cis-Dichloro(α,ω-diamino carboxylate ethyl ester)palladium(II) as Palladium(II) *versus* Platinum(II) Model Anticancer Drugs: Synthesis, Solution Equilibria of Their Aqua, Hydroxo, and/or Chloro Species, and *in Vitro/in Vivo* DNA-Binding Properties

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cis-Dichloro(*d*,*l*-2,3-diaminopropionate ethyl ester)palladium(II), *cis*-[Pd(Etdap)Cl_2] (**I**), and *cis*-dichloro(*d*,*l*-2,4-diaminobutyrate ethyl ester)palladium(II), cis-[Pd(Etdab)Cl_2] (**II**), were synthesized and characterized by elemental analysis, IR spectroscopy, and TG-DTA thermal analysis. The equilibrium model and log β_{pqr} constants of hydrolytic species of **I** and **II** were also investigated by potentiometric methods (**I** = 0.15 M ($NaClO_4$) and 37 °C). Aqueous solutions of *cis*-[Pd(Etdaa)$(H_2O)_2$]$(ClO_4)_2$ (Etdaa = Etdap (**III**) or Etdab (**IV**)) were prepared by stoichiometric reaction of **I** or **II** with $AgClO_4$. Thus, log β_{pqr} of the corresponding *cis*-aquahydroxo (*pqr* = 1,0,−1) and di(μ-hydroxo) (*pqr* = 2,0,−2) species were obtained from $E(H^+)$ data of alkalimetric titrations of solutions of **III** or **IV**. Such constants were then used as fixed values to obtain log β_{pqr} of chloro-containing species (*cis*-dichloro (1,2,0), *cis*-chloroaqua (1,1,0), and *cis*-chlorohydroxo (1,1,−1)) from $E(H^+)$ and $E(Cl^-)$ data pairs simultaneously obtained by titration of solutions of **III** or **IV** with NaOH and NaCl. All log β_{pqr} were fitted by the SUPERQUAD program. Appropriate log β_{pqr} sets give good simulations of experimental titration curves and several species distribution diagrams. In addition the interaction of **I** and/or **II** with DNA *in vitro* and *in vivo* was studied by various methods. UV spectral data and melting and renaturation curves indicate that both compounds destabilize the DNA helicoidal structure. Compound **II** disrupts *in vivo* the structure and transcription process of polytene chromosomes of *Drosophila hydei*.

Introduction

Research into *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) and related compounds has determined several structural features that favor anticancer activity.[2,3] Most active Pt(II) drugs are neutral at plasma pH and chloride ion concentration (104 mM) but give positively charged species upon hydrolytic reactions at physiological pH and low $[Cl^-]$ = 4 mM within cells. Consequently, many active Pt(II) drugs have two *cis* leaving groups (Cl^-, NO_3^-, dicarboxylato) as well as two inert groups (NH_3, chelating diammines). Primary and secondary amines (with N−H groups which form hydrogen bonds) are also preferred.[3]

The remarkable analogy between Pt(II) and Pd(II) coordination stereochemistry has promoted studies of palladium(II) compounds as anticancer drugs.[4] Recent advances in this sense have been focused on Pd(II) complexes with one *N*,*N*-diamino[5−12] or *N*,*S*-aminothioether[13−19] chelating ligand, which impels the *cis*-coordination to the more labile ligand (two chloro[5−11,13−19] or nitrato[7] or one dicarboxylato.[7] In such cases, the chelating ligand prevents *cis* → *trans* isomerization around Pd(II) in spite of its higher lability (approximately up to 10^5-fold) relative to its analogous Pt(II). This essential kinetic difference promotes the use of Pd(II) compounds as models for the Pt(II) ones in mechanistic and kinetic[4] or potentiometric[5,9] and/or spectrophotometric studies.[12,20]

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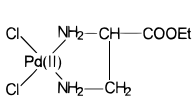
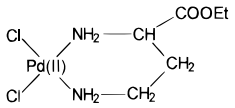
Inagaki *et al.*[21] have prepared *cis*-dichloroplatinum(II) complexes with α,ω-diaminocarboxylic acids (namely, 2,3-diaminopropionic (Hdap), 2,4-diaminobutyric (Hdab), and 2,3-diaminosuccinic (H_2dasa) acids) as well as their ethyl ester derivatives (Etdap, Etdab, Et_2dasa) as N,N-chelating inert groups. The reactivity of these latter esterified complexes with calf thymus DNA (CT-DNA) and the antitumor properties against leukemia L1210 were explained on the basis of their noncharged nature and their expected ability to permeate into cells. In contrast, the negatively charged *cis*-dichloro(diaminocarboxylato)platinum(II) complexes exhibit lower reactivity with CT-DNA and the absence of antitumor activity.

We have recently reported the synthesis of *cis*-[Pd(H_2dasa)Cl_2] and *cis*-[Pd(Et_2dasa)Cl_2] as well as the molecular structure of the latter and that of [Pd(2,2′-bipy)(dasa)]·$3H_2O$ (bipy = 2,2′-bipyridine).[22] Both *cis*-dichloropalladium(II) complexes have been shown to induce conformational changes in the covalent closed circular form of pUC8 plasmid. Both compounds showed significant cytotoxicity against MDA-MB468 and HL-60 human cancer cell lines.[22] In these and closely related *cis*-dichloro(chelating diammine)palladium(II) compounds it can be assumed that the chelating ligand remains effectively bounded to Pd(II) in a wide pH range,[4,7,10,12,23,24] so that is called a "spectator" ligand.[4] However, at physiological pH 7.4 and ionic strength $I = 0.15$ M such *cis*-dichloro complexes would give a variety of aqua and/or hydroxo and/or chloro species (including certain polynuclear ones), depending on the low (inside of cells) or rich (plasma or blood) chloride ion concentration and on the drug concentration itself. We think that equilibrium studies of such labile systems would be instructive for a better understanding of the modeling role of Pd(II) complexes for the inert Pt(II) ones. In this sense, Hohmann and van Eldik[9] have reported interesting potentiometric and spectrophotometric results for water by chloride substitution reactions on *cis*-diaqua(ethylenediamine)palladium(II), which include the use of a selective electrode to measure the free-chloride ion concentration at equilibrium. In our laboratories we investigated selected *cis*-dichloropalladium(II) complexes as potential anticancer drugs[25] as well as model systems for their platinum(II) analogues. For these purposes we have attempted to develop a more general experimental methodology in order to fit the complexation model and accurate formation constants for the aqua and/or hydroxo and/or chloro species in solutions of *cis*-dichloropalladium(II) drugs. This general procedure supposes the use of the SUPERQUAD program[26] to fit, as a first step, formation constants log β_{pqr} of the hydroxo species from $E(H^+)$ potentiometric data of alkalimetric titrations of *cis*-diaqua complex solutions. In a second step the above formation constants log β_{pqr} (as fixed values) and the referred program are used with series of $E(H^+)$ and $E(Cl^-)$ data pairs, simultaneously obtained from several titrations of the corresponding diaqua complex solutions, to fit log β_{pqr} for the chloro-containing species. In this paper we report the synthesis and some DNA binding properties of *cis*-dichloro(*d*,*l*-2,3-diaminopropionate ethyl ester)-palladium(II) (**I**) and *cis*-dichloro(*d*,*l*-2,4-diaminobutyrate ethyl ester)palladium(II) (**II**). We also apply our potentiometric procedure to compounds **I** and **II** in order to explain their biochemical and biological properties on the basis of the nature of their species in solution.



Compound I



Compound II

Experimental Section

Synthesis of 2,3-Diaminopropionate Ethyl Ester Dihydrochloride (Etdap·2HCl) and 2,4-Diaminobutyrate Ethyl Ester Dihydrochloride (Etdab·2HCl). A mixture of *d*,*l*-2,3-diaminopropionic acid hydrochloride (5 g; Sigma) or of *d*,*l*-2,4-diaminobutyric acid dihydrochloride (5 g; Sigma) in absolute ethanol (500 mL) was stirred and refluxed for 24 h under a flow of dried HCl until a clear solution was obtained. The hot solution was filtered. After cooling with ice and NaCl (−15 °C), the desired diamino ester dihydrochloride precipitates as a white polycrystalline product, which was filtered, washed with absolute ethanol and acetone, and air-dried: yield, >95%. Anal. Calcd for Etdap·2HCl ($C_5H_{14}Cl_2N_2O_2$): C, 29.29; H, 6.88; N, 14.00. Found: C, 29.37; H, 7.25; N, 13.67. Mp(dec) = 160 °C. Calcd for Etdab·2HCl ($C_6H_{16}Cl_2N_2O_2$): C, 32.82; H, 7.34; N, 12.76. Found: C, 33.06; H, 8.02; N, 12.81. Mp (dec) = 182 °C.

Synthesis of *cis*-[Pd(Etdap)Cl_2] (I) and *cis*-[Pd(Etdab)$_2$Cl] (II). Yellow-orange polycrystalline samples of compounds **I** and **II** were obtained by equimolar reactions of K_2PdCl_4 in water with Etdap·2HCl and Etdab·2HCl, respectively, followed by neutralization with $NaHCO_3$. A similar procedure was reported for *cis*-dichloro(diaminosuccinate diethyl ester)palladium(II) in ref 21: yield. >80%; Calcd for **I**, $C_5H_{12}Cl_2N_2O_2Pd$: C, 19.41; H, 3.91; N, 9.05; Cl, 22.91. Found: C, 19.51; H, 3.91; N, 9.18; Cl, 23.90. Mp (dec) 230 °C. Calcd for **II**, $C_6H_{14}Cl_2N_2O_2Pd$: C, 22.28; H, 4.36; N, 8.66; Cl, 21.92. Found: C, 22.30; H, 4.32; N, 8.25; Cl, 20.39. Mp (dec) = 245 ° C. Both complexes can also be prepared by reacting a solution of $PdCl_2$ (4%) in hot 0.1 M HCl with an equimolar amount of each diaminoacidate ethyl ester dihydrochloride. The pH of the resulting solutions is adjusted to 6−7 with aqueous $NaHCO_3$. The desired products precipitate during 24 h at room temperature as yellow powders which can be recrystallized from dilute NaCl aqueous solutions.

Physicochemical Characterization of the Products. Solid samples of diaminoacidate ethyl ester dihydrochlorides, and the corresponding *cis*-dichloropalladium(II) complexes (**I** and **II**) were characterized by infrared spectroscopy and TG-DTA thermal analysis as described in ref 22.

Potentiometric Titrations, Complexation Model, and Complex Formation Constant Refinement. All required solutions were prepared with CO_2-free doubly distilled water. The ionic strength of solutions of the complexes (see below) as well as titrant reagents (0.1 M NaOH or NaCl) were adjusted to $I = 0.15$ M ($NaClO_4$). The temperature of the sample in the double wall reaction cell was maintained at 37.00 ± 0.05 °C by circulating temperature-controlled water. A stream of N_2 presaturated with 0.15 M $NaClO_4$ flowed over the tested solutions. Aqueous solutions of *cis*-diaquapalladium(II) complexes with Etdap or Etdab were prepared as perchlorate salts by the reaction of carefully measured amounts of **I** or **II**, respectively, dissolved in the smallest possible volume of water with 2 equiv of a fresh and standardized $AgClO_4$ aqueous solution (working with topaz stained glass material in a darkroom and removing AgCl by filtration). Clear solutions of *cis*-[Pd(Etdap)$(H_2O)_2$]$(ClO_4)_2$ (**III**) and *cis*-[Pd(Etdab)$(H_2O)_2$]$(ClO_4)_2$ (**IV**) were diluted with appropriate amounts of a stock solution of $NaClO_4$ and water to obtain virtually chloride-free solutions (estimated $[Cl^-] \ll 10^{-5}$ M) of **III** or **IV** salts with $I = 0.15$ M ($NaClO_4$). These solutions alone, or diluted with 0.15 M background electrolyte, form the initial solutions for the two steps of the potentiometric study. As a first step, aliquots (50 mL) of three initial solutions of **III** (3.079×10^{-3}, 2.207×10^{-3}, and 1.333×10^{-3} M) and of **IV** (3.277×10^{-3}, 2.344×10^{-3}, and 1.390×10^{-3} M) were titrated with standardized 0.1 M NaOH in a Metrohm Dosimat 665 Titroprocessor equipped with a digital (pH/mV)-meter Crison 2002 with a glass electrode (Ingold 10-401-3664) and a (Ag/AgCl) reference electrode (Ingold 373-90-WTE-ISE-S7) with an intermediate electrolyte

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chamber. The intermediate electrolyte (0.15 M $NaClO_4$) was replaced daily. The standard electrode potential $E°$, the concentration of the NaOH titrant, and $K_w = (4.57 \pm 1) \times 10^{-14}$ were checked before and after each experiment by the titration of a known amount (50 mL) of $\sim 10^{-2}$ M $HClO_4$ in 0.15 M $NaClO_4$. This calibration procedure of the potentiometric system is similar to that described by Leporati[27] and was proved successful in a system involving Cu(II)-chelated hydroxo-complex formation.[28] The equilibrium model and the corresponding hydroxo-complex formation constants ($\log \beta_{pqr}$) can be defined by the general equilibrium 1 (charges omitted for simplicity)

$$p\mathrm{PdL(H_2O)_2} + q\mathrm{Cl} \pm r\mathrm{H} \rightleftarrows (\mathrm{PdL})_p\mathrm{Cl}_q(\mathrm{H_2O})_2\mathrm{H}_{\pm r} \qquad (1)$$

where L represents Etdap or Etdab, $q = 0$ for chloride-free species, $pqr = 1,0,0$ corresponds to *cis*-$[\mathrm{PdL(H_2O)_2}] = \mathrm{PdL(H_2O)_2}$ and, for example, $pqr = 1,0,-2$ indicates $\mathrm{PdL(OH)_2} =$ *cis*-$[\mathrm{PdL(OH)_2}]$. Obviously equilibrium 1 can also be used for reactions involving chloro ($q = 1$) and dichloro species ($q = 2$). The formation constants of the hydroxo complexes reported herein were obtained from 158 or 139 $E(\mathrm{H^+})$ data of three solutions of diaqua-complex salts (**III** or **IV**) treated with the method of rigorous least-squares by means of the SUPERQUAD program.[26] In these calculations we use $\sigma_v = 0.002$ mL and $\sigma_E = 0.1$ mV. These potentiometric data correspond to the range $3.5 \leq \mathrm{pH} \leq 7$. This upper pH limit was decided upon because in the alkaline range it becomes evident that there is an overlap of the ester hydrolysis reaction with the formation of dihydroxo species type $\mathrm{PdL(OH)_2}$, which prevents the calculation of $\log \beta_{1,0,-2}$. Fortunately, these uncharged species $\mathrm{PdL(OH)_2}$ have no significant abundance at physiological pH values. The second step was to use the formation constants of aquahydroxo and di(μ-hydroxo) species as fixed values to fit the chloro-complex formation constants ($\log \beta_{pqr}$, $q = 1$ or 2). In this step, we have simultaneously used two potentiometric systems which measure $E(\mathrm{H^+})$ (as described above) and $E(\mathrm{Cl^-})$ potential data pairs. For this purpose the titroprocessor was equipped with a second digital (pH/mV) meter with a ($\mathrm{Ag_2S/AgCl}$) chloride ion selective electrode (Ingold 15-213-3000) and the above-mentioned reference electrode with an intermediate electrolyte chamber (salt bridge 0.15 M $NaClO_4$, replaced daily). When necessary, we replaced the alkali titrant container with another one to add aliquots of 0.1 M NaCl in 0.15 M $NaClO_4$ (second titrant reagent). Using these two potentiometric systems simultaneously, we carried out a variety of experimental procedures, changing the order of titrant (NaOH or NaCl) additions. The best results correspond to the addition of NaOH to reach the first buffer pH range of the titration (pH $\sim$ 4.5) before making successive small additions of the NaCl solution. Each of these additions can produce changes in both $E(\mathrm{Cl^-})$ and $E(\mathrm{H^+})$ potentials if the H_2O or OH^- ligand of the aqua and/or hydroxo species undergo substitution reactions by Cl^- ions to give chloro-containing species. $E(\mathrm{H^+})$ and $E(\mathrm{Cl^-})$ data pairs were measured after each of these additions. From 1.745×10^{-3} and 3.490×10^{-3} M solutions of compound **III** and from 1.750×10^{-3} and 3.500×10^{-3} M solutions of compound **IV** we treated 127 and 70 $E(\mathrm{H^+})$ and $E(\mathrm{Cl^-})$ pairs of data, respectively, by the SUPERQUAD program to fit the formation constants of the *cis*-chloroaqua, *cis*-chlorohydroxo, and *cis*-dichloro species ($pqr = 1,1,0$; $1,1,-1$; and 1,2,0, respectively) formed from *cis*-$[\mathrm{PdL(H_2O)_2}]$ (L = Etdap or Etdab). These calculations were performed with $\sigma_v = 0.002$ mL, $\sigma_E = 0.1$ mV for H^+, and 0.2 mV for Cl^-. The entire set of fitted $\log \beta_{pqr}$ values for Etdap or Etdab Pd(II) complexes were used to simulate the alkalimetric titrations of **I** or **III** and of **II** or **IV**, respectively, as well as to obtain a variety of diagrams which illustrate the species abundance in the studied systems as a function of pH, $\mathrm{pCl} = -\log[\mathrm{Cl^-}]$ and total molar complex concentration.

DNA Binding Studies. A comparative study of the interaction of compounds *cis*-DDP, *cis*-$[\mathrm{Pd(Etdap)Cl_2}]$ (**I**), and *cis*-$[\mathrm{Pd(Etdab)Cl_2}]$ (**II**) with native DNA samples was carried out *in vitro* by differential spectrophotometry methods. In addition to measuring the UV difference spectral data of drug:DNA complexes, melting profiles and renaturation curves of DNA samples treated with the metal complexes at several molar ratios *r* (complex/nucleotide of DNA) were recorded. We used DNA from salmon sperm or *Streptomyces sp.* sources (this latter having 80% of GC pairs). Drug:DNA complexes were formed by incubation at 37 °C of compounds *cis*-DDP, **I**, or **II** and DNA in a buffered solution (pH = 7.2) 0.1 × SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate). The periods of complex formation were 15 min and 48 h (so that the equilibria with *cis*-DDP were also reached). The UV spectra were recorded on a Beckman Acta III spectrophotometer attached to a temperature programmer. Melting profiles were recorded at 258 nm using a rate of temperature increase of 1 °C/min from 25 to 90 °C. In the renaturation assays a phosphate buffer (pH = 7.2) was used. Salmon sperm DNA was denaturated by heating it for 20 min at 100 °C and then allowing it to renature at 10 °C below the melting temperature (T_m) of native DNA. The activity *in vivo* of the drugs was studied by analysis of the transcription process using as a model system the polytene chromosomes of *Drosophila hydei*. For this purpose larvae from the third instar were microinjected with 0.5 μL of a 10^{-6} M solution of compound **II**. Four hours after the microinjection the chromosomes were isolated in physiological Ringer solution following standard methods, pre-fixed with ethanol:acetic acid (3:1), fixed with 50% acetic acid, flattened between cover glass, and frozen in liquid nitrogen. After the cover glass was removed, the chromosomes on the slide were introduced into absolute ethanol for 10 min before being stained with lactoacetic orcein. Transcription was followed by the detection of chromosomal nascent RNA visualized by the formation of DNA:RNA hybrids *in situ* according to Alcover *et al.*[29] The hybrids were detected by an anti-DNA:RNA antibody followed by a fluorescent antibody. The chromosomes were photographed in a Zeiss fluorescence microscope using Tri-X Pan Kodak film. The amount of fluorescence was determined by densitometry of 10 chromosomes in control as well as in treated chromosomes as a measurement of transcription.

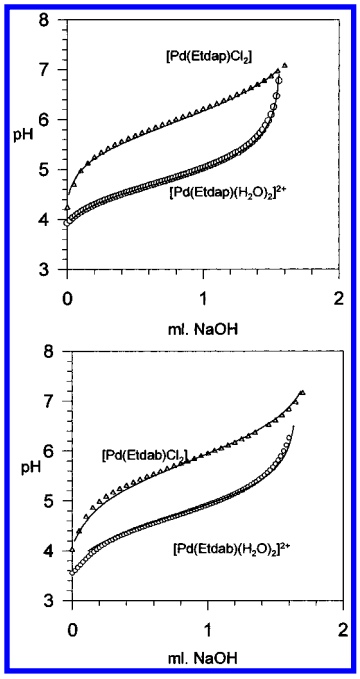

Figure 1. Experimental potentiometric data points for 3.079 mM *cis*-$[\mathrm{Pd(Etdap)(H_2O)_2}]^{2+}$ (lower), 3.489 mM *cis*-$[\mathrm{Pd(Etdap)Cl_2}]$ (upper), 3.277 mM *cis*-$[\mathrm{Pd(Etdab)(H_2O)_2}]^{2+}$ (lower), and 3.506 mM *cis*-$[\mathrm{Pd(Etdab)Cl_2}]$ (upper) solutions and the corresponding simulated titrations (unbroken lines) with NaOH (0.0992, 0.0994, 0.0988, and 0.0983 M, respectively). $V_0 = 50$ mL.

Results and Discussion

Chemical Characterization. The IR spectra ($\mathrm{cm^{-1}}$) of Etdap·2HCl and Etdab·2HCl show bands of the modes ν(C=O) (1742 and 1735, respectively) of $-\mathrm{COOEt}$ and δ_d ($\sim$1600) and δ_s (1475−1500 $\mathrm{cm^{-1}}$) of $-\mathrm{NH_3^+}$ groups. The IR spectra of complexes **I** and **II** show a double ν_{as} band (3240 and 3170, **I**; 3270 and 3200, **II**) and a ν_s band (3090, **I**; 3120, **II**) of $-\mathrm{NH_2}$

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Table 1. Formation Constants (log β_{pqr})[a] of the Hydroxo and/or Chloro Species in Aqueous Solutions of *cis*-Dichloro(diaminoacidate ethyl ester)palladium(II), *cis*-[Pd(Etdaa)Cl_2], for $I = 0.15$ M ($NaClO_4$) and $t = 37$ °C

	Etdaa = Etdap		Etdaa = Etdab		Etdaa = Et_2dasa[d]
complex[b] (*pqr*)	log β	stat. D[c]	log β	stat. D[c]	log β[d]
aquahydroxo (10-1)	−5.99(2)	$Z = 158$	−5.06(1)	$Z = 139$	−5.25
di(μ-hydroxo) (20-2)	−7.14(4)	$\sigma = 1.09$	−7.35(2)	$\sigma = 3.50$	−6.55
		$\chi^2 = 8.68$		$\chi^2 = 9.89$	
dichloro (120)	5.78(1)	$Z = 127$	5.98(2)	$Z = 70$	5.86
chloroaqua (110)	3.38(1)	$\sigma = 1.88$	3.32(1)	$\sigma = 2.32$	3.65
chlorohydroxo (11-1)	−2.97(4)	$\chi^2 = 9.75$	−2.37(2)	$\chi^2 = 10.40$	−2.68

[a] Values in parentheses are standard deviations. [b] Univalent ligands bounded to one ($p = 1$) or two ($p = 2$) Pd(Etdaa) moieties. [c] Statistical data for accuracy: Z = total number of experimental data points used in the refinement; χ^2 = observed statistical parameter based on weighted residuals of emf readings (values < 12.6 represent a confidence level of >95%);[27] σ = ratio of the mean square root of the weighted residuals to the estimated error in the actual working conditions (σ_v and σ_E). [d] log β_{pqr} values of the hydrolytic species of *cis*-dichloro(diaminosuccinate diethyl ester)palladium(II), *cis*-[Pd(Et_2dasa)Cl_2].[31]

groups, two weak bands of ν(Pd−N) (560 and 520, **I**; 550 and 510, **II**) and a typical one of ν(Pd−Cl) near 300 cm^{-1}. These bands are absent in the ligand dihydrochloride spectra. Other expected absorptions such as the ν(C═O) stretching (1735) and $\delta(NH_2)$ bands (1555, **I**; 1565, **II**) were also observed. These assignments are consistent with those reported for Etdap−Pt(II) and Etdab−Pt(II) analogues in ref 21. The TG-DTA diagrams reveal that compounds **I** and **II** are stable in an air atmosphere below 230 and 245 °C, respectively. At higher temperatures the pyrolysis of the organic ligand probably produces a crude residue of $PdCl_2$ (near 600 °C).

Hydrolytic Species of *cis*-[Pd(Etdaa)Cl_2] (I and II) and *cis*-[Pd(Etdaa)$(H_2O)_2$]$(ClO_4)_2$ (III and IV). Alkalimetric titrations of **I** and **II** and the corresponding diaqua complexes **III** and **IV** (perchlorate salts) show a defined inflection to $a = 1$ (a = equiv of base/mol Pd(II), Figure 1) followed by another one to $a = 2$ (extremely smooth, not shown). These behaviors are consistent with the titration of two groups with different acidities, in separate steps, to give hydroxo species with OH/Pd(II) ratios 1:1 and 2:1, respectively. In Cl^--free solutions of **III** or **IV** (diaqua complexes, $pqr = 1,0,0$) the assumption that only mononuclear hydroxo complexes ($pqr = 1,0,-1$) are formed for $a \leq 1$ does not seem reasonable because of the remarkable difference in their two acidities. The formation of dinuclear di-(μ-hydroxo) complexes have been suggested in the hydrolysis of related complexes.[5,8,9,30−32] However, the formation of only such dinuclear di-(μ-hydroxo) complexes [(Etdaa)-$Pd(OH)_2$ Pd(Etdaa)]$^{2+}$ ($pqr = 2,0,-2$) also seems unreasonable because the "apparent" pK_1 value (the pH value after the addition of a half of alkali equivalent) does not remain as a "constant" but decreases as the complex concentration increases. Thus, the first hydrolysis of **III** and **IV** will involve at least the overlapped formation of mononuclear hydroxo and di-(μ-hydroxo) complexes (and perhaps other ones).[30−32] These features preclude all attempts to estimate a constant pK_a for Pd(II)−H_2O groups. In the second step ($1 \leq a \leq 2$, pH > 7) [Pd(Etdaa)$(OH)_2$] will be formed, but a slow and persistent lowering of pH (by ester hydrolysis) prevents the calculation of log $\beta_{1,0,-2}$.

Previous suggestions were taken into account in the fitting of the hydrolytic model and formation constants (log β_{pqr}) by the SUPERQUAD program (Table 1). In the experimental conditions of the present work, only mononuclear ($pqr = 1,0,-1$) and dinuclear species ($pqr = 2,0,-2$) seem involved in the first hydrolysis of **III** and **IV**. These two hydroxo complexes as well as dichloro, chloroaqua, and chlorohydroxo ones ($pqr = 1,2,0$; 1,1,0; and 1,1,−1) will be formed in the presence of Cl^- (Table 1). Other di- or polynuclear species can be formed in more concentrated complex solutions.

The fitness of both sets of formation constants reported herein are shown in the remarkable agreement of the simulated and experimental titrations (Figure 1). These constants can be compared with those reported for related diaminopalladium(II) complexes.[8,9,20,31,32] Our log β_{pqr} data are very similar to those[31] of the closely related *cis*-[Pd(Et_2dasa)Cl_2] and *cis*-[Pd(Et_2dasa)-$(H_2O)_2]^{2+}$ (Table 1). However, the dimerizing ability of the aquahydroxo complexes by the general equilibrium

$$2[Pd(Etdaa)(H_2O)(OH)]^+ \rightleftarrows [(Etdaa)Pd(\mu\text{-}OH)_2Pd(Etdaa)]^{2+} + 2H_2O$$

are different depending on the spectator chelating ligand.[4] Indeed the dimerization constants log K_D = log $\beta_{2,0,-2}$ − 2 log $\beta_{1,0,-1}$ are in the order of log $K_D = 4.84$, 3.95, and 2.77 for daa = Etdap, Et_2dasa, and Etdab, respectively. Thus, such dimerization ability seems mainly related to the chelate ring size formed by the spectator ligand (Pd(Etdab) moiety with a larger six-membered ring should better hinder the dimerization than the Pd(Etdap) and Pd(Et_2dasa) ones with shorter five-membered rings).

The hydrolysis of **III** and **IV** depends on pH, [Cl^-], and the diaqua complex concentration. A variety of illustrative diagrams are given in Figures 2−4. In acid to neutral solutions, the hydrolysis of *cis*-[Pd(Etdaa)$(H_2O)_2]^{2+}$ (100, Etdaa = Etdap or Etdab) produces *cis*-aquahydroxo and di(μ-hydroxo) species (10−1 and 20−2). The mole fraction of the dimeric species depends on the diaqua complex concentration because dilution favors dissociation (Figure 2). In addition, as expected for log K_D values, the hydrolysis of *cis*-[Pd(Etdap)$_2(H_2O)]^{2+}$ produces a higher proportion of di-(μ-hydroxo) complex than that of *cis*-[Pd(Etdab)$(H_2O)_2]^{2+}$.

As an illustrative case, Figure 3 shows the distribution diagram of species for 1 mM *cis*-[Pd(Etdab)$(H_2O)_2](ClO_4)_2$ at the physiological pH 7.4 as a function of pCl = −log [Cl^-]. In such conditions, at [Cl^-] = 1 M (pCl = 0) the abundance of the neutral dichloro complex becomes 90%, while the remaining 10% corresponds to the also neutral chlorohydroxo one. A decrease in [Cl^-] produces an increasing hydrolysis which gives the chlorohydroxo complex (1,1,−1) as the predominant species as well as positively charged aquahydroxo and di(μ-hydroxo) ones (1,0,−1 and 2,0,−2). Note that in a wide range of [Cl^-] the chloroaqua complex cation (1,1,0) does not have significance at physiological conditions. At [Cl^-] = 104 mM (plasma) all of the species formed are neutral chloro ones (1,2,0 and 1,1,−1). In clear contrast, at [Cl^-] = 4 mM (inside of cells) the neutral chlorohydroxo species and two positively charged ones (1,0,−1 and 2,0,−2) are formed.

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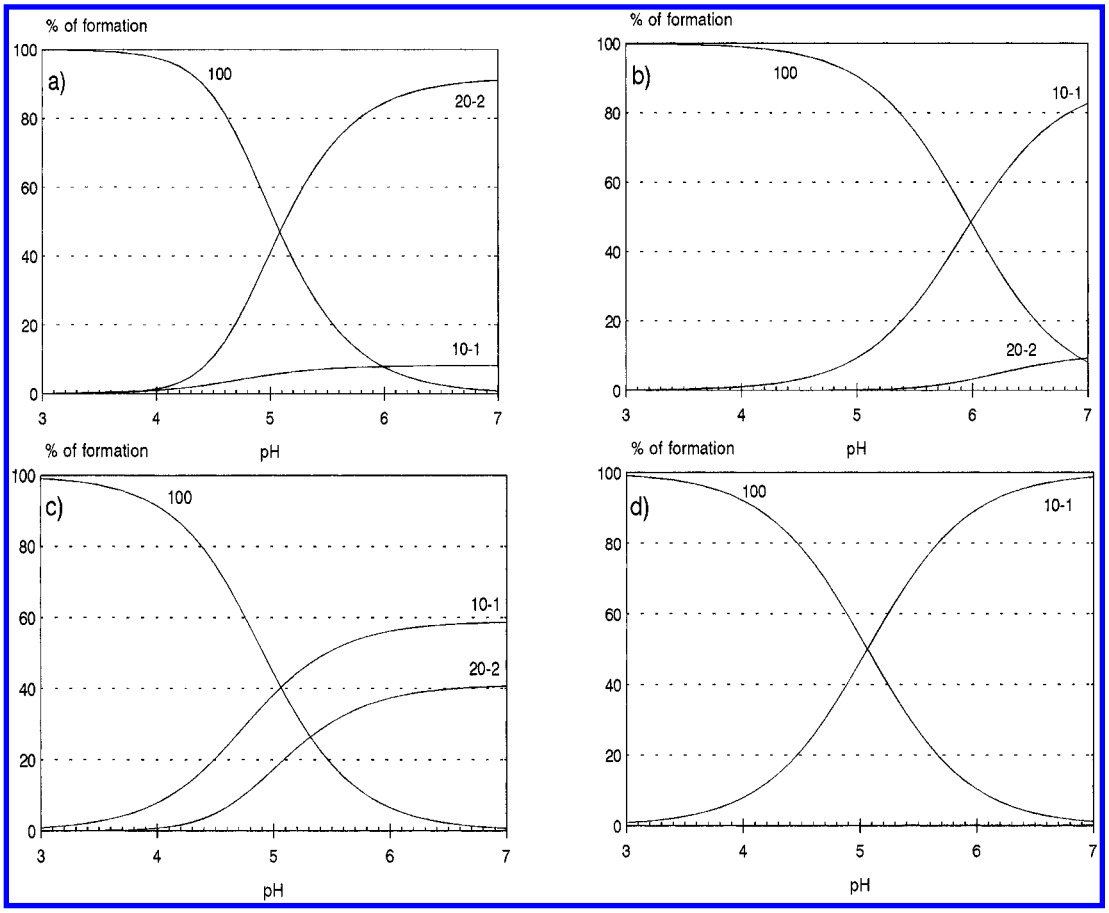

Figure 2. Distribution diagrams for hydrolytic species (indicated as *pqr* codes) of the diaqua complexes as a function of pH and complex concentration. Solutions of *cis*-$[Pd(Etdap)(H_2O)_2]^{2+}$ (**III**) at 1 mM (a) or 1 μM (c) and solutions of *cis*-$[Pd(Etdab)(H_2O)_2]^{2+}$ (**IV**) at 1 mM (b) or 1 μM (d).

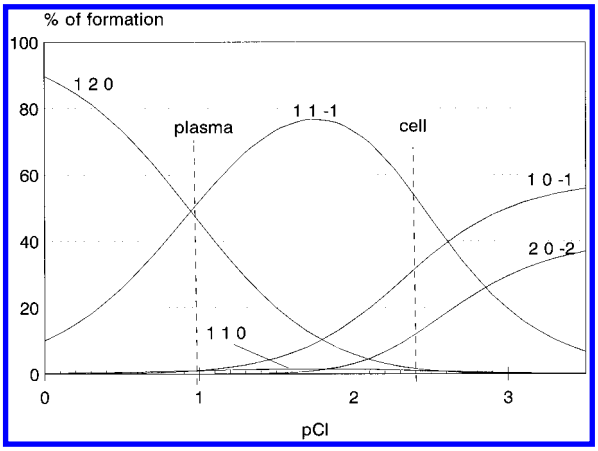

Figure 3. Distribution of species (indicated by *pqr* codes) in solutions of 1 mM *cis*-$[Pd(Etdab)(H_2O)_2](ClO_4)_2$ at pH = 7.4 as a function of chloride ion concentration (pCl = $-\log[Cl^-]$). Dotted lines correspond to plasma (104 mM) and into cell 4 mM Cl^- ion concentrations.

In view of the reactivity with DNA, it is interesting to estimate the total percentage of neutral and charged species in physiological conditions (Figure 4). Noteworthy, both diaqua complexes **III** and **IV** will produce >96% of neutral species in plasma (1 mM to 1 μM complex concentrations). In contrast, they produce abundant cationic species (~24−72% for [Pd(Etdap)$(H_2O)_2]^{2+}$ and ~34−45% for [Pd(Etdab)$(H_2O)^{2+}]_2$) at the same conditions. Such a pathway can be significant for analogous inert platinum(II) compounds (where μ-hydoxo oligomers seem resposiblie of toxicity) but not for kinetically labile palladium(II) ones which can quickly displace a variety of complex formation equilibria. Our results are very suitable for the diffusion of compounds **I** and **II** across biological membranes as well as for interaction with DNA into cells.

DNA Binding Studies. The data shown in Table 2 indicate the spectral behavior of the DNA bound to the complexes tested in this study. The data (λ_{max}, H, and $\Delta A_{270}/\Delta A_{295}$) obtained for the DNA−*cis*-DDP complex (used as a reference system) are in agreement with other data reported in the literature.[21,33,34] The UV spectral pattern of the DNA samples treated with compounds **I** and **II** was, in general, similar to that obtained for the DNA−*cis*-DDP complex. Bathochromic shifts accompanied by hyperchromic effects are generally observed. However, our data reveal that the interaction between *cis*-DDP and DNA increases with the incubation time, while the interactions of the Pd(II) complexes (**I** and **II**) with DNA are time independent (the data obtained at 15 min or 48 h of incubation for Pd(II) complexes are the same). These features are related to the kinetic differences existing between Pt(II) and Pd(II) complexes. Thus, the latter can react up to 10^5-fold faster than the Pt(II) ones. Furthermore, it was interesting to note that the effects induced by binding of compounds **I** and **II** are stronger than those induced by *cis*-DDP binding. Indeed, concentrations of compounds **I** or **II** (*i.e.*, $r = 0.0025$) at even 100-fold lower than *cis*-DDP (*i.e.*, $r = 0.25$) give similar or higher values of λ_{max}, *H*, and $\Delta A_{270}/\Delta A_{295}$. Particularly interesting is the $\Delta A_{270}/\Delta A_{295} = 1.8$ value presented by compound **II** (at $r = 0.0025$), quite similar to the $\Delta A_{270}/\Delta A_{295} = 2$ value presented by the antitumoral agent *cis*-DDP at $r = 0.25$. These features suggest that the secondary structure of DNA may be disrupted in the presence of compounds **I** and **II** to an extent similar to or greater than that produced by *cis*-DDP.

The data obtained from the melting curves of DNA samples treated with compound **I**, **II**, or *cis*-DDP (reference) are

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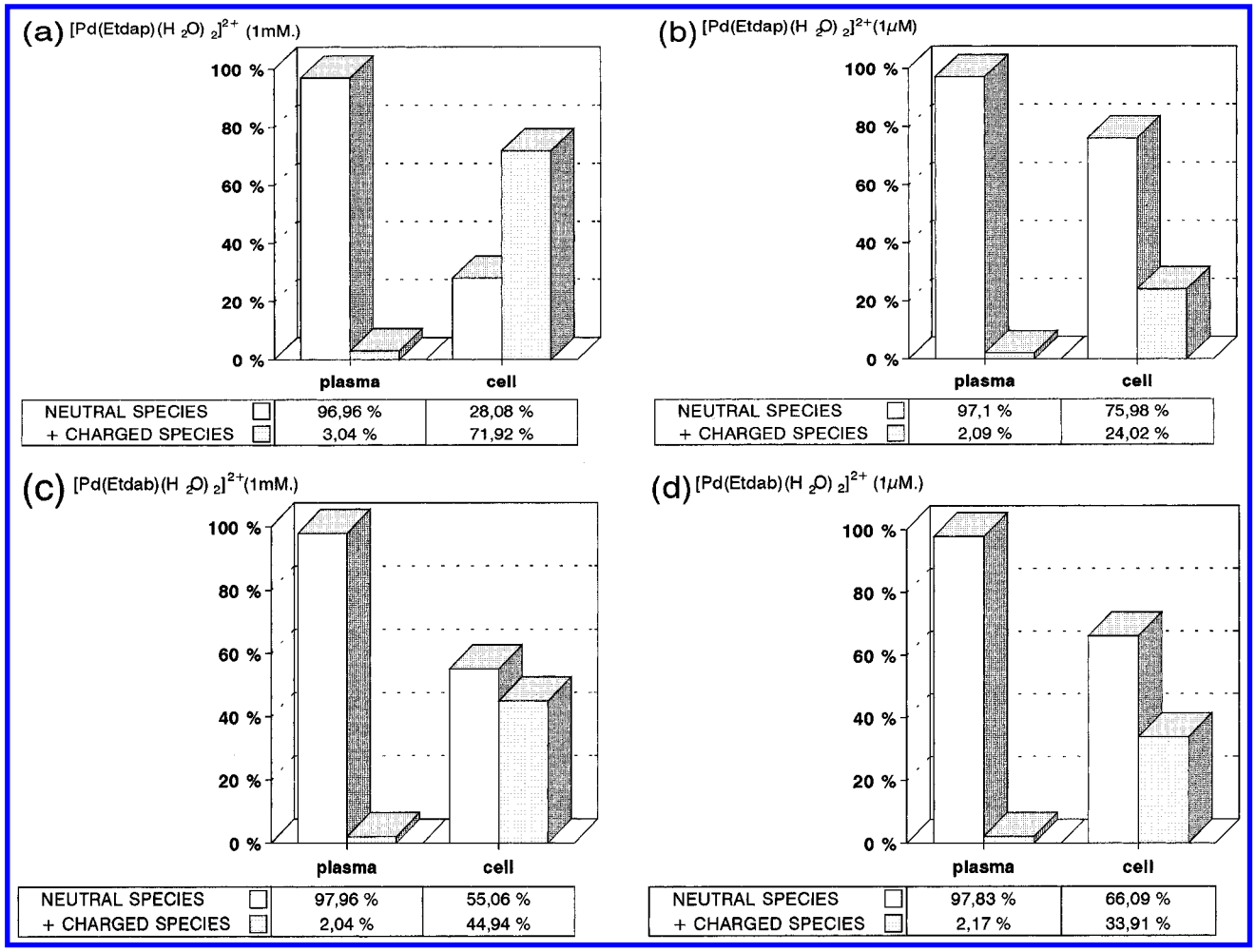


Figure 4. Total percentage of neutral and charged species in diaqua complex solutions at pH = 7.4 and chloride concentrations as in plasma or within cells: 1 mM (a) and 1 μM (b) *cis*-$[Pd(Etdap)(H_2O)_2]^{2+}$ 1mM (a) and 1μM (b) and 1 mM (c) and 1 μM (d) *cis*-$[Pd(Etdab)(H_2O)_2]^{2+}$.

Table 2. Ultraviolet Spectral Data[a] of Native DNA and DNA Samples after the Addition of Pd(II) Complexes **I** and **II** and *cis*-DDP[b]

systems	*r*	λ_{max}[a] (nm)	*H*[a] (%)	$\Delta A_{270}/\Delta A_{295}$[a]
native DNA		258		
DNA−compound **II**	0.0005	259	1.4	1.06
	0.0010	260.5	4.0	1.56
	0.0015	261	11.5	1.6
	0.0020	261.3	13.4	1.65
	0.0025	262	22.1	1.8
DNA−compound **I**	0.0025	258	7.5	1.4
DNA−*cis*-DDP	0.25(15 min)	260	(−0.8)	1.3
	0.25(24 h)	263	4.5	2.0

[a] λ_{max} = wavelength at which maximal ultraviolet absorption is produced; *H* (%) = hyperchromic (or hypochromic, if negative) effect, as percentage of the native DNA; $\Delta A_{270}/\Delta A_{295}$ = increase in the absorption ratio at 270 versus 295 nm. [b] Compounds **I** and **II** (Pd(II) complexes) show time independent effects on DNA, whereas those of *cis*-DDP are time dependent.

Table 3. Melting Temperature (T_m, °C), Hyperchromicity (*H*, %), and Width of Transition 2σ (°C) of Native DNA and DNA Complex Systems

systems	*r*	T_m (°C)[a]	*H*[b] (%)	2σ[c] (°C)
native DNA		68.9	33.5	8.8
DNA−compound **II**	0.0005	68.9	28.4	9.1
	0.0010	65.7	24.4	9.9
	0.0015	47.8	11.0	9.2
	0.0020	40.6	10.1	5.4
DNA−compound **I**	0.0020	48.0	16.3	6.0
DNA−*cis*-DDP	0.2500 (t = 15 min)	65.7	35.4	9.9
	0.2500 (t = 48 h)	64.9	28.2	10.1
DNA (*Streptomyces sp.*)		84.7	27	5.1
DNA−compound **II**	0.0020	53.4	11.9	16.0

[a] T_m = temperature value at half denaturation. [b] *H* = hyperchromicity by heating. [c] 2σ = temperature range corresponding to 15 and 85% denaturation.

summarized in Table 3. The melting temperature (T_m) and hyperchromicity (*H*) values of DNA−compound **II**, DNA−compound **I**, and DNA−*cis*-DDP systems are lower than the T_m = 68.9 °C and *H* = 33.5% values of the native DNA and decrease with the increase of the drug concentrations (*r*). This behavior reveals the metal−complex interaction with the DNA bases leading to the destabilization of its double helix with respect to heat denaturation. The destabilization is greater as the concentration of metal−complexes increases. The effects produced by the Pd(II) complexes on the melting profiles of DNA are independent of the incubation time, in contrast with the effects of *cis*-DDP that increase with the incubation time. The data for T_m and *H* obtained for compounds **I** and **II** even at *r* = 0.001 are quite similar to those of *cis*-DDP (*r* = 0.25, incubation time 48 h). Thus, lower concentrations of Pd(II) drugs can produce DNA destabilization similar to that of *cis*-DDP. Both compounds at *r* = 0.0025 produce an almost complete DNA denaturation. With GC 80% rich DNA (*Streptomyces sp*) the behavior seems different. The width transition values (2σ) of this native DNA increase from 5.1 to 16.0 (*r* = 0.002) for its DNA−compound **II** complex, while this parameter decreases from 8.8 for native DNA (salmon sperm) to 5.4 for its DNA−compound **II** complex (*r* = 0.002). These results

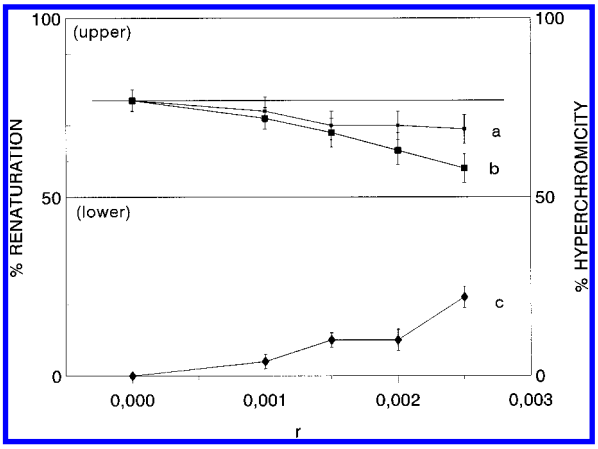


Figure 5. (Upper) DNA renaturation (%) of DNA treated with different concentrations (r) of *cis*-[Pd(Etdab)Cl_2] (**II**): (a) Addition of complex to heat denatured DNA; (b) addition of complex before DNA denaturation process. (Lower, c) Hyperchromicity (%) of DNA treated with different concentrations of *cis*-[Pd(Etdab)Cl_2] (**II**) (T = 25 °C).

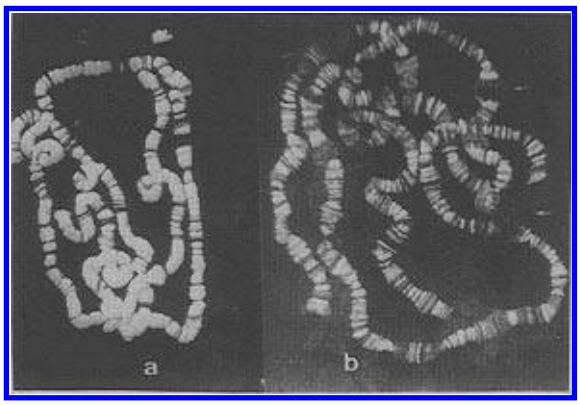
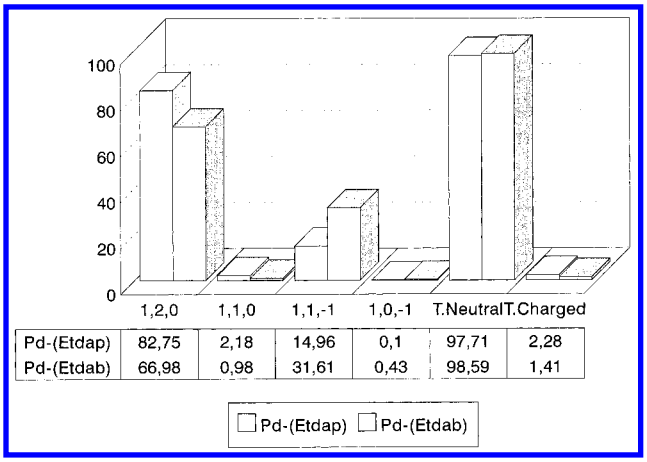


Figure 6. Photographic images of polytene chromosomes extracted from salivary glands of *Drosophila hydei* larvae and stained with lactoacetic orcein: (a) chromosome of a larva microinjected with 0.5 μL of a solution of 1 μM *cis*-[Pd(Etdab)Cl_2] (**II**); (b) chromosome control (untreated).

indicate that the interaction of drug **II** with DNA depends upon the GC content, with the GC-rich DNA being destabilized more effectively.

The results of the DNA renaturation assays are given in Figure 5. The addition of compound **II** to a heat denatured DNA (line a) does not modify the DNA renaturation percentage, this being practically complete even at a complex concentration corresponding to $r = 0.0025$. However, the addition of the complex before the DNA denaturation process produces an inhibition of the DNA renaturation (line b). This effect increases with the value of r. Since the inhibition percentage of the DNA renaturation (line b) is similar to the hyperchromicity percentage caused by the drug on native DNA (at the same drug concentration) (line c), it is likely that the interaction of drug **II** with DNA stimulates its denaturation, while it partially blocks its renaturation.

The effects of compound **II** upon chromatin structure and transcription were revealed by analysis of the polytene chromosomes of *Drosophila hydei* (Figure 6). It was observed that the chromatin of the treated chromosomes appeared more compact than that of the untreated ones. In particular the actively transcribing regions (puffs) present in the untreated chromosmes (Figure 6b) collapsed after *in vivo* administration of compound **II** (Figure 6a). That in the treated chromosomes these regions were transcriptionally inactive was confirmed by



Pd-(Etdap)	82,75	2,18	14,96	0,1	97,71	2,28
Pd-(Etdab)	66,98	0,98	31,61	0,43	98,59	1,41

Figure 7. Abundance (%) of hydrolytic species and total of neutral and charged species for *cis*-[Pd(Etdap)Cl_2] and *cis*-[Pd(Etdab)Cl_2] in experimental conditions of *in vitro* DNA binding studies (pH = 7.2, [Cl^-] = 0.15 M, [complex] = 1 μM). Species indicated by *pqr* codes.

in vivo incorporaction of 3*H*-uridine. It was observed that these regions did not incorporate the precursor, while as expected the puffed regions in the control chromosomes did (data not shown). The immunograms of the nascent RNA transcripts as detected by DNA:RNA fluorescence indicated that the control chromosomes accumulated 4-fold more RNA than the treated ones after 4 h of incubation with compound **II** as an indication that the drug partially inhibited RNA transcription.

It is instructive to correlate the biochemical results with the hydrolytic behavior of Pd(II)−complexes **I** and **II**. Figure 7 shows the percentage of species formed in the hydrolysis of the Pd complexes under the experimental conditions of the DNA binding studies *in vitro* (pH = 7.2, [Cl^-] = 0.15 M and [Pd(II) complex] $\leq 10^{-6}$ M). On the basis of Inagaki's suggestions,[21] we think that the charged species of drugs **I** and **II** would be the more active ones in the interaction mechanism of these drugs with DNA. However, such cationic species arise from the hydrolysis of both Pd(II) complexes (drugs **I** and **II**) in very low proportions (2.28 and 1.41% for **I** and **II**, respectively). This fact does not suppose a low interaction with DNA because the hydrolytic equilibria of Pd(II) complexes are labile, and consequently the equilibrium displacement could provide new amounts of the charged species to interact with DNA, as the biochemical experiments reveal. In contrast, the hydrolytic equilibria of analogous Pt(II) complexes are not labile. The kinetic differences of the Pt(II) complexes with respect to the Pd(II) ones and not their thermodynamic resemblances are more probably responsible for the hydrolysis of Pt(II) complexes making a high proportion of charged species available to produce an important interaction with DNA.[21]

The available biochemical and/or biological data indicate that compound **II** has a DNA binding capacity higher than that of compound **I**, although the first produces a total percentage of charged species lower than the last one when both compounds are hydrolyzed (Figure 7). Perhaps, in addition to charged species, different neutral species (dichloro or chlorohydroxo ones) of the studied drugs may influence their reactivity with DNA by different ways. For example, we can assume that the chlorohydroxo species (1,1,−1) could form hydrogen bonds. This feature will favor the activity of drug **II** with respect to drug **I**, although the total neutral species for both drugs is similar.

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