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Interaction of Paraquat with Calf Thymus DNA: A Terbium(III) Luminescent Probe and Multispectral Study

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Terbium(III), as a good luminescent probe, was developed for the study of the interaction between paraquat and calf thymus DNA (ctDNA) when the binding mode of small molecules to DNA was electrostatic binding. This interaction was further investigated using an ethidium bromide (EB) probe, UV absorption spectra, and circular dichroism spectra. On the basis of Scatchard plots constructed from fluorescence titration data of the ctDNA−Tb^{3+} system in the presence of paraquat, the binding constants between paraquat and ctDNA were obtained. The results showed that the electrostatic attraction between positively charged sodium ion and negatively charged phosphate groups could inhibit the binding of paraquat to ctDNA, and competitive inhibition between Tb^{3+} and paraquat also existed when they were bound to ctDNA. The effects of paraquat on the fluorescence intensity of the EB−ctDNA system indicated that the intercalation binding of paraquat to ctDNA could be excluded. This conclusion could be further supported by both the absorption spectra of paraquat in the presence of ctDNA and the CD spectra of the paraquat−ctDNA system.

KEYWORDS: Paraquat; calf thymus DNA; Tb^{3+}; luminescent probe; interaction

INTRODUCTION

Although many investigations on the interaction of small molecules with biomacromolecules (such as protein, DNA) have been carried out, which is helpful to clarify the mechanism of these compounds' effects on the structure and physical properties of biomacromolecules (*1*−*5*), almost all of studies reported in the literatures are concentrated on the interaction of drugs with biomacromolecules; there are very few reported studies are relative to pollutants (*6*, *7*), especially the interaction of pollutants with DNA. Paraquat (1,1′-dimethyl-4,4′-dipyridinium chloride, Par) (**Figure 1**) is a nonselective herbicide that is widely used in many countries. Because of its poor bioavailability, its resistance to microbial degradation, and the difficulty of its decomposition by light, paraquat has a long lifetime in the environment and it can be transferred from foliage to foodstuffs (*8*). This pollutant is extremely toxic, which can cause lethal responses in both animals and humans after acute exposure (*9*). Chronic exposure to paraquat may cause pulmonary fibrosis, a stiffening of the lung tissue (*10*). It has been classified as a possible human carcinogen and a weakly genotoxic pollutant by the U.S. EPA. However, the interaction of paraquat with DNA has not been thoroughly investigated. To better understand the molecular mechanism of paraquat's toxicity, a study of the interaction of paraquat with the genomic species of DNA is highly demanded.

Nowadays various techniques have been used to investigate the interaction of small molecules with DNA including UV absorption spectra (*11*), circular dichroism (CD) (*12*), Raman spectroscopy (*13*), gel electrophoresis (*14*), infrared spectroscopy (IR) (*15*), nuclear magnetic resonance spectroscopy (NMR) (*16*), and so on. Compared to these techniques, the fluorometric methods have attracted more attention because of their high sensitivity and high selectivity. It is well-known that the fluorescence intensity of DNA itself is very weak, and the direct use of fluorescence emission properties to investigate biological properties has been limited. Generally, fluorescent probes including organic dyes such as ethidium bromide (EB) (*17*), Hoechst 33258 (*18*), Phosphin 3R (*19*), and metal ions are employed to investigate DNA. Due to the luminescence characteristics of rare-earth ions (*20*), especially Tb^{3+} and Eu^{3+}, more attention has been directed toward these two rare-earth cations because of their resonance energy levels overlap with ultraviolet light (*21*), so they are widely used as luminescent probes to study DNA. In recent years, the coordination complexes of metal ions, especially rare-earth ions, as a probe to study DNA have attracted much attention (*22*, *23*). Although many luminescent probes with high sensitivity can be used to quantitatively determine DNA, not all of the DNA probes, even with low toxicity and high selectivity and sensitivity, are suitable to be used for the study of small molecules with DNA. It is required that when a fluorescence probe of DNA is used for the study of the interaction of small molecules with DNA, the interaction of the probe itself with DNA should not influence the judgment of the interaction mechanism of the specific small molecule with DNA. Similar to the luminescent probes of the coordination complexes of metal ions, they are not suitable for the study

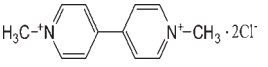

Figure 1. Molecular structure of paraquat.

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of the interaction between the specific small molecule and DNA. This is because both the ligands and metal ions of the metal complex participate in the interaction with DNA, which makes it difficult to discuss the interaction mechanism of the objective compound with DNA.

At present, it is well-known that EB (*17*) is an intercalation binding probe of DNA and Hoechst 33258 (*18*) is a groove-binding probe. In this study, one of the objectives was that a terbium(III) luminescent probe was chosen to examine if it could be used as an electrostatic-binding probe for the study of the interaction of the ionic compound of paraquat with calf thymus DNA (ctDNA). Another objective was that the interaction of paraquat with ctDNA using UV absorption spectra, fluorescence spectra, and circular dichroism spectra was investigated in detail. The work should be valuable in ecotoxicology, for the binding study of paraquat with ctDNA has toxicological importance.

MATERIALS AND METHODS

Apparatus. The fluorescence spectra and intensities were acquired on an F-2500 spectrofluorometer (Hitachi, Japan) with a quartz cell (1 cm × 1 cm cross section) equipped with a xenon lamp (150 W) and dual monochromators. CD spectra were acquired on a J-815 CD spectrometer (JASCO, Japan). All absorption spectra were recorded with a UV-2401 PC spectrophotometer (Shimadzu, Japan). All pH measurements were made with an MP 220 pH-meter (Mettler Toledo, China).

Reagents. Commercially available ctDNA (Sigma Chemical Co.) was suspended directly in water at a final concentration of 100 μg mL^{-1} as stock solution. After the absorbance ratio A_{260}/A_{280} had been established in the range of 1.80−1.90 for DNA, the concentration of DNA was determined according to its absorbance at λ_{max} = 260 nm by using ε_{DNA} = 6600 L mol^{-1} cm^{-1}. The calculated concentration of DNA stock solution was 2.82 × 10^{-4} mol L^{-1}. All diluted solutions of DNA were used within 24 h after their preparation. All of the stock solutions and their diluted solutions of DNA were stored in a refrigerator at 4 °C. An amount of 0.01 mol L^{-1} stock standard solution of Tb^{3+} was prepared by dissolving the corresponding oxide (Tb_4O_7, 99.99%) in 1:1 (v/v) hydrochloric acid, and the solution was evaporated to near dryness. The residue was then dissolved in water and diluted to the concentration of 1.0 × 10^{-2} mol L^{-1}. The stock solution of paraquat (1.0 × 10^{-3} mol L^{-1}) was prepared by dissolving the corresponding paraquat (Sigma) in water. A 0.2 mol L^{-1} Tris-HCl buffer solution was prepared by dissolving the corresponding tris(hydroxymethyl)aminomethane (Tris) in water and adjusting the pH with 1.0 mol L^{-1} hydrochloric acid to give a final total volume of 500 mL (pH 7.4). All chemicals used were of analytical purity, and all solutions were prepared with doubly distilled water.

Fluorescence Spectra Measurements. One milliliter of a 0.2 mol L^{-1} Tris-HCl buffer solution, 1.0 mL of a 2.82 × 10^{-4} mol L^{-1} ctDNA solution, 1.0 mL of a 8.0 × 10^{-5} mol L^{-1} Tb^{3+} solution, and a known volume of paraquat solution with appropriate concentration were transferred to a 10 mL colorimetric tube. The mixture was diluted to 10.0 mL with water, shaken thoroughly, and allowed to stand for 10 min. The fluorescence spectra were recorded in the range of 460−560 nm at an excitation wavelength of 290 nm. The entrance and exit slits for all fluorescence measurements were both maintained at 5 nm. In the experiments for the determination of binding constants, the concentration of ctDNA was fixed. When paraquat was fixed at a certain concentration, the fluorescence titration experiments were taken as follows: to a 10 mL colorimetric tube were added 1.0 mL of a 0.2 mol L^{-1} Tris-HCl buffer solution (pH 7.4), 1.0 mL of a 2.82 × 10^{-4} mol L^{-1} ctDNA solution, and 1.0 mL of a paraquat solution with a certain concentration, diluted to 10.0 mL with water, and then shaken gently to uniformity. The colorimetric tube containing 10.0 mL of mixture solution of 0.02 mol L^{-1} Tris-HCl buffer, 2.82 × 10^{-5} mol L^{-1} ctDNA solution, and some concentration of paraquat was allowed to stand for 10 min at room temperature. The mixture solution was titrated by successive additions of 1.0 × 10^{-3} mol L^{-1} stock solution of Tb^{3+} into it. For each addition, the mixture solution was shaken and allowed to stand for 10 min, and then the fluorescence intensities were measured with an excitation wavelength of 290 nm; the emission spectra were read at 545 nm. After every determination, the residue solution in the 1 cm quartz cell should be returned to the colorimetric tube. In the meantime, the absorptions of the mixture solution were measured at λ = 290 and 545 nm, respectively, for the use of correcting observed fluorescence intensities. When the concentration of paraquat was changed, the same procedures were carried out. In the course of successive additions, a concentrated stock solution of Tb^{3+} was chosen so that volume increment (the total increment volume was 0.12 mL) was negligible compared with the 10.0 mL mixture solution. Titrations were done manually by using a trace syringe.

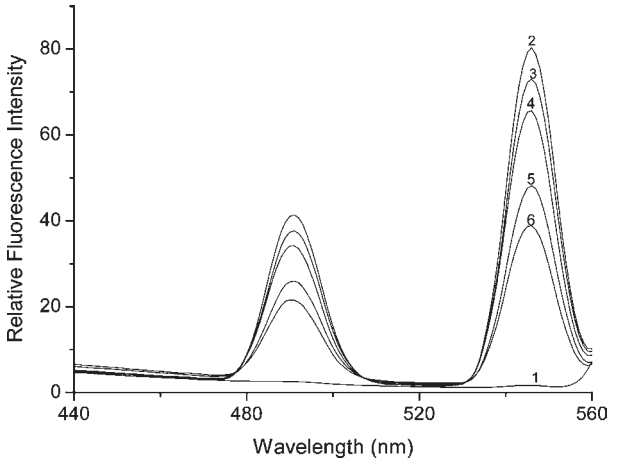

Figure 2. Fluorescence emission spectra (λ_{ex} = 290 nm): (1) Tb^{3+}; (2) Tb^{3+}−ctDNA; (3−6) Tb^{3+}−ctDNA−paraquat, the concentrations of paraquat being 7, 14, 28, and 42 μmol L^{-1}, respectively. Conditions: Tb^{3+}, 8.0 μmol L^{-1}; ctDNA, 28.2 μmol L^{-1}; Tris, 0.02 mol L^{-1}, pH 7.4.

Because of the partial overlap between the UV absorption spectra of paraquat (258 nm) and the excitation spectra of the Tb^{3+}−ctDNA system (290 nm), the inner filter effects (IFEs) should be considered. To evaluate existing IFEs of Tb^{3+}−ctDNA and paraquat, absorbance measurements were performed at excitation and emission wavelengths of Tb^{3+}−ctDNA. Observed F values were corrected using eq 1 (*24*)

$$F_{cor} = F_{obsd}\, 10^{(A_{290}+A_{545})/2} \quad (1)$$

where A_{290} and A_{545} are the sum of the absorbances of Tb^{3+}−ctDNA and paraquat at excitation and emission wavelengths, respectively.

Absorption Spectra Measurements. One milliliter of a 0.2 mol L^{-1} Tris-HCl buffer solution, 1.0 mL of a 1.0 × 10^{-4} mol L^{-1} paraquat solution, and a known volume of ctDNA solution with appropriate concentration were transferred to a 10 mL colorimetric tube. The mixture was diluted to 10.0 mL with water, shaken thoroughly, and allowed to stand for 10 min. The pure paraquat solution was prepared according to the above procedure without ctDNA. The absorption spectra of the mixture solution and pure paraquat solution were measured on a UV-2401PC spectrophotometer. All of the absorption spectra reflect subtraction of the background absorption from all of the reagents by using a corresponding solution without paraquat as a reference solution.

Circular Dichroism Spectra. CD spectroscopy was performed on a Jasco J-815 CD spectrometer in a 0.5 cm cell at room temperature. Bandwidth was 5 nm, and scanning speed was 100 nm/min. Each measurement was the average of three repeated scans, and the background was subtracted from all of the reagents by using a corresponding solution without ctDNA as a reference solution.

RESULTS AND DISCUSSION

Fluorescence Spectra. It is well-known that Tb^{3+} as a lanthanide ion has some good characteristics such as narrow spectral width, long luminescence lifetime, large stokes shift, and strong binding with biological molecules (*25*). When Tb^{3+} stayed in a water solution, it could coordinate with H_2O and form the complex of Tb^{3+}−$(H_2O)_n$, which emitted the very weak fluorescence of Tb^{3+} (see Figure**2**, curve 1). When single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) existed in the

solution, $Tb^{3+}-(H_2O)_n$ would coordinate with the phosphate group of dsDNA or O-6 and N-7 of guanine base in the ssDNA (*26*), which was accompanied with the release of a H_2O molecule in the $DNA-Tb^{3+}-(H_2O)_n$ complex. Thus, the nonradiative energy loss through O−H vibration of the H_2O molecule in the $DNA-Tb^{3+}-(H_2O)_n$ complex would be decreased and the fluorescence of the system would be enhanced by DNA (see **Figure 2**, curve 2). The fluorescence spectrum of the DNA−Tb^{3+} system showed the characteristic spectrum of Tb^{3+}. The emission peaks were at 490 and 545 nm, which corresponded to the transitions from the $^5D_4$ level of Tb^{3+} to the $^7F_6$ and $^7F_5$ level, respectively. The fluorescence intensity at 545 nm was stronger than that at 490 nm; therefore, the fluorescence intensity at 545 nm was used for the measurements.

Figure 2 shows that when different concentrations of paraquat were added to the ctDNA−Tb^{3+} system, the fluorescence intensities of the system were regularly extinguished. The reasons why paraquat could extinguish the fluorescence of Tb^{3+} were explained as follows: Because paraquat was a cation, it would compete with Tb^{3+} for the binding sites of ctDNA, which caused part of the Tb^{3+} ions to be released from the DNA−Tb^{3+} complex and resulted in the fluorescence of Tb^{3+} decreasing. On the basis of the above results, it was feasible that the terbium(III) luminescent probe could be used to study the interaction on paraquat with ctDNA.

Fluorescence Quenching Mechanism. Figure 2 shows that the fluorescence intensity of the ctDNA−Tb^{3+} system decreased regularly with the concentration increase of paraquat, which was called the fluorescence quenching effect. The fluorescence quenching effect might result from a variety of processes such as excited state reactions, ground state complex formations, and collisional processes. The static quenching was due to the formation of ground state complex between fluorophores and quencher. Because the collisional quenching or dynamic quenching resulted from the collisions between fluorophores and quencher, and it could be mathematically expressed by the Stern−Volmer equation (eq 2 (*27*))

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \qquad (2)$$

where F_0 and F are the fluorescence intensities of the fluorophores in the absence and presence of quencher, respectively. K_q is a quenching rate constant, K_{SV} is a dynamic quenching constant, τ_0 is the average lifetime of the molecule without quencher, and [Q] is the concentration of quencher.

The graph plotted according to the Stern−Volmer equation is shown in **Figure 3**. **Figure 3** shows that the graph was nonlinear, which indicated that the possibility of the collisional quenching or dynamic quenching resulted from the collisions was excluded. These results indicate the fluorescence quenching effect of paraquat was not initiated by the dynamic collision, but was caused by the static quenching of the compounds' formation (*28*).

Measurement of Binding Constant by Tb^{3+} Luminescent Probe. At the different concentrations of paraquat, the changes of the fluorescence intensity of the system caused by the titration of Tb^{3+} are shown in **Figure 4**. **Figure 4** shows that when Tb^{3+} was at the same concentration, the fluorescence intensity of the ctDNA−Tb^{3+} system decreased gradually with the increasing concentration of paraquat. When the concentration of Tb^{3+} reached 6.0×10^{-6} mol L^{-1}, the fluorescence quenching effect became the strongest. This indicated that the binding of paraquat with ctDNA was obviously inhibited by paraquat.

Fluorescence Scatchard plots for the binding of Tb^{3+} to ctDNA in the presence of various concentrations of paraquat

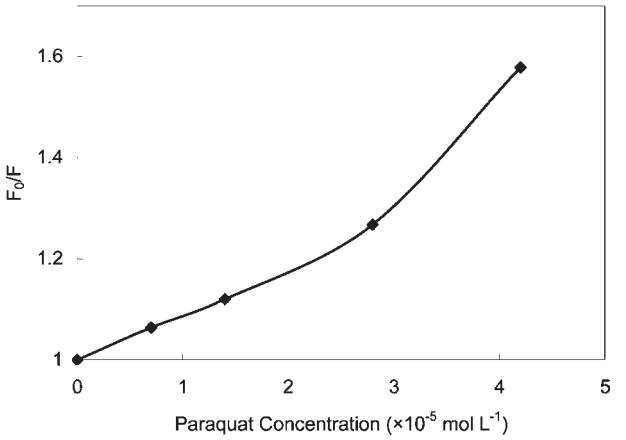

Figure 3. Stern−Volmer plots of the fluorescence quenching of the ctDNA−Tb^{3+} system treated with different paraquat concentrations. Conditions: Tb^{3+}, 8.0 μmol L^{-1}; ctDNA, 28.2 μmol L^{-1}; Tris, 0.02 mol L^{-1}; pH 7.4.

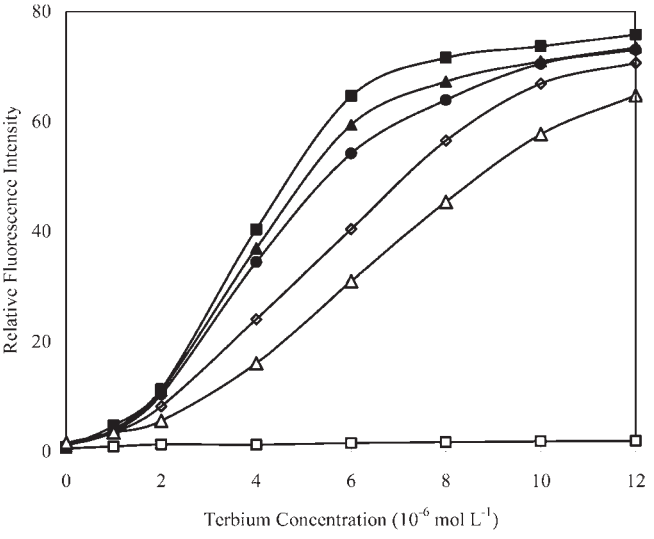

Figure 4. Competitive binding between paraquat and terbium(III) to ctDNA: (□) Tb^{3+}; (■) Tb^{3+} + 28.2 μmol L^{-1} ctDNA; (▲, ●, ◇, △) in the presence of both Tb^{3+} + 28.2 μmol L^{-1} ctDNA and increasing amounts of paraquat (7, 14, 28, and 42 μmol L^{-1}, respectively). Conditions: Tris, 0.02 mol L^{-1}; pH 7.4.

were obtained according to eq 3 (*29*,*30*). Equation 3 expresses the binding of Tb^{3+} to ctDNA in the presence of paraquat (Par).

$$\frac{r_{Tb}}{C_{Tb}} = (n - r_{Tb})\left[\frac{K_{Tb}}{1 + K_{Par}C_{Par}}\right] \qquad (3)$$

Here r_{Tb} is the ratio of bound Tb^{3+} to total nucleotide concentration, C_{Tb} is the concentration of free Tb^{3+}, n is the maximum value of r_{Tb}, K_{Tb} and K_{Par} are intrinsic binding constants for Tb^{3+} and paraquat to DNA, respectively, and C_{Par} is the concentration of free paraquat. Using fluorescence to determine r_{Tb}, binding isotherms were measured in the presence of paraquat and the corresponding Scatchard plots constructed; the results are shown in **Figure 4** and **Table 1**.

From eq 3, the following relationship could be obtained.

$$K_{obsd} = \frac{K_{Tb}}{1 + K_{Par}C_{Par}} \qquad (4)$$

Table 1. Binding of Tb(III) with ctDNA in the Presence of Increasing Concentrations of Paraquat

paraquat/ctDNA ratio	K_{obsd} ($\times 10^6$ L mol^{-1})	n
0.00	4.61	0.222
0.25	3.97	0.209
0.50	2.70	0.208
1.00	1.03	0.216
1.50	0.52	0.219

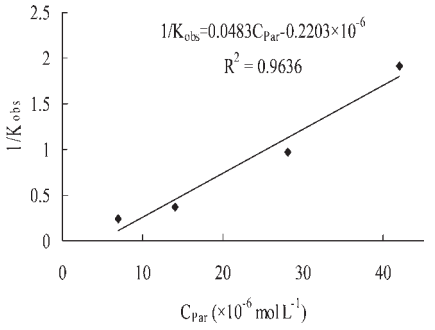

Figure 5. Plot of the relationships between $1/K_{obsd}$ and the concentrations of paraquat.

A straight-line relationship can be obtained by the reciprocal of eq 4 and is shown below.

$$\frac{1}{K_{\text{obsd}}} = \left(\frac{K_{\text{Par}}}{K_{\text{Tb}}}\right) C_{\text{Par}} + \frac{1}{K_{\text{Tb}}} \quad (5)$$

The concentration of free paraquat had been approximated by the total paraquat concentration at the start of each titration when the concentration of paraquat was much higher than that of DNA. The data obtained from **Table 1** were plotted according to eq 5 (see **Figure 5**), and the linear−regression equation was $1/K_{\text{obsd}} = 0.048C_{\text{Par}} - 2.2 \times 10^{-7}$ ($r = 0.982$, $N = 4$). The binding constant of paraquat to ctDNA was then estimated to be 2.21×10^5 L mol^{-1}. n, the amount of Tb^{3+} bound per base pair of DNA, was found to be about 0.22 mol of Tb^{3+}/mol of base pair DNA. In addition, the Scatchard plots are usually used to deduce the binding mode. The research results show that when K_{obsd} is flexible and n is constant, indicating that they (paraquat and Tb^{3+}) exists the competitive binding for the binding sites of DNA (*31*, *32*). As shown in **Table 1**, with the addition of paraquat, the slope (K_{obsd}) decreased with increase in the concentrations of paraquat, but the intercept on the abscissa, n (number of binding sites per nucleotides) was maintained as a constant, indicating there was competitive inhibition between Tb^{3+} and paraquat when they bound to ctDNA.

Effect of the Ionic Strength on the Binding of Paraquat with ctDNA. To further explore the binding mode between paraquat and ctDNA, the effect of the ionic strength on the binding of paraquat with ctDNA was investigated. The binding constants between paraquat and ctDNA in the absence or presence of sodium chloride are listed in **Table 2**. It could be deduced from this table that sodium ions significantly inhibited the binding of paraquat to ctDNA. When the concentration of sodium ions was up to 2.5 mM, the binding constant was decreased 89% from 2.21×10^5 to 2.5×10^4 L mol^{-1}. This could be explained by the fact that positively charged sodium ions could bind to negatively charged phosphate groups on the helix bones of ctDNA via electrostatic attraction and thus neutralize the negative charges of the helix bones of ctDNA, which weakened the binding ability of paraquat to ctDNA. Considering the cation structural nature of paraquat, the possible binding mode of paraquat to ctDNA was thus believed to be electrostatic attraction.

Table 2. Effect of Sodium Ions on the Binding Constants between Paraquat and ctDNA

sodium ion concentration (mmol L^{-1})	K_{Par} ($\times 10^5$ L mol^{-1})
0	2.21
1.25	0.44
2.50	0.25

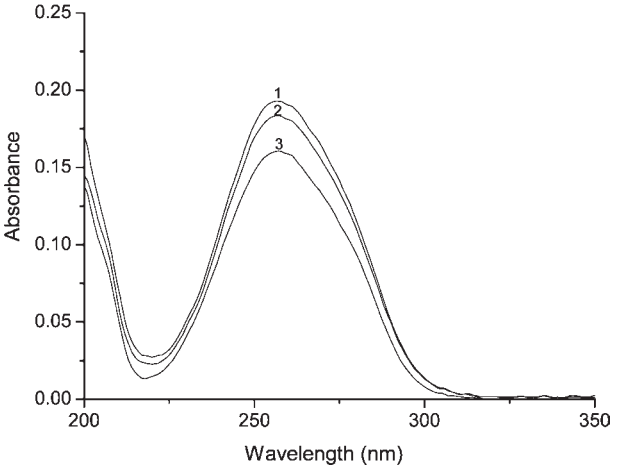

Figure 6. Absorption spectra of paraquat in the presence of ctDNA: (1) no ctDNA; (2, 3) in the presence of 14.1 and 28.2 μmol L^{-1} ctDNA, respectively. Conditions: paraquat, 10.0 μmol L^{-1}; Tris, 0.02 mol L^{-1}; pH 7.4.

Effect of Paraquat on the Fluorescence Intensity of the EB−ctDNA System. To examine whether other binding modes such as intercalation binding existed or not, the effect of paraquat on the fluorescence intensity of the EB−ctDNA system (EB, 10 μmol L^{-1}; ctDNA, 28.2 μmol L^{-1}) was investigated. The results showed almost no change in the fluorescence of the EB−ctDNA system within the paraquat concentration range of 1.0−10 μmol L^{-1}, which indicated that the intercalation binding of paraquat to ctDNA could be excluded. This conclusion could be further supported by the following study on the absorption spectra of paraquat.

Absorption Spectra. Absorption spectra as a convenient technology are usually used to study the interaction of small molecules with nucleic acid. Absorption spectra of paraquat in the presence of various amounts of ctDNA are shown in **Figure 6**. **Figure 6** shows that paraquat had the characteristic absorption peak at 258 nm due to $\pi-\pi^*$ transition. With increasing amounts of ctDNA added to the solution containing a fixed concentration of paraquat (10 μmol L^{-1}), an obvious hyperchromic effect was observed. Generally, red shift and hypochromic effect were observed in the absorption spectra of small molecules if they intercalated into DNA (*11*). The obvious hyperchromic effect indicated a strong interaction between the paraquat and ctDNA. Meanwhile, it was suggested that the intercalation binding of paraquat to ctDNA was excluded. On the basis of the above results, the possible binding mode of paraquat to ctDNA was electrostatic attraction.

CD Spectra. CD spectroscopy could also verify the above assumption. The circular dichroism of nucleic acid resulted from the interaction of the transition dipoles in the component of the helical structure, and circular dichroism of the perturbed DNA has become a good method to monitor the conformational changes caused by the interacting molecules. The CD spectra as

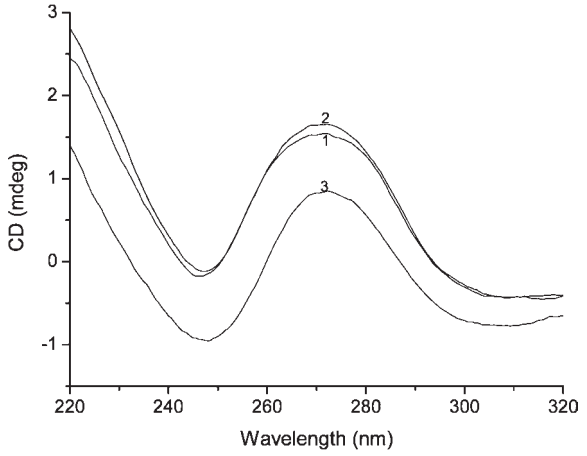

Figure 7. CD spectra of the paraquat−ctDNA system at room temperature: (1) ctDNA; (2) ctDNA + paraquat; (3) denatured ctDNA. Conditions: ctDNA, 28.2 μmol L^{-1}; denatured ctDNA, 28.2 μmol L^{-1}; paraquat, 20.0 μmol L^{-1}; Tris, 0.02 mol L^{-1}; pH 7.4.

an important analytical method were usually used to study the interaction of small molecules with DNA (*33*). The well-known CD spectrum of ctDNA was a conservative pattern with a positive ellipticity at approximately 275 nm and a negative ellipticity at approximately 245 nm (*1*). The positive absorption band at 275 nm was due to base stacking, and the negative band at 245 nm was due to the conformation of B-DNA (*34*). **Figure 7** shows that the CD spectrum of thermally denatured ctDNA was similar to that of native ctDNA with a positive band at 273 nm and a negative band at 247 nm. Generally, when small molecules are intercalated into DNA, the $\pi-\pi$ stacking interaction between the base pairs in the DNA double helix weakens, and this makes the DNA double helix become looser. Accordingly, this resulted in decreasing CD intensity of the positive band, occasionally accompanied by red shift of the positive band. As shown in **Figure 7**, when paraquat was added to the DNA solution, the CD intensity increased, which indicated that the intercalation binding of paraquat to ctDNA was excluded. It could be also suggested that the possible binding mode of paraquat to ctDNA was electrostatic attraction, because paraquat as a cation might bind to the phosphate group of the DNA helix by electrostatic attraction, which could stabilize the structure of DNA and make the CD intensity of ctDNA increase. The above conclusion obtained from CD spectra was consistent with that of absorption spectra.

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