBA solutions increased the toxicity, suggesting the formation of intermediate products with higher cytotoxicity effects.

Keywords—Nitro compounds Physicochemical properties Cytotoxicity Risk assessment

INTRODUCTION

The N-substituted aromatic compounds are environmental contaminants associated with anthropogenic activities such as production and use of dyes, explosives, pesticides, and pharmaceuticals, among others. Physicochemical characterization of these compounds often is required for the development of correlations between chemical properties and biological activities. Because nitro-substituted aromatic compounds have strong electron withdrawing groups, they are poorly biodegradable in aerobic treatment systems [1]. The oxidation of such compounds can be performed by using advanced oxidation processes based on photochemical reactions. These techniques, currently applied to degradation of organic pollutants [2], are based on the use of ultraviolet (UV), vacuum UV, or visible radiation (usually combined with O_2, O_3, or H_2O_2) to produce highly reactive species. We have recently shown that physicochemical properties of the organic pollutant (i.e., absorption coefficients, rate constants, and pH influence, among others) must be known to develop reaction models capable of predicting the efficiency of oxidation, [3–5].

In many works where these procedures have been applied, the depletion of the pollutant was used as a measure of the success of the treatment but no attention has been paid to the evolution of the toxicity of the solutions. In fact, in the study of the UV/H_2O_2 photodegradation of 4-chloro-3,5-dinitrobenzoic acid (CDNBA) and other nitroaromatic derivatives [3–5], intermediate reaction products such as oxalic acid, formic acid, phenolic derivatives, and nitrate remain in the reaction mixture after the complete disappearance of the pollutant. Thus, the depletion of the primary substrate does not assure the elimination of organic matter.

Because many hazardous intermediate by-products may be formed during advanced oxidation process treatments, toxicological studies on irradiated solutions become relevant. Toxicity screening tests can be performed with the protozoon *Tetrahymena pyriformis GL* [6,7], a freshwater ciliate that possesses features of both single eucaryotic cells and multicellular organisms. For more than 40 years, toxicity assays with *T. pyriformis* have proven extremely useful for evaluations of environmental impact. This single-celled eucaryote is relatively easy to handle and grows in axenic laboratory cultures (i.e., in absence of any other organism) with a short generation time, and its biology and general responses are well known. ?2

In the present study, we performed studies on a chloro-nitro-aromatic derivative, CDNBA (Chemical Abstracts Service 118-97-8). This compound is an intermediate in the manufacture of dyes and in the pharmaceutical industry.

The model compound is irritating on contact with skin and eyes, and may be harmful to mucous membranes and the upper respiratory tract [8]. 4-Chloro-3,5-dinitrobenzoic acid has been to be subject to dehalogenation in lake water as a result of

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microbial growth in the presence of light without nutrients [9], but also in darkness in the presence of acetate anions. Axenic bacterial cultures and nonaxenic *Chlamydomonas* cultures also release chloride from CDNBA, resulting in the production of hydromuconic derivatives [9].

Several physicochemical properties of CDNBA are not available in the literature and its toxicological effects have not been thoroughly investigated, although many nitro-aromatic compounds are known to be harmful. In a recent work [3], we reported the photochemical degradation of CDNBA in aqueous solutions by employing different advanced oxidation process techniques (i.e., UV, UV/H_2O_2, and vacuum UV). We observed, in all cases, that complete dissolved organic carbon (DOC) removal can be achieved, and chloride and nitrate ions are released as inorganic products. However, the nitrogen and carbon balance suggested that traces of organic intermediates are formed during the advanced oxidation processes. Therefore, toxicity screening tests with *T. pyiriformis* can be used for detection and quantification of environmental effects of CDNBA photolyzed solutions.

The aim of this work was to study several physicochemical properties of CDNBA (solubility, acid–base equilibrium, and kinetic behavior in alkaline media) and the toxicological effects of CDNBA and UV-irradiated CDNBA aqueous solutions.

MATERIALS AND METHODS

Chemicals

4-Chloro-3,5-dinitrobenzoic acid (97%, Aldrich, Milwaukee, WI, USA) is a yellow solid at room temperature. However, it slowly decomposes during storage, as shown by changes in color (yellow to brownish) and melting point. Therefore, as recommended in the literature [10] CDNBA was purified by several recrystallizations from mixtures of ethyl alcohol and water, until the melting temperature was in the range from 159 to 162°C. 4-Chloro-3,5-dinitrobenzoic acid is only slightly soluble in water, with a solubility of approximately 2.5 g/L at 25°C.

Kinetic studies were performed in alkaline solutions with NaOH (Merck, Darmstadt, Germany) concentrations ranging from 0.1 to 1 mol/L. Ionic strength (μ) was regulated by using $NaClO_4$ (Merck). These chemicals were used without further purification.

Analyses

Absorption spectra were registered on a Cary 3 spectrophotometer (Varian, Palo Alto, CA, USA). Measurements of pH of the irradiated solutions were performed with a pH meter (Radiometer, Westlake, OH, USA). Dissolved organic carbon measurements (Beckman Tocamaster 915-B, Beckman Instuments, Fullerton, CA, USA) and oven temperature (950°C) measurements also were carried out on various samples. The experimental error of DOC values was ±8%.

Kinetic experiments

Kinetics measurements were performed by using the kinetic mode of the Cary 3 spectrophotometer and a unit SFA-20 A (Hi-tech Scientific, Salisbury, UK) specially developed for this spectrophotometer. Temperature was regulated with a MGW-Lauda NB-D8/17 thermostat connected to a Krymotat TK 30-D cryostat (MGW-Lauda, Messgeratewerk Lauda/Tauber, Lauda, Germany). In all the kinetic experiments, the initial CDNBA concentration was held constant (1.22×10^{-4} mol/L). The analysis of the rate constant dependence with μ was performed.

Flash photolysis experiments

Conventional flash photolysis equipment was used (Xenon, Woburn, MA, USA) to determine the nature of the first intermediate product formed during UV treatment of CDNBA solutions. The irradiation was performed with two FP-8-100C lamps (Xenon) as source of 80-μs polychromatic light pulses. The description of the employed equipment is reported elsewhere [11]. Solutions were irradiated at selected wavelengths by using acetone as a cutoff filter ($\lambda < 320$ nm) to avoid radiation of high energy.

Photochemical reactor

The UV photolysis of CDNBA solutions was performed in an annular photochemical reactor Nr 1326, DEMA (Mangels, Bornheim-Roisdorf, Germany). The reactor had a volume of 750 ml and was equipped with a medium pressure Hg arc HPK 125 W (Philips, Eindhoven, The Netherlands), positioned in the axis of the reactor in a quartz well. The reactor was equipped with ports for sample withdrawing. The purging gas was introduced from below through a glass frit. Solutions were continuously purged with synthetic air (21% O_2 in N_2) and temperature was maintained at 25 ± 1°C.

Solubility measurements

Solubility measurements were performed by using a well-known standard procedure [12]. In all experiments, CDNBA solutions were initially saturated at 70°C and then cooled. The temperature was regulated by using a thermostatic bath until equilibrium was reached. Samples at different temperatures were carefully taken from the stock solution (to avoid crystallization and withdrawal of crystals of the stock solution during sampling, a hot filtrating system was used). The concentrations of CDNBA were measured by a standard titration procedure.

Dissociation constant

The equilibrium constant K_a is given by the following equation:

$$K_a = \frac{a_{H^+} a_{CDNB^-}}{a_{CDNBA}} = \frac{[H^+]\gamma_{H^+}[CDNB^-]\gamma_{CDNB^-}}{[CDNBA]\gamma_{CDNBA}} \cong K_c \gamma_{\pm}^2 \quad (1)$$

where K_c is the acid dissociation constant involving concentrations and $\gamma_{\pm}$s are the mean activity coefficients. The last equality holds because for diluted solutions, $\gamma_{CDNBA} \cong 1$. We have applied two different methods to estimate the value of K_c: conductimetry and titration by using the modified Gran method. ?3

Conductivity method

The conductivity of CDNBA solutions was measured as a function of the acid concentration. For a weak acid, the molar conductivity depends on α, the dissociation degree, as stated by the well-known Arrhenius expression [12–15]

$$\Lambda_m = \alpha \Lambda_m^{\infty} \quad (2)$$

Here Λ_m^{∞}, the molar conductivity at infinite dilution, for this compound is unknown. Ostwald's dilution law [15] can be rearranged to give an alternative expression for estimating a starting value of Λ_m^{∞}. The following expression holds strictly for weak electrolytes

$$\Lambda_m = \Lambda_m^{\infty} - \frac{\Lambda_m^2 \cdot c}{\Lambda_m^{\infty} \cdot K_c} \quad (3)$$

Modified Gran method

Aqueous CDNBA solutions of concentration C_a and volume V_a were titrated by adding V_b milliliters of NaOH of concentration C_b. The relationship between the former quantities can be obtained combining Equation 1 with the mass balance for CDNBA and the electroneutrality expression for the solution [16]

$$V_c[H^+] = K_c(V_e - V_c) \quad (4)$$

where $V_c = (V_b + V \cdot [H^+]/C_b)$, and where $V = V_a + V_b$. Equation 4, Schwartz's modification of the Gran method [17,18], states that a plot of $V_c[H^+]$ against V_c should yield a straight line with slope K_c. Values of $[H^+]$ were calculated from the measured pH values and the activity coefficients estimated by Davies equation ($\mu = 0.10$ molal).

Toxicity assays

Cultures of *T. pyriformis GL* (University Blaise-Pascal, Clermont-Ferrand, France) were used. The precultures were preserved in a sterile medium (0.75% proteose peptone enriched with yeast extract and inorganic salts [PPYS]) under axenic conditions and renewed every two to four weeks. In both acute and inhibition of growth assays, a minimum of four different concentrations of each test material with three replicate flasks of each concentration were performed.

Acute toxicity assays

The CDNBA concentrations of the compound to be tested ranged from 25.3 to 1,014 μM. Two methods were used, one without pH control, and another adjusting the pH of CDNBA solutions to 6.0 with NaOH. In both methods, 1.3 ml of the CDNBA solutions were inoculated with 0.2 ml of precultured *T. pyriformis* (48 h). The initial concentration of *T. pyriformis* was 2.4×10^6 cells/ml. The mobility of the organisms was then monitored by using a stereoscopic magnifying glass (×10–50) at different times. The immobile organisms were transferred to distilled water and observed after 10 min and 24 h (they were considered dead in case of immobility after 24 h). Blank experiments were performed with distilled water.

As previously reported [3], the quantum efficiency of CDNBA disappearance when using UV polychromatic irradiation source is very low, that is, $(1.7 \pm 0.2) \times 10^{-4}$. Therefore, CDNBA can be stored in a glass flask for large periods of time [3]. Furthermore, in our experimental condition, inoculated solutions were stored in darkness.

Inhibition of growth assays

The procedure of Yoshioka et al. [19] was employed. A volume of 8 ml of 2% PPYS medium was mixed with 0.2 ml of precultured *Tetrahymena* suspension (exponential growth, 24–48 h) and 1.8 ml of the test solutions. Final CDNBA concentrations ranged from 18.3 to 292.7 μM. The tubes were incubated in vertical position at 28 ± 1°C during 24 and 48 h. The counting was carried out with an optic microscope at ×100 magnification in a Fuchs-Rosenthal camera by using aliquots of 1 ml fixed with Bouin solution. The initial number of cells was calculated from the blank test. The relative growth rate was calculated as the ratio of the number of cells cultured with CDNBA and the number cultivated in the blank. To compare results, analysis of variance (ANOVA) [20] was performed and variance homogeneity was verified with the F_{max} test. The the median effective concentration (EC50; the concentration of CDNBA at which the proliferation of cells was restricted to one half of the blank test) values were obtained by plotting the relative growth rate against the decimal logarithm of CDNBA concentration by using the inhibition concentration approach [21].

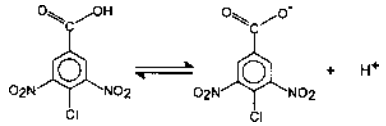

Fig. 1. Structure and acid–base equilibrium of 4-chloro-3,5-dinitrobenzoic acid.

RESULTS

4-Chloro-3,5-dinitrobenzoic acid is slightly soluble in water at room temperature. As shown in Figure 1, CDNBA presents an acid–base equilibrium. Because the extinction coefficients of the species involved in this equilibrium are not the same, the absorption spectrum changes with the pH of the solutions. These changes are reversible in solutions with pH lower than 7. On the other hand as previously reported [3], under alkaline conditions, solutions show irreversible changes in the absorption spectrum, developing an intense yellow coloration with a maximum of absorption at 425 nm. It is noteworthy that flash photolysis experiments showed an intermediate transient spectrum with an absorption band in the same wavelength region.

Solubility of CDNBA in water at various temperatures

Water solubility is a critical property in risk assessments because it is one of the most important factors controlling fate pathways and mass transport processes for organic contaminants in the environment. Highly soluble materials are quickly distributed and diluted. On the other hand, insoluble materials are more likely to adsorb on solids, or to cross biological membranes and accumulate in biota. Natural degradation processes also are concentration-dependent, so insoluble contaminants are more slowly transformed.

To the best of our knowledge, measurements of the solubility of CDNBA have not been reported, although estimated values that use semiempirical or empirical correlations are available in the literature [22].

Table 1 shows the experimental solubility, in the molality scale, at various temperatures. From the plot of ln S versus 1/*t* (where S is solubility and *t* is temperature in °C), a value of 23.2 ± 2.5 kJ/mol resulted for the enthalpy of solution of CDNBA. ?4

Determination of the dissociation constant

As expected, solutions of CDNBA are acidic. However, because of the electron-withdrawing ability of the $-NO_2$ sub-

Table 1. Solubility (S) of 4-chloro-3,5-dinitrobenzoic acid at different temperatures. Experimental errors are indicated

t (°C)	$10^2 \times$ S (Molal)
26.6	1.00 ± 0.07
34.6	1.44 ± 0.05
43.5	1.73 ± 0.1
52.5	2.45 ± 0.2
62.3	2.65 ± 0.1

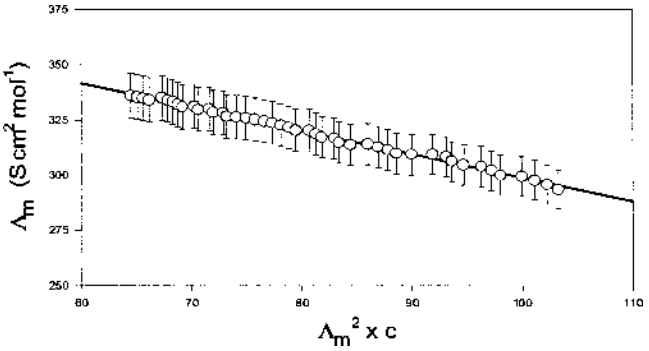

Fig. 2. Determination of the dissociation constant involving concentrations (K_c) by using conductivity measurements. Experimental error bars are shown.

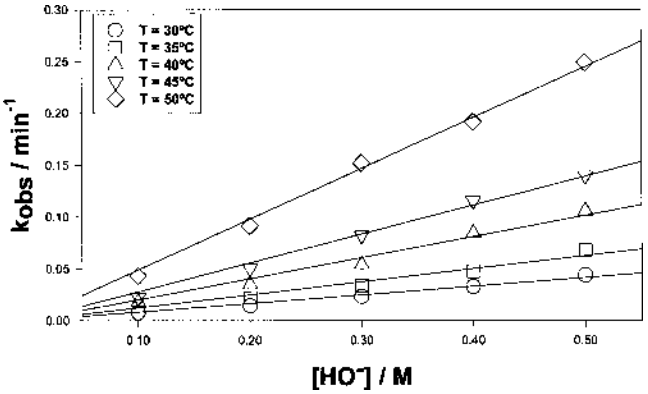

Fig. 3. Apparent rate constants (k_{app}s) at different NaOH concentrations and temperatures. The size of the dots represents the experimental errors. HO = hydroxyl ion; T = temperature.

stituents, the dissociation constant (pK_a) of CDNBA is lower than the pK_as reported for other carboxylic acids [13,14]. The CDNBA is partially dissociated in aqueous solutions, as depicted in Figure 1.

By applying the modified Gran method (Eqn. 4), a value of 3.75×10^{-3} mol/L was obtained for K_c. By using Equation 1, a value of 2.26×10^{-3} resulted for K_a at 25°C.

On the other hand, Figure 2 shows the experimental values of Λ_m against $\Lambda_m^2 \cdot c$. A value of 378.3 cm²/Ω/mol was estimated for Λ_m^∞ by an iterative procedure [23]. From the slope of the regression (Fig. 2), an average value for K_c of 2.45×10^{-3} mol/L was found in the experimental domain. By using this value and the mean activity coefficients calculated by Davies equation [24] (average $\mu = 2.6 \times 10^{-4}$ molal), a value of 2.36×10^{-3} was obtained for K_a.

Stability of CDNBA in alkaline media

The plot of $\ln[(A_\infty - A_t)/(A_t - A_0)]$ versus time at each wavelength gives straight lines. In all cases, the observed slope was independent of wavelength, suggesting that only a simple process is observed. Furthermore, factor analysis [25,26] of the time-resolved spectra showed that only two species contribute to the observed behavior. The relative concentration profiles were calculated by using bilinear regression analysis and the initial and final spectra [26]. The time evolution of CDNBA alkaline solutions was analyzed at different experimental conditions. In all cases, a first-order kinetic decay was observed. The apparent rate constant (k_{app}) obtained depends on the temperature, ionic strength, and HO^- concentration.

Figure 3 shows the k_{app}s as a function of [OH^-] obtained at constant ionic strength for different temperatures. From the slope of the curves, the bimolecular rate constants for the reaction of HO^- with $CDNB^-$ were calculated at each temperature. The results are shown in Table 2.

?5

The Arrhenius plot for the data listed in Table 2 yielded an average activation energy of 70.7 ± 3.4 kJ/mol.

We also have studied the dependence of the k_{app} (t = 40°C and [HO^-] = 0.1 mol/L) as a function of ionic strength. As expected from the Debye–Hückel theory for a chemical reaction between ions [24], a linear behavior of log k_{app} against $\mu^{0.5}$ was observed, that is, $\log k_{app} = -2.69 + 0.30\mu^{0.5}$ [24] ($r^2 = 0.992$).

Photochemical transformation of CDNBA

We previously reported that during photostationary irradiation of CDNBA solutions at 254 nm, the substrate is depleted at the same rate that chlorine anions are released [3]. When using conventional flash photolysis experiments, the intermediate spectrum of Figure 4 was obtained. The strong absorption band around 415 nm compares to that obtained in alkaline media. In addition, chlorine anions also were detected after irradiation.

Ecotoxicity tests

Acute toxicity assays. Two sets of experiments were performed to study the lethal effects of CDNBA on *T. pyriformis*. The first series was carried out without control of pH and in the second series the pH of the test solutions was adjusted with drops of concentrated NaOH before inoculation.

The experiments without regulation of pH showed that 100% of the organisms die after 60 min of exposure from a concentration of 101.4 μM; at higher concentrations the mortality was instantaneous. In a solution of CDNBA at 50.6 μM, only very few cells survive after 48 h of exposition (~ 1%), but in solutions of 25.3 μM most cells remain alive even after 48 h. These results suggest a strong correlation between mortality and the pH change induced by CDNBA (see pK_a measurements). In the experiments with controlled pH, for concentrations between 49.4 μM and 989.4 μM, the organisms suffer a shock and lose mobility almost completely during 24 h. However, after 48 h, they recover and acquire the same mobility as observed in the blank test.

Inhibition of growth assays. The EC50, defined as the concentration of CDNBA at which the proliferation of *T. pyiriformis* was restricted to one half of the blank test, was determined to be 104.7 ± 5.7 μM by the inhibition concentration method. The pH of the cultures ranged between 6.5 and 6.6 because of the buffer capacity of the PPYS medium. The ANOVA between treatments showed a highly significant variation ($p = 10^{-6}$) due to the effects of the treatment, whereas the

Table 2. Rate constants (k) and standard deviations for the reaction of 4-chloro-3,5-dinitrobenzoic acid with NaOH at various temperatures

t (°C)	$k \times 10^3$/M/seg
30.0	1.38 ± 0.06
35.0	2.09 ± 0.10
40.0	3.38 ± 0.15
45.0	4.64 ± 0.13
50.0	8.15 ± 0.26

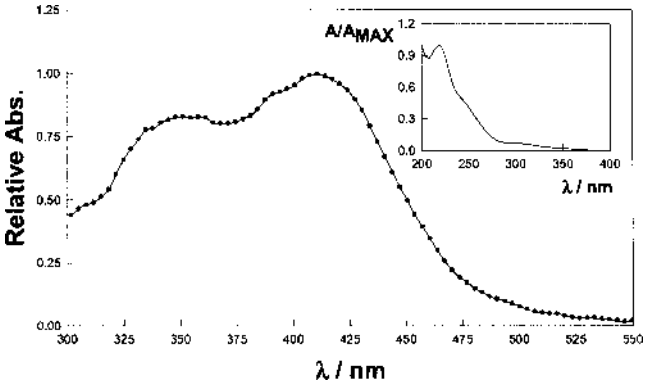

Fig. 4. Relative absorption spectrum of the intermediate obtained by flash photolysis. This spectrum was obtained from the average of at least 10 different flashes to diminish the experimental error. The standard deviation is below 5% in the wavelength range showed in this spectrum. The normalized absorption spectra of 4-chloro-3,5-dinitrobenzoic acid at pH 3.5 and 25°C is shown as an insert. The experimental error in the absorbance measurements is below 5×10^{-4} in absorbance (Abs) units.

differences within treatments were not significant (in all cases, $p > 0.09$). The results obtained are shown in Table 3.

Inhibition of growth with CDNBA photolysis products. The inhibition assays with products generated by UV photolysis of CDNBA showed an increase in the toxicity of the solutions with irradiation time. The ANOVA yielded significant results ($p < 10^{-6}$) for the treatment, that is, UV-photolysis products greatly affected the growth of *T. pyriformis*. Figure 5 compares the time evolution of DOC with the inhibition of growth results.

Changes in cellular morphology due to intoxication. The microscopic analysis reveals changes in the morphology of the cells after exposure to CDNBA or its photolysis products. The appearances of the cells exposed during 96 h to CDNBA and the blank test population are presented in Figure 6A and B, respectively (96 h, ×160, CDNBA = 219 μM, pH = 5.9; Fernández–Galiano silver impregnation method [27]).

In the cells exposed to CDNBA (Fig. 6A), an area of small refractive material can be seen. This area, located between the oral area and the nucleus, is not observed in the blank test cells (Fig. 6B).

DISCUSSION

The solubility measured for CDNBA is lower than the solubility of benzoic acid. In Table 4, the solubilities of several structurally related compounds at 25°C are presented. Comparison of the data shows that nitro-groups do not affect solubility to a great extent, whereas chloro-groups are associated with an important decrease of the solubility. Because the enthalpies of solution for CDNBA and benzoic acid are quite

Table 3. Inhibition of growth by 4-chloro-3,5-dinitrobenzoic acid (CDNBA) after 48 h of exposure. The experimental *F* and *p* values were obtained from the analysis of variance

[CDNBA] (μM)	Cell growth (cells/ml)	% Inhibition	*F*	*p*
0	57,390	0	1.5	0.29
36.6	45,694	20.4	3.8	0.09
73.2	35,069	38.9	1.7	0.26
146.3	21,493	62.5	1.6	0.27
292.7	5,902	89.7	1.1	0.37

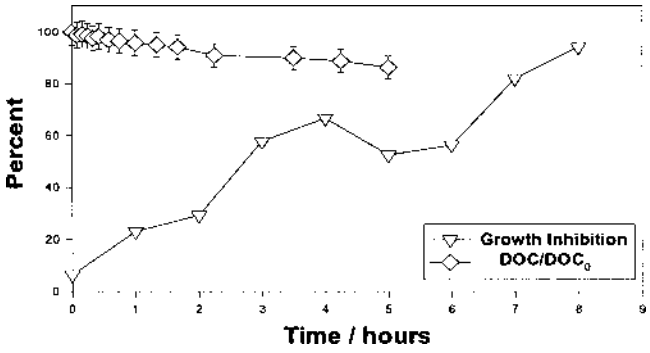

Fig. 5. Comparison of dissolved organic carbon (DOC) and inhibition of growth profiles. The figure shows the experimental DOC error bars; significance level *p* values for inhibition data are listed in Table 3.

similar (23.2 kJ and 28.9 kJ [28], respectively), the low solubility observed for CDNBA suggests that chloro-substituents may affect the entropy of solution, that is, a lower entropy of solution may be expected for CDNBA in comparison with benzoic acid.

The pK_a obtained for CDNBA (2.64) is lower than the reported values for other carboxylic derivatives [13,14]. The presence of two nitro-groups withdraws negative charge from the aromatic ring, making the carboxyl group more acidic. An estimation of the pK_a value of CDNBA was performed by using the Hammett equation [29], assuming σ parameters to be additive. As shown in Table 5, the pK_a calculated for CDNBA and other metha and para derivatives of benzoic acid are in good agreement with the experimental values.

Base hydrolysis recently has been proposed for the destruction of explosive aromatic nitro-compounds as an alternative procedure to open burning or open detonation [30]. The base usually attacks several functional groups and the reactions of aromatic nitro-compounds under these conditions take place with intense color changes in the solution. Because base hydrolysis has been proposed for the treatment of several risky materials, the knowledge of kinetics parameters and the influence of the medium on the reaction rate are very important for risk assessment and technical developments.

Aqueous solutions of CDNBA suffer important spectral changes at pH higher than 7 [3]. Time-resolved spectra show the development of an absorption band with a maximum at 425 nm.

Chloro-aromatic derivatives are well known to suffer nucleophilic substitution reactions with several nucleophiles, such as OH^- and ethoxy anions (SNAr reaction) [31–35]. ?6 Stopped flow spectrophotometric studies have shown that the reaction involves the formation of a transient σ-complex intermediate [33] (i.e., a Jackson–Meisenheimer's complex) followed by its decomposition. The generally accepted reaction scheme is

$$ArX + Y^- \leftrightarrow ArXY^- \rightarrow ArY + X^-$$

where Y^- is a nucleophile and X^- is the leaving group. The first step is usually, but not always, rate-determining.

The occurrence of σ-complexes in the substitution of the chlorine atom of CDNBA to yield 4-hydroxy-3,5-dinitrobenzoate has been previously observed in aqueous dimethyl sulfoxide [31], but no activation parameters were reported. In addition, kinetic studies of the alkaline hydrolysis of picryl chloride (an isoelectronic species of the $CDNBA^-$ anion) showed that the σ-complex decomposes to give Cl^- and picryl

A B

Fig. 6. Cells from a population exposed during 96 h to (**A**) 4-chloro-3,5-dinitrobenzoic acid (CDNBA) and (**B**) the blank test (96 h, ×160, CDNBA = 219 μM, pH = 5.9).

acid [33,34]. Thus, a phenollike compound, probably 4-hydroxy-3,5-dinitrobenzoate, may be the expected product for CDNBA alkaline nucleophilic substitution in aqueous media.

In the present study, the rate-limiting step for the SNAr reaction involved two negative charges. As expected, the ionic strength affects the k_{app} and a positive slope is obtained from log k_{app} versus $\mu^{1/2}$. Applying the transition state theory [36,37], a negative entropy of activation for this reaction (-75 ± 10 J/K·mol) can be estimated, suggesting that solvent molecules in the neighborhood of the double-charged transition state are acted on by strong electrostatic forces (electrostriction effect [37]), which restrict their freedom of motion.

The spectra of the intermediates produced in flash photolysis experiments show absorption bands in the same spectral region as the products detected in the hydrolysis experiments. Because the release of Cl^- ion as product was observed in both cases and the development of absorption bands between 350 and 500 nm is typical of phenollike derivatives, the observed behavior suggests that the carbon–chlorine (C−Cl) bond is broken after UV light absorption and a phenollike intermediate may be generated. Previous studies have shown that dehalogenation can take place either by homolytic cleavage of the chlorine atom [3] or by photoenhanced nucleophilic substitution [35].

A significant inhibition of growth of *T. pyriformis* was observed for CDNBA concentrations higher than 73 μM, with the resultant EC50 of 104.47 μM.

The appearance of granules, in response to a decreased rate of growth, is a common feature in cells subjected to some stress [6] (e.g., heavy metals, drugs, lack of oxygen, and pH or temperature changes). These granules resemble those observed in cells of higher organisms during the exposition to heavy metals. Studies carried out with *T. pyriformis* have demonstrated that these granules may be involved in the binding of toxic substances [6]. This suggests an adaptation of the microorganisms, presumably through a mechanism related with the formation of electron-dense particles.

Cultures of *T. pyriformis* exposed to irradiated solutions of CDNBA showed a decreased growth rate. These results suggest the formation of substances that, in general, contribute to an increase in toxicity. Several N-substitued aromatic compounds have been tested with *T. pyriformis*. According to Yoshioka et al. [19], EC50 values for aniline, nitrobenzene, 2,6-dinitrotoluene, and 4-nitrophenol are 2,000, 790, 550, and 40 μM, respectively. Thus, the formation of phenollike derivatives during UV irradiation may be associated with the increase of growth inhibition. Because the application of UV radiation in the range 200 to 280 nm (region C of the UV range) to contaminated waters can lead to the formation of undesirable toxic compounds, the depletion of the pollutant itself does not assure the success of the treatment. Therefore, a very important task, when advanced oxidation process techniques are applied to water remediation, is to perform toxicity tests before the final disposal of the treated effluent.

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Table 4. Solubilities of structurally related compounds taken from Hodgman et al. [28]. The experimental value for 4-chloro-3,5-dinitrobenzoic acid (CDNBA) reported in this paper was taken at 25°C

Compound	Solubility (molal)	Reference
p-Chlorobenzoic acid	4.5×10^{-4}	[28]
m-Chlorobenzoic acid[a]	5.8×10^{-3}	[28]
CDNBA	0.010	This work
o-Chlorobenzoic acid	0.013	[28]
Benzoic acid	0.028	[28]
3,5-Dinitrobenzoic acid[a]	0.029	[28]
3,4-Dinitrobenzoic acid	0.032	[28]

[a] Values were estimated from other temperatures by assuming a change in enthalpy of 23.2 kJ/mol.

Table 5. Dissociation constant (pK_a) values of structurally related compounds taken from Lide and Frederikse [14]. The experimental value for 4-chloro-3,5-dinitrobenzoic acid (CDNBA) is as reported in this paper

Compound	pK_a measured	pK_a calculated	Reference
o-Nitrobenzoic acid	2.16	3.38[a]	[14]
CDNBA	2.66	2.53	This work
o-Chlorobenzoic acid	2.92	3.99[a]	[14]
p-Nitrobenzoic acid	3.41	3.38	[14]
m-Nitrobenzoic acid	3.47	3.48	[14]
m-Chlorobenzoic acid	3.82	3.82	[14]
p-Chlorobenzoic acid	3.98	3.95	[14]
Benzoic acid	4.19	4.19	[14]

[a] Hammett correlation usually fails with ortho-substituents.

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