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ORIGINAL PAPER

New Fluorescent Antitumour Cisplatin Analogue Complexes. Study of the Characteristics of their Binding to DNA by Flow Injection Analysis

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Abstract The flow injection technique is applied to study the binding to DNA of new platinum complexes—E_1: ethylenediaminechlorocholylglycinateplatinum(II): [PtCl(CG)(en)], $C_{54}H_{92}O_{12}Pt$ and E_2: ethylenediaminebischolylglycinateplatinum(II): [Pt$(CG)_2$(en)], $C_{28}H_{50}ClN_3O_6Pt$—derived from cisplatin in which the exchangeable ligands were replaced by bile acids, such that these anticancer drugs have less toxicity and less resistance is developed towards them. Both compounds are fluorescent and their fluorescence is enhanced when they form adducts with DNA, a property that is extremely useful for monitoring the cytotoxic activity and their mechanisms of action. The binding parameters to DNA of E_1 [apparent intrinsic binding constant K_{E1}: $(11.2\pm0.4)\times10^3$ M^{-1} and maximum number of binding sites per nucleotide, n_{E1}: $0.121\pm2\times10^{-3}$) and E_2 (K_{E2}: $9.2\pm0.7)\times10^3$ M^{-1} and n_{E2} $0.098\pm2\times10^{-3}$] were determined following the Scatchard method and the type of binding was studied experimentally through the modifications introduced by each of the compounds into the ethidium bromide–DNA bond.

Keywords Ethylenediaminechlorocholylglycinateplatinum (II) · Ethylenediaminebischolylglycinateplatinum(II) · Ethidium bromide · DNA · Flow injection analysis · Fluorimetry

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Introduction

Since the discovery by Rosenberg et al. [1] of the anticancer activity of platinum complexes, cis-diamminedichloroplatinum(II) (cisplatin) [2] has been one of the most widely used anticancer agents, and the great success of its use in the clinical treatment of human malignancies has stimulated research in the area of inorganic antitumour agents [3, 4].

The design of new metalloorganic anticancer drugs, with a broader spectrum of activity than those already existing and with fewer side effects for patients, requires exhaustive knowledge of the mechanisms of drug transport to the biological target and of the interaction with this latter. In this regard, attempts have and continue to be made to find methods for monitoring the metabolic pathways and different aspects of the intracellular accumulation of metalloorganic compounds. Some of the techniques employed, not always with the expected success, are X-ray diffraction and atomic absorption spectrophotometry. Owing to their relative simplicity, fluorimetric methods with different types of markers, mainly lanthanides [5], have also had some success.

Molenaar et al. [6] used fluorescence microscopy to study the metabolic pathway and intracellular distribution of Pt compounds up to the time of their binding to DNA of living cells. They designed a derivative of (1-aminomethyl-1,2-ethylendiammine)-dichloroplatinum(II) with a diacetate of carboxyfluoresceine, which, although not fluorescent, is hydrolyzed by intracellular esterases to generate a fluorescent species that binds to different DNAs. In this way it was possible to perform a dynamic study in vivo that afforded knowledge about the zones of accumulation of Pt complexes by recording images over time.

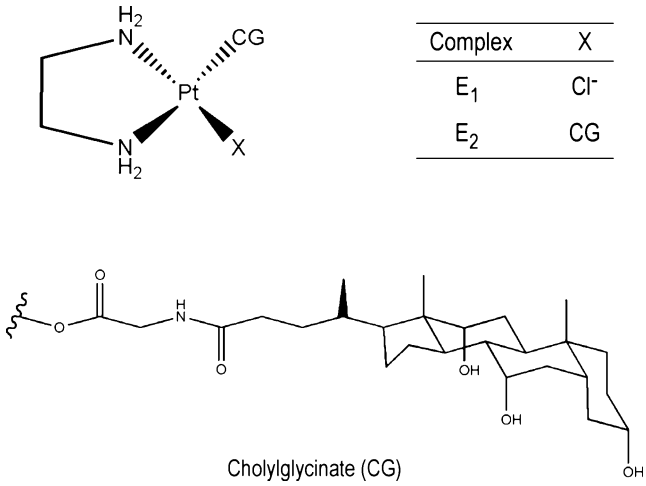
This new technique, with a very promising future, has the drawback that to date the complexes used, even though

of use owing to the similarity of their structures to those of Pt derivatives, contain fluorophores that may alter their extracellular and intracellular behaviour in some way. The ideal situation would be to have available Pt compounds that, having antitumour activity, are both fluorescent and have the ability to increase their fluorescence upon forming adducts with DNA.

To gain insight into the binding characteristics, the apparent binding constant and the number of binding sites per nucleotide between an antitumour complex and DNA, and to determine the type of bond (covalent or not, intercalating or not) that such complexes forms, the most widely used technique consists of studying the competition exerted by such compounds on the ethidium bromide bond by measuring the fluorescence of ethidium free in solution and that of the Etd–DNA complex. This method, the Scatchard method [7], is widely agreed to be useful [8] and has been employed successfully for cisplatin by using the flow injection analysis (FIA) technique [9].

In light of the usefulness of the FIA technique for study of the binding characteristics of compounds that bind to DNA, regardless of whether they have antitumour characteristics or not, by fluorometric monitoring of the Edt^+ bond with DNA, in the present work this analytical methodology was applied to two new Pt (II) complexes synthesised by a multidisciplinary team at the University of Salamanca [10]. Both of them are classic derivatives of cisplatin in which the exchangeable ligands have been replaced by bile acids, which are less labile than chloride, with an amphiphilic nature and with vectoriality towards DNA, such that they would presumably act as anticancer drugs with less toxicity and with less resistance to them (Scheme 1).

Although the above research group has already synthesised other Pt complexes with cytostatic activity [11–14], none of them shares common characteristics with the two compounds addressed here: these emit fluorescence at room temperature, which is enhanced when they bind to DNA. This property, together with their cytostatic activity, means that they can be seen as extremely useful tools for monitoring the cytotoxic activity of Pt(II) complexes, affording better insight into their mechanisms of action.



Scheme 1 Structures of the E_1 and E_2 complexes

Experimental

Reagents

Complex E_1 Ethylene diaminechlorocholylglycinateplatinum(II): [PtCl(CG)(en)] $C_{54}H_{92}O_{12}Pt$ was obtained from $[Pt(en)_2Cl_2]$ with NaCG, liquid–liquid extraction in C_{18} cartridges, and purification by thin layer chromatography (TLC). Its characterisation was accomplished with MNR. The compound is a yellowish solid, soluble in water, methanol and dimethylsulphoxide. The quantum yield in water, with respect to quinine sulphate, was $\Phi_{E2}=0.11$.

Complex E_2 Ethylenediaminebischolylglycinateplatinum (II), $[Pt(CG)_2(en)]$, $C_{28}H_{50}ClN_3O_6Pt$. This was also obtained from [Pt $(en)_2$ (Cl) $_2$] but with a larger amount of NaCG. Purification was carried out with TLC and the compound was characterised by NMR. The compound is a yellow solid, and it is soluble in water, methanol, and dimethylsulphoxide. The quantum yield in water with respect to quinine sulphate was $\Phi_{E2}=0.10$.

A solution 0.05 M of Tris–HCl [(Tris(hydroxymethyl aminomethane) $NH_2(CH_2OH)_3$, >99% (Fisher Chemicals)], at pH=7.5.

A concentrated solution of 2.5 M ethidium bromide in Tris–HCl buffer. The exact concentration was determined by dilution and measurement of absorbance at 480 nm (ε: 5.6×10^3 M^{-1} cm^{-1}). Ethidium bromide is an irritant and is potentially mutagenic and hence should be handled with care.

A solution of DNA 3.0×10^{-1}M (Sigma, 9007-49-2), prepared by dissolving the disodium salt in Tris–HCl. By diluting this with buffer, the exact concentration in base-pairs (bp) was determined, measuring absorbance at 260 nm (ε: 1.3×10^4 M^{-1} cm^{-1}).

The working solutions of the different complexes of platinum were prepared from the solid product dissolved under conditions of stirring in Tris–HCl buffer.

Apparatus and materials

Minipuls HP4 (Gilson, France) peristaltic pumps with silicone or vinyl pump tubes and PTFE sample-injection valves (Rheodyne) were used. Detection was performed with a RF-5000 spectrofluorimeter (Shimadzu, Japan) fitted

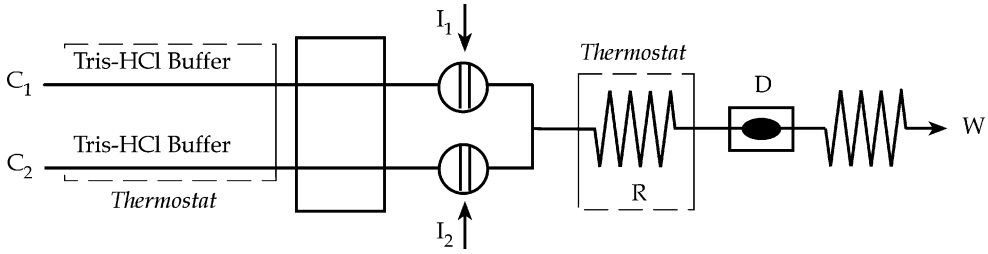


Fig. 1 FI system in the merging zones and stopped-flow method. C_1 and C_2 Channels, I_1 and I_2 simultaneously operated injection valves, R reaction coil, D detector, W waste

with a DR-15 data processor and a 25-µl flow-cell (Hellma, Germany). PTFE tubing of 0.5 mm internal diameter with standard tube fittings, connectors (Upchurch Scientific, Inc., USA), and a water bath thermostatted at 27°C were also used.

Flow system

The flow injection systems used in the experiments are shown in Fig. 1.

The system has two channels, C_1 and C_2, through which the solution of 0.05 M Tris–HCl buffer solution, pH 7.5, with 10 mM NaCl, which acts as carrier, flows at rates of 1.0 ml min^{-1}. Before the merging of both channels, a 10 µl bolus of the solution of the corresponding agent (V_1) and 20 µl of the DNA solution (V_2) are injected simultaneously into C_1 and C_2, respectively. Both solutions merge in a reaction coil of 50 cm length thermostatted at 27°C, like all the solutions introduced into the system. The reagent bolus is mixed with the central portion of the DNA (V_1 is inserted in V_2), favouring a reaction between both. When the 12 µl flow cell is reached, which occurs 6 s after the simultaneous injection, the pumps are stopped, and the recording of the intensity of the emitted fluorescence is continued. If EtBr is injected, the wavelengths are λ_{ex}=501 nm and λ_{em}=586 nm, but if either of the complexes is injected, the values are λ_{ex}=350 nm and λ_{em}=437 nm. When the flow is stopped, a 3 m long restriction coil is connected to the exit from the cell in order to minimise the inertia of the flow after the stop. The stopped-flow mode allows the evolution of the reaction to be recorded over time.

Results and discussion

Fluorescence spectra

In order to choose the optimum conditions for fluorescence measurements, multispectra of solutions of E_1 and E_2 complex were obtained. EtBr shows fluorescence spectra with excitation and emission maxima at λ_{ex}=501 nm and λ_{em}=586 nm, which were chosen as the working wavelengths for the reagent.

The E_1 and E_2 complexes emit broad emission bands, with maxima at 384 and 437 nm respectively for their corresponding wavelengths of maximum excitation (301 nm for E_1 and 350 nm for E_2, and it was observed that the maximum emission of E_1 was greater (Fig. 2). However, since UV light was used with a fixed wavelength of 350 nm in the studies of cytotoxicity and flow cytometry to check the antitumour activity of the complexes, the wavelengths of λ_{ex}=350 and λ_{em}=437 nm were used as the most appropriate for carrying out the measurements. Under such conditions, the emission spectra of both complexes were similar, although the emission of E_1 was more intense. An important observation was that neither of the complexes emitted at the wavelengths of the maximum fluorescence of EtdBr.

The salmon DNA used in the studies did not fluoresce either at the optimum wavelengths for EtdBr or at those chosen for both platinum complexes.

Optimisation of the experimental conditions

Preliminary studies

The kinetic evolution of the reactions of DNA with the E_1 and E_2 coordination complexes and ethidium bromide was

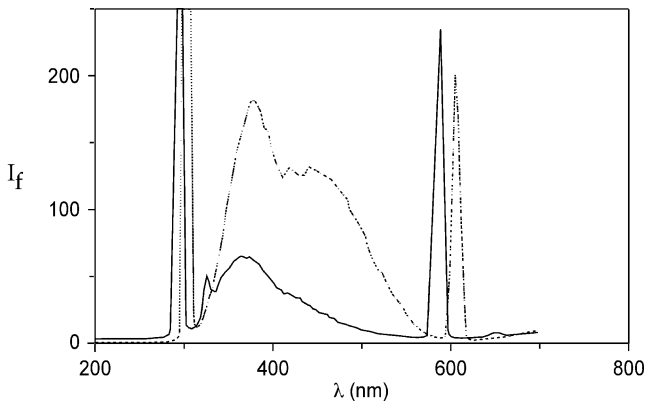


Fig. 2 Fluorescence emission spectra of the E_1 and E_2 complexes. 8×10^{-4} M solutions of each complex in Tris–HCl buffer: E_1 (*broken line*), λ_{ex}=301 nm, and E_2 (*solid line*), λ_{ex}=350 nm; slit widths: 5 nm

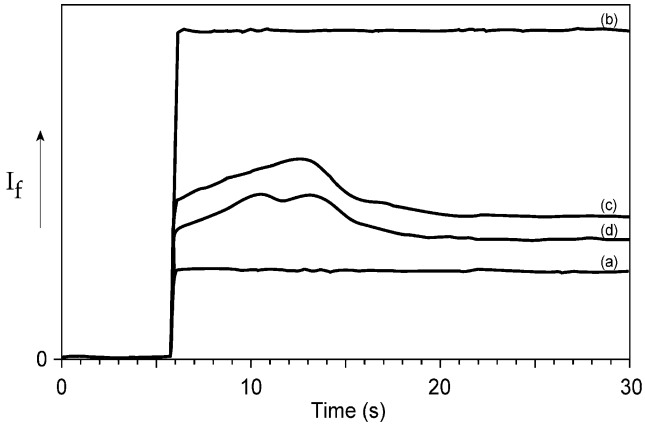


Fig. 3 Comparative scheme of the kinetic development of the reactions at 27°C: *(a)* EtdBr, *(b)* Etd–DNA, *(c)* E_1–DNA, *(d)* E_2–DNA

studied in the flow systems shown schematically in Fig. 1, operating in the merging-zones and stopped-flow modes. Figure 3 shows the kinetic development obtained in the presence of excess DNA so that this latter would not be the limiting reagent.

When only EtBr was injected (Fig. 3a), the absence of reaction was confirmed by the recording of a constant signal with greater intensity than that corresponding to the baseline recorded before flow had been stopped. This indicated not only that EtBr was stable but also that the time at which flow was stopped was optimum and, more importantly, that there was no inertia in the flow after the stop.

The injection of EtBr and DNA led to the formation of an adduct, which was recorded as an increase in the fluorescence intensity emitted (Fig. 3b). The reaction showed fast kinetics, since at the time when flow was stopped the maximum signal had already been reached (the reaction time was less than 6 s).

The reaction kinetics of the E_1 (Fig. 3c) and E_2 (Fig. 3d) complexes with DNA, which also lead to the formation of adducts, were more complex, reaching constant fluorescence values as from 24 s of reaction time. These reactions probably occur in successive stages of approach, binding and reorganisation.

The E_1–DNA reaction

Binding of [PtCl(CG)(en)] to DNA. Scatchards plots

In a solution containing a given concentration of DNA and the fluorescent complex E_1 in excess, the observed fluorescence intensity is the sum of the fluorescence intensities due to the free E_1, I_f, and the E_1 bound to the DNA (E_1–DNA complex), I_b [8].

$$I_{obs} = I_b + I_f \tag{1}$$

This equation can be written as:

$$I_{obs} = K_b C_b + K_f C_f \tag{2}$$

That is,

$$I_{obs} = K_b C_b + K_f (C_{total} - C_b) \tag{3}$$

where K_f and K_b are the proportionality constants for free E_1 and that bound to the DNA, respectively; C is the total concentration of E_1, corresponding to the initial one. This equation can be used to determine the amounts of free complex and that bind to the DNA, which allows study of the antitumour efficiency of the complex. The proportionality constants and C_b can be determined experimentally; the determination of C_b requires extrapolation to high added DNA concentrations.

Many methods have been developed for the determination of the amount, r, of complex bound per unit of DNA and the choice of one or another depends on the mode of binding. For simple binding models, the data can be fitted to the following Scatchard equation [7]:

$$\frac{r}{C_f} = nK - rK \tag{4}$$

where r is the ratio between the concentration of the E_1–DNA complex and the concentration of DNA, expressed in base-pairs (bp) or nucleotides; when this ratio is maximum it is designated n. K is the intrinsic binding constant between E_1 and DNA.

By plotting the values of r/C_f against r, it is possible to fit them to a straight line. From this equation, K—the slope value—can be obtained, together with the value of n, which is the value taken by r at the intersection of the fitting line with the abscissa. n represents the maximum number of binding sites to each DNA double helix bp.

Determination of the proportionality constant of the free E_1 complex, $K_f^{E_1}$, and when bound to DNA, $K_b^{E_1}$

First, we determined the proportionality constant, $K_f^{E_1}$, between the concentration of the E_1 complex and the intensity of the fluorescence emitted at the indicated wavelengths. The experimental study was conducted in the flow system depicted in Fig. 2, in which 10 μl of the platinum complex E_1 was injected at concentrations ranging between 20 and 900 mM, measuring the fluorescence intensity at λ_{ex}=350 nm and λ_{em}=437 nm. Channel C_2 contained the buffer solution and no solution was injected into it.

The variation in I_f with the concentration of E_1 can be fitted to the straight line equation: I_f=(0.36±0.01)+

$(6.5\times10^4\pm20)$ M^{-1} $[E_1]$; r^2=1.000, n=10, whose slope is the proportionality constant of free E_1:

$$K_f^{E1} = \left(6.500 \times 10^4 \pm 20\right)M^{-1}$$

To obtain the value of the proportionality constant between the fluorescence intensity and the concentration of the bound complex, $K_b^{E_1}$, equivalent to the concentration of E_1–DNA adduct, ten sets of experiments were carried out in the flow system shown schematically in Fig. 1, each at a different concentration of E_1 between 2.0 and 36 μM, in all cases varying the DNA concentration between 4.0 and 500 μM. The pairs of values obtained ([E_1–DNA], I_f) are plotted in Fig. 4.

For each concentration of E_1, the fluorescence intensity increased with the DNA concentration, since increasingly more adduct was formed, whose fluorescence was more intense than that of the free E_1 complex. When all the E_1 added initially formed the adduct, the fluorescence intensity became almost constant. In the flat segment of the curve the I_f value is obtained, corresponding to the concentration of the E_1–DNA adduct, which in each series corresponds to the concentration of E_1 injected. It should be noted, however, that in the experiments in which the concentrations of E_1 injected were ≥ to 22 μM, the final segment of the curve did not maintain a constant value: a line was obtained whose slope increased slightly with the concentration of DNA injected. This is because at those DNA concentrations, that remaining free in solution emits appreciable fluorescence at the working wavelengths. In these cases, the value of I_f of the adduct was obtained at the intersection between the final segment and the tangent traced through the points close to the stoichiometric point.

The values of I_f obtained for each maximum concentration of adduct, $C_b^{E_1}$, have a linear relationship:

$$I_f = (0.03 \pm 0.09) + \left(3.979 \times 10^6 \pm 9 \times 10^3\right)M^{-1}[E_1];$$

$$r^2 = 1.000,\ n = 11$$

$$K_b^{E1} = \left(3.979 \times 10^6 \pm 9 \times 10^3\right)M^{-1}$$

Binding parameters of E_1 to DNA

With knowledge of the values of $K_f^{E_1}$ and $K_b^{E_1}$, it is possible to perform the experiments required to determine the binding parameters between E_1 and DNA. 30 μl of solutions of DNA at concentrations of 120 or 180 μM are injected simultaneously with 10 μl of E_1 at varying concentrations between 41 and 558 μM for the first series and between 58 and 776 μM for the second one. Under these conditions, in the reaction bolus, obtained by the merging of the two injections, the E_1–DNA adducts and the free complex will always coexist, it never being possible for the latter to become completely transformed into adduct. The I_f values measured are therefore the sum of the fluorescence due to the free E_1 complex and the E_1–DNA monoadduct formed.

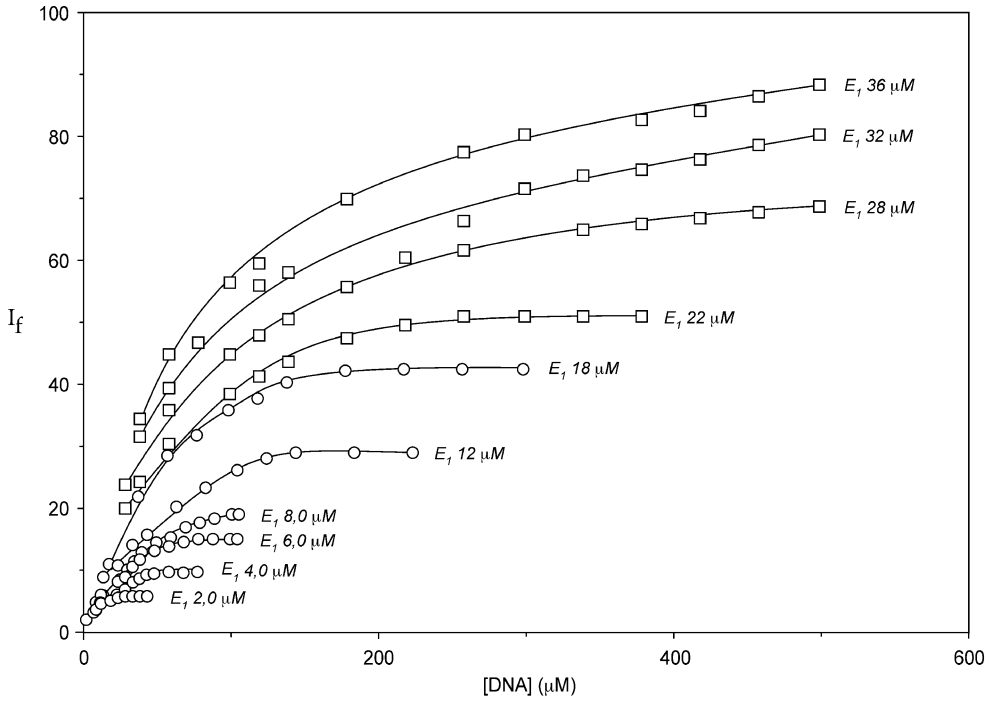


Fig. 4 Variation in fluorescence intensity with the concentration of E_1 and of DNA. C_1 and C_2: 0.05 M solution of Tris–HCl buffer, pH 7.5, with 10 mM NaCl; Q_1=Q_2=0.50 ml min^{-1}; I_1 10 μl of E_1 solutions of different concentrations between 4.0 and 500 μM; I_2 30 μl of DNA solutions with concentrations varying between 2.0 and 36 μM; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}= 350 nm and λ_{em}=437 nm

Table 1 Determination of the E_1–DNA binding parameters by the Scatchard method

	I_f	[E_1] (μM)			r	r/C_f (μM^{-1})
		C_0	C_b	C_f		
DNA 120 μM	12.16	41	4.3	36.7	0.036	9.7×10^{-4}
	14.63	52	5.1	46.9	0.042	9.0×10^{-4}
	15.35	56	5.3	50.7	0.044	8.7×10^{-4}
	18.86	76	6.3	69.7	0.052	7.5×10^{-4}
	24.92	114	7.9	106.1	0.066	6.2×10^{-4}
	33.77	189	9.7	179.3	0.081	4.5×10^{-4}
	39.49	250	10.5	239.5	0.087	3.6×10^{-4}
	53.47	417	11.9	405.1	0.099	2.4×10^{-4}
	59.84	502	12.3	489.7	0.102	2.1×10^{-4}
	64.40	558	12.7	545.3	0.106	1.9×10^{-4}
DNA 180 μM	20.82	58	7.7	50.3	0.043	8.5×10^{-4}
	27.50	87	9.9	77.1	0.055	7.1×10^{-4}
	36.48	139	12.4	126.6	0.069	5.4×10^{-4}
	40.68	166	13.5	152.5	0.075	4.9×10^{-4}
	46.92	215	14.9	200.1	0.083	4.1×10^{-4}
	52.00	258	15.9	242.1	0.088	3.6×10^{-4}
	57.53	316	16.7	299.3	0.093	3.1×10^{-4}
	70.01	474	17.7	456.3	0.098	2.2×10^{-4}
	79.90	585	18.9	566.1	0.105	1.9×10^{-4}
	93.62	776	19.5	756.5	0.108	1.4×10^{-4}

Applying the linearization method of Scatchard (Table 1), and plotting both series together (Fig. 5), the equation of a straight line is obtained:

$$r/C_f = \left(1.34\times10^3 \pm 30\right)M^{-1} - \left(1.12\times10^4 \pm 8\times10^2\right)M^{-1} r;$$

$$r^2 = 0.995, \quad n = 20$$

The slope and the intersection with the abscissa axis provide, respectively, the values of the binding parameters of the E_1 complex, [PtCl(en)CG], to DNA to form the corresponding adduct (Table 2).

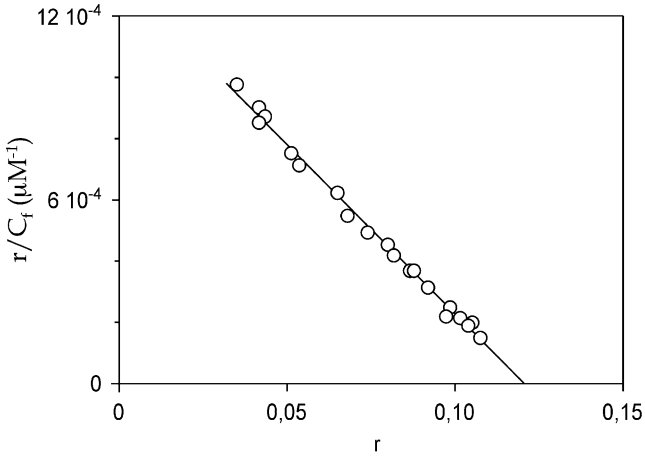


Fig. 5 Scatchard plots for E_1–DNA binding. C_1 and C_2 0.05 M solution of Tris–HCl buffer, pH 7.5 with 10 mM NaCl; Q_1=Q_2= 0.50 ml min^{-1}; I_1 10 μl of solutions of E_1 of varying concentrations between 40 and 800 μM; I_2 30 μl of DNA solutions with concentrations of 120 or 180 μM; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}=350 nm and λ_{em}=437 nm

Mode of binding of E_1 to DNA

Once the binding parameters—K_{E_1} and n_{E_1}—were known, we attempted to determine the type of binding between the complex and DNA. To do so, we studied experimentally how Etd–DNA binding is affected in the presence of E_1. Working with the usual flow system, Fig. 2, at the maximum emission wavelength of the Etd–DNA adduct, λ_{ex}=501 nm and λ_{em}=586 nm, it was possible to determine the modifications occurring in the adduct since neither E_1 nor its DNA adduct emit detectable fluorescence. Thus, 30 μl of 30 μM DNA and 10 μl of solutions of Etd with concentrations in the 7.8 to 20 μM range, which in turn were free of or contained different concentrations of E_1 (between 10 and 60 μM), were injected simultaneously. Under these conditions, both Etd^+ and E_1 reacted at the same time with DNA, such

Table 2 Binding parameters of the E_1, [PtCl(CG)(en)], complex to DNA

Binding parameters E_1−DNA		
K_{E_1}	$(1.12\times10^4\pm4\times10^2)$ M^{-1}	$(1.12\times10^{-2}\pm4\times10^{-4})$ μM^{-1}
n_{E_1}	$(0.121\pm2\times10^{-3})$	

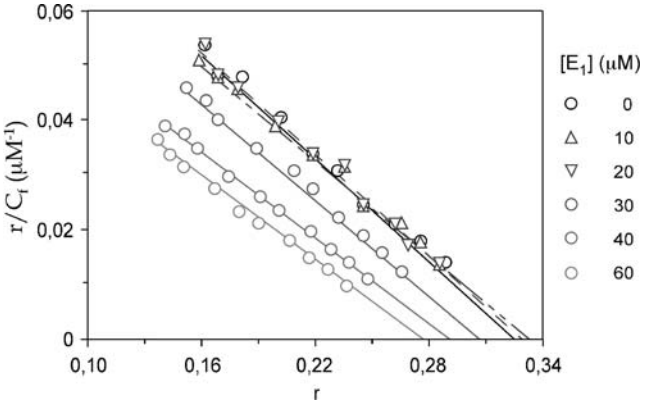


Fig. 6 Scatchard plots with ethidium bromide in the presence of E_1. C_1 and C_2 0.05 M Tris–HCl buffer solution at pH 7.5 with 10 mM NaCl; Q_1=Q_2=0.50 ml min^{-1}; I_1 10 μl of solutions of EtdBr of varying concentrations between 7.8 and 30 μM, containing E_1 at varying concentrations between 0 and 60 μM; I_2 30 μl of 30 μM DNA; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}=350 nm and λ_{em}=437 nm

that the modification of I_f depends on the type of E_1–DNA bond and on the sites where it occurs.

The experimental data were linearised using the Scatchard method and from the straight lines thus obtained (Fig. 6) it was possible to deduce the values of the Etd–DNA binding constant, K_{obs}, and of the number of binding sites per nucleotide, n, with a reliability of 95% (Table 3).

For this probability, it may be affirmed that up to values lower than 20 μM of E_1, the slopes, K_{obs}, and the value of n do not different significantly. Accordingly, in this concentration range E_1, [PtCl(CG)(en)], behaves as a C class compound, the class to which cisplatin belongs; i.e., the covalent bond predominates over any other type of interaction. However, for concentrations equal to or greater than 30 μM the slopes differ significantly, decreasing with increasing concentration. The behaviour of E_1 at higher concentrations tended towards a B type behaviour; apart from the covalent bond the complex studied establishes other types of interaction with DNA, mainly hydrogen bridges, and Van der Waals forces, competitive inhibition with the Etd–DNA binding taking place.

The E_2–DNA reaction

Binding of [PtCl(CG)$_2$(en)] to DNA

The platinum complex designated E_2, [PtCl(CG)$_2$(en)], like its analogue E_1 ([PtCl(CG)(en)]), is fluorescent at λ_{ex}= 350 nm and λ_{em}=437 nm and when it binds to DNA it forms adducts in which the fluorescence is enhanced with respect to the free compound. This serves, as in the case of E_1, to determine the binding parameters of E_2 with DNA with no need to use ethidium bromide as a fluorescent marker.

Proportionality constant of the free E_2 complex, $K_f^{E_2}$, and when bound to DNA, $K_b^{E_2}$

Following a similar procedure to that used previously with the E_1 complex, we first determined the proportionality constant, $K_f^{E_2}$, between the E_2 complex and the intensity of the fluorescence emitted at the indicated wavelengths. In the flow system depicted in Fig. 1, 10 μl of solutions of E_2 at varying concentrations ranging from 30 to 860 μM were passed through channel C_1, while buffer solution was passed through C_2 with no injection of any solution. Under these conditions, the values of I_f were recorded and it was observed that the pairs of values ([E_2], I_f) fitted a straight line with an equation of r^2=1,000, n=10, whose slope is the proportionality constant sought.

$$K_f^{E_2} = 4.90 \times 10^4 \pm 2 \times 10^2 \mathrm{M}^{-1}$$

This value is slightly lower than that obtained for the E_1 complex at the same working wavelengths the intrinsic fluorescence of E_2 is therefore greater than that of E_1.

Knowing $K_f^{E_2}$, we studied the value of the proportionality constant between the concentration of the bound complex or E_2–DNA monoadduct and the fluorescence intensity, $K_b^{E_2}$. In the flow system used, 10 μl of solutions of E_2, at different concentrations in the 3–52 μM range, were injected into C_1 and at the same time, but in C_2, 30 μl of DNA between 4.0 and 720 μM for each concentration of E_2.

The values of I_f measured for each series (Fig. 7) increased with the concentration of DNA until an almost

Table 3 Binding parameters between DNA and ethidium bromide in the presence of the E_1 platinum complex

[E_1] (μM)	Equation	r^2	K_{obs} (μM^{-1})	n
–	r/C_f=(1.02×10^5±5×10^3)–(3.11×10^5±2×10^3) r	0.991	0.311±2×10^{-3}	0.327±9×10^{-3}
10	r/C_f=(9.6×10^4±5×10^3)–(2.9×10^5±2×10^4) r	0.992	0.29±0.02	0.332±9×10^{-3}
20	r/C_f=(1.02 10^5±6×10^3)–(3.1×10^5±3×10^4) r	0.990	0.31±0.03	0.324±9×10^{-3}
30	r/C_f=(9.0×10^4±3×10^3)–(2.9×10^5±1×10^4) r	0.996	0.29±0.01	0.306±5×10^{-3}
40	r/C_f=(7.6×10^4±2×10^3)–(2.6×10^5±1×10^4) r	0.998	0.26±0.01	0.290±4×10^{-3}
60	r/C_f=(7.1×10^4±3×10^3)–(2.6×10^5±1×10^4) r	0.995	0.26±0.01	0.277±5×10^{-3}

The equation is given in molar concentrations; K_{obs} intrinsic binding constant; n number of binding sites per nucleotide or bp.

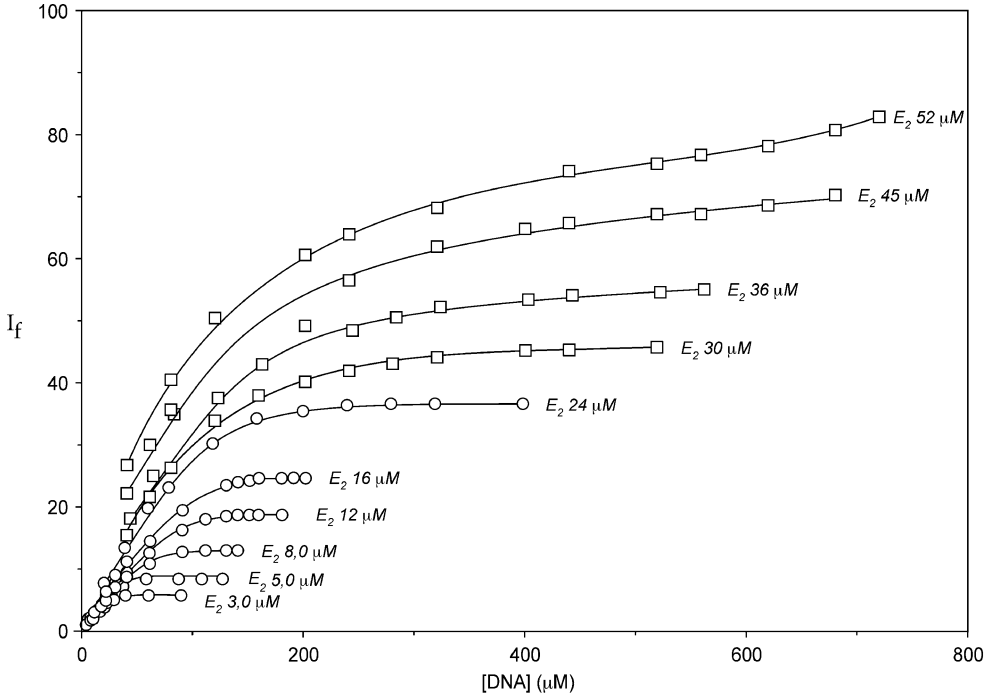


Fig. 7 Variation in fluorescence intensity with the concentration of E_2 and of DNA. C_1 and C_2 0.05 M solution of Tris–HCl buffer, pH 7.5, with 10 mM NaCl; $Q_1=Q_2=0.50$ ml min^{-1}; I_1 10 μl of E_2 solutions of different concentrations between 3.0 and 52 μM; I_2 30 μL of DNA solutions with concentrations varying between 4.0 and 720 μM; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}= 350 nm and λ_{em}=437 nm

constant value was reached; it may thus be affirmed that the concentration of the adduct formed is maximum and equal to the initial concentration of E_2. When the concentrations of E_2 were ≥ 30 μM, the amount of DNA added towards the end of the series was considerable, such that the curve did not become flat but, instead, showed a slight ascent as a result of the fluorescence of the DNA; the value of I_f was determined with the intersection of the straight lines determined by the pairs of experimental values prior to and after the fixed point of stoichiometry.

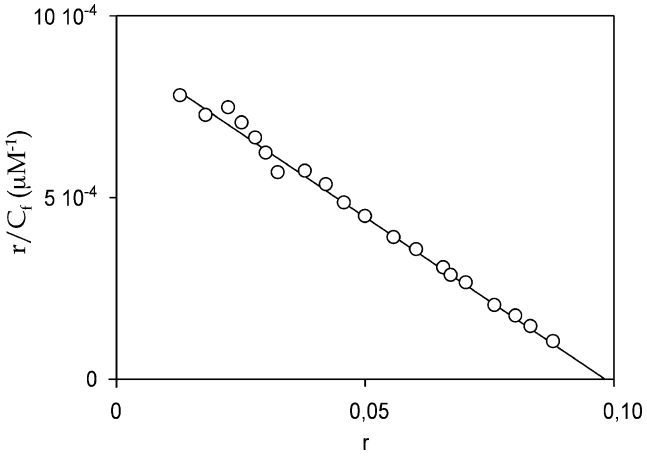


Fig. 8 Scatchard plot for E_2–DNA binding. C_1 and C_2 0.05 M solution of Tris–HCl buffer, pH 7.5 with 10 mM NaCl; $Q_1=Q_2=$ 0.50 ml min^{-1}; I_1 10 μl of solutions of E_2 of varying concentrations between 16 and 888 μM; I_2 30 μl of solutions of DNA with concentrations of 90 and 140 μM; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}=350 nm, y λ_{em}=437 nm

The values of I_f for each concentration of E_2 have a linear correlation:

$$I_f = (1.23 \pm 0.08) + \left(1.469 \times 10^6 \pm 3 \times 10^3\right)\mathrm{M}^{-1}[\mathrm{E_2}]$$
$$r^2 = 1.000,\ n = 11$$
$$K_b^{E2} = \left(1.469 \times 10^6 \pm 3 \times 10^3\right)\mathrm{M}^{-1}$$

It may be seen that the value of the slope, $K_b^{E_2}$, is lower than that obtained for the E_1–DNA adduct.

Binding parameters of E_2 to DNA

With knowledge of the values of $K_f^{E_2}$ and $K_b^{E_2}$, we carried out experiments aimed at collecting the Scatchard plot that would allow us to know the E_2–DNA binding parameters: K_{E_2} and n_{E_2}. Thus, two series of measurements were carried out, each of them at a different DNA concentration: 90 and 140 μM. In each series, 30 μl of the corresponding solution of DNA was injected simultaneously with 10 μl of solutions of E_2 at varying concentrations between 26 and 888 μM for the first series and between 16 and 666 μM for the second one. The concentrations of both reagents were

Table 4 Binding parameters of the E_2, [Pt(CG)$_2$(en)], complex to DNA

Binding parameters E_2−DNA		
K_{E_2}	(9.2±0.7) 10^3 M^{-1}	(9.2±0.7)·10^{-3} μM^{-1}
n_{E_2}	(0.098±2×10^{-3})	

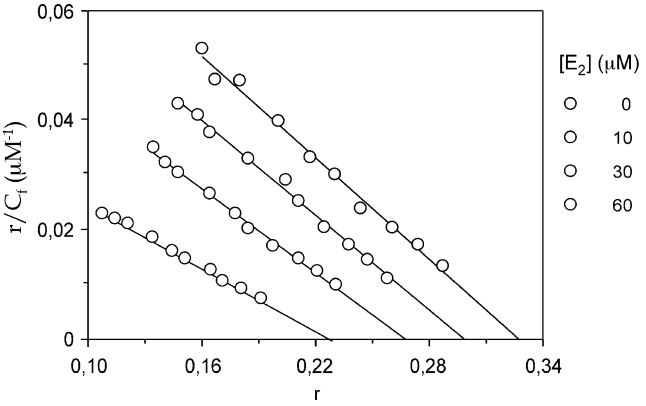


Fig. 9 Scatchard plots for ethidium bromide in the presence of E_2. C_1 and C_2 0.05 M solution of Tris–HCl buffer, pH 7.5, with 10 mM NaCl; Q_1=Q_2=0,50 ml min^{-1}; I_1 10 μl of solutions of EtdBr of varying concentrations between 7.8 and 30 μM and E_2 between 0 and 60 μM; I_2 30 μl of DNA at 30 μM concentrations 30 μM; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}=350 nm, y λ_{em}=437 nm

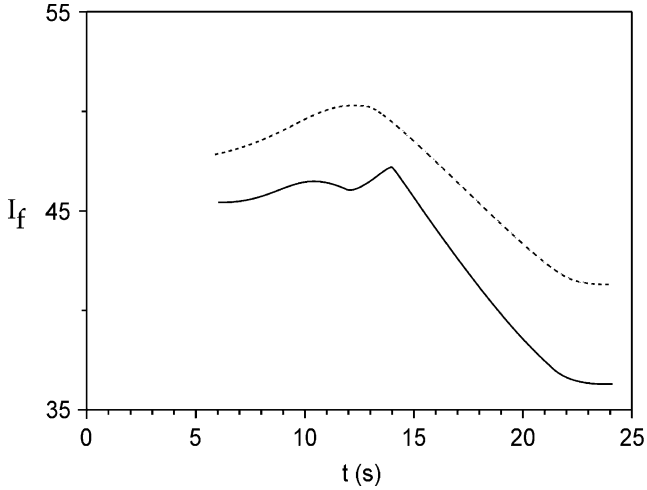


Fig. 10 Kinetic evolution of the binding of the E_1 and E_2 complexes to DNA: E_1–DNA (*broken line*) and E_2–DNA (*solid line*). C_1 and C_2: 0.05 M solution of Tris–HCl buffer, pH 7.5, with 10 mM NaCl; Q_1=Q_2=0.50 ml min^{-1}; I_1 10 μL of solutions of 18 μM E_1 or of 24 μM E_2; I_2 30 μl of solutions of DNA at concentrations of 200 or 300 μM, respectively; R reactor coil, 200 cm in length. T 27°C; D λ_{ex}=350 nm, y λ_{em}=437 nm

such that at all times the E_2–DNA adduct and the free complex coexisted in the reaction bolus.

The experimental data obtained fitted a Scatchard plot (Fig. 8) with an equation of:

$$r/C_f = (1.34\times10^3 \pm 30)\mathrm{M}^{-1} - (1.12\times10^4 \pm 8\times10^2)\mathrm{M}^{-1} r;$$
$$r^2 = 0.995,\ n = 20$$

The slope and intersection with the abscissa axis provided, respectively, the values of the binding parameters of the E_2 complex [PtCl(CG)$_2$(en)], to the DNA to form the corresponding adduct (Table 4).

The apparent binding constant of E_2 is slightly lower than that of E_1, as is the case of the values of n.

Mode of binding of E_2 to DNA

To determine whether the E_2 complex bound to DNA in the same way as its homologue, E_1, we obtained Scatchard plots in the presence of EtdBr, which acts as a fluorescent marker of certain binding sites.

Thirty microliters of 30 μM DNA and 10 μl of solutions with varying concentrations of E_2 in the 10–60 μM range and of EtdBr between 7.8 and 30.0 μM were injected simultaneously into the flow system. The detection wavelengths were optimum for the ethidium bromide and its adducts with DNA: λ_{ex}=350 nm and λ_{em}=437 nm.

The data calculated for the Scatchard plot are shown in Fig. 9. The set of straight lines obtained shows that both the slope, K_{obs}, and the intersection with the abscissa axis, n, decrease when the concentration of the E_2 complex increases (Table 5).

The E_2 complex, [PtCl(CG)$_2$(en)], clearly belongs to class B, a set of compounds that non-competitively inhibit the binding of Etd$^+$ to DNA [7]. E_2 binds covalently to the DNA at sites other than those occupied by the ethidium, but it prevents the binding of the latter as a result of other interactions with the DNA molecule.

Kinetic studies

Although the E_1 and E_2 complexes only differ in the nature of one of their ligands, Cl^- and cholylglycinate (CG) respectively, both hydrolysable in aqueous medium, the studies with ethidium bromide to determine the mode of

Table 5 Binding parameters between DNA and ethidium bromide in the presence of the E_2 platinum complex

[E_2] (μM)	Equation	r^2	K_{obs} (μM^{-1})	n
–	r/C_f=(1.02×10^5±5×10^3)–(3.11×10^5±2×10^3) r	0.991	0.311±2×10^{-3}	0.327±9×10^{-3}
10	r/C_f=(8.07×10^4±4×10^3)–(2.9×10^5±2×10^4) r	0.992	0.29±0.02	0.332±9×10^{-3}
30	r/C_f=(6.9×10^4±3×10^3)–(2.6×10^5±2×10^4) r	0.996	0.29±0.02	0.306±5×10^{-3}
60	r/C_f=(4.4×10^4±1×10^3)–(1.9×10^5±1×10^4) r	0.995	0.26±0.01	0.277±5×10^{-3}

The equation is given in molar concentrations; K_{obs} intrinsic binding constant; n number of binding sites per nucleotide or bp.

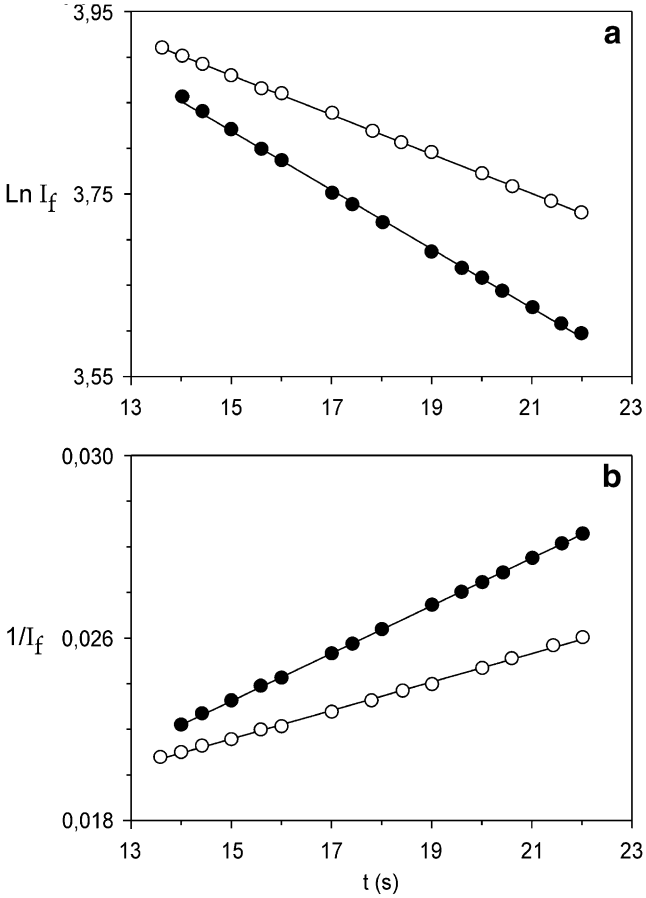


Fig. 11 Determination of the order of the reactions of the formation of the E_1–DNA (*open circle*) and E_2–DNA adducts (*filled circle*). Reaction order 1 (**a**) and order 2 (**b**). C_1 and C_2 0.05 M solution of Tris–HCl buffer, pH 7.5, with 10 mM NaCl; $Q_1=Q_2=0.50$ ml min^{-1}; I_1 10 μl of solutions of 18 μM E_1 or of 24 μM E_2; I_2 30 μl of solutions of DNA at concentrations of 200 or 300 μM, respectively; *R* reactor coil, 200 cm in length. *T* 27°C; *D* λ_{ex}=350 nm, *y* λ_{em}=437 nm

binding to DNA revealed differences. In order to obtain further information about the kinetics of the reactions of E_1 and E_2 with DNA, we performed a new study under a stopped flow regimen. 10 μl of a solution of E_1 with a concentration of 18 μM or of E_2 with a concentration of 24 μM and 30 μl of a DNA solution at 200 μM or 30 μM, respectively, were injected simultaneously into the system depicted in Fig. 1. Six seconds after injection, the peristaltic pump was stopped, when the reaction bolus was in the flow cell, where the fluorescence intensity emitted at λ_{ex}= 350 nm and λ_{em}=437 nm is recorded up to 24 s.

The shape of the curves recorded (Fig. 10) suggests that the binding between the DNA and the E_1 and E_2 complexes occurs in four clearly differentiated phases: a first step involving the approach of the species, during which I_f increases, followed by the formation of an initial bond (I_f almost constant), its rearrangement (I_f decreases), and consolidation of the bond (I_f constant) due to formation of the DNA-complex adduct.

The initial phases of approach and the establishment of some interaction between the DNA molecule and the corresponding complex must to a large extent depend on the hydrolysis processes of the complexes. For the sake of simplicity, it may be considered that this leads to the substitution of the most easily hydrolysable ligand by a water molecule:

$$[\mathrm{PtC1(CG)(en)}] \leftrightarrows [\mathrm{Pt(CG)(en)(H_2O)}]^+ + \mathrm{Cl}^-$$

$$[\mathrm{Pt(CG)_2(en)}] \leftrightarrows [\mathrm{Pt(CG)(en)(H_2O)}]^+ + \mathrm{CG}^-$$

However, the hydrolysis process must be more complex, in which the loss of the other CG^- ligand or deprotonation of the water molecule should not be ruled out.

The bond between the cationic species and the DNA is probably covalent, analogous to that formed between cisplatin and DNA, although in the case of the E_1 and E_2 complexes studied it seems certain that electrostatic interactions and hydrogen bridges would be involved.

The phase of rearrangement of the bond seems to be related to the establishment of new interactions of the cholylglycinate bound to the Pt with DNA sites that could even be on the strand opposite the one that Pt binds to. The fluorescence data corresponding to this phase fit first- and second-order reactions (Fig. 11), which is not uncommon in very fast reactions. The rate (pseudo) constant is greater with respect to the bond between the E_2 complex and the DNA (Table 6).

Although the precise mechanisms involved in the formation of the adducts of the complexes studied with DNA remain obscure, one undoubted practical aspect is that they are very stable since their fluorescence has a half-life of several weeks.

Table 6 Fitting equations of the order of formation of the E_1–DNA and E_2–DNA adducts

	Complex	Equation	r^2	k
Ord. 1	E_1	Ln I_f=−(2.15×10^{-2}±2×10^{-4}) t+(4.203±4×10^{-3})	1.000	(2.15×10^{-2}±2×10^{-4}) s^{-1}
	E_2	Ln I_f=−(3.25×10^{-2}±6×10^{-4}) t+(4.31±1×10^{-2})	0.999	(3.25×10^{-2}±6×10^{-4}) s^{-1}
Ord. 2	E_1	$1/I_f$=(4.72×10^{-4}±9×10^{-6}) t+(1.36×10^{-2}±2×10^{-4})	0.999	(4.72×10^{-4}±9×10^{-6}) l mol^{-1} s^{-1}
	E_2	$1/I_f$=(7.85×10^{-4}±3×10^{-6}) t+(1.014×10^{-2}±6×10^{-5})	1.000	(7.85×10^{-4}±3×10^{-6}) l mol^{-1} s^{-1}

t Time in seconds, *k* rate constant.

Conclusions

Flow Injection Analysis has proved to be an analytical technique of great use for the study of the binding parameters between different organic molecules, regardless of their antitumour activity, and DNA. The merging-zones mode allows the reactions to be studied as from initial times and, on injecting small amounts of reagents, antitumour complexes and DNA, it permits the reactions to be studied in continuous mode with low reagent consumption.

Additionally, the merging-zones mode with stopped flow allows the kinetic curves of the reactions to be obtained, whose shape provides some clues to the mechanisms of binding of the substances studied to DNA.

The methodology discussed was applied to study of the binding of two new platinum complexes, [PtCl(CG)(en)] and [Pt$(CG)_2$(en)], to salmon DNA. The peculiarity of the study is that the complexes are fluorescent and that their fluorescence is enhanced when they form adducts with DNA.

We determined the binding parameters of both complexes following the Scatchard procedure with fluorimetric detection. Using ethidium bromide, we established that the mode of binding is different for each complex. Thus, the [PtCl(CG)(en)] bond evolves from an essentially covalent bond (class C) at low concentrations to a more complex type of bond at higher concentrations: it establishes interactions through hydrogen bridges or Van der Waals forces able to competitively inhibit the intercalative ethidium bond (class B). The E_2 complex, [Pt$(CG)_2$(en)], in turn, always shows the same type of behaviour; class B: a covalent bond and electrostatic interactions.

Another difference between both complexes is DNA binding mechanism, which seems to occur in several phases: approach, initial binding, rearrangement and formation of the stable adduct. Unfortunately, the data on fluorescence intensity during the rearrangement phase do not allow us to discern whether both reactions are of the same order or not.

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