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Electron microscopic measurement of chain flexibility of poly(dG-dC).poly(dG-dC) modified by cis-diamminedichloroplatinum(II)

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ABSTRACT

The antitumor drug cis-diamminedichloroplatinum (II) (cis-Pt) forms bidentate adducts with guanine residues of poly(dG-dC).poly(dG-dC). The secondary structure of the polymer is altered. In this work, high resolution pictures of naked molecules, obtained by dark field electron microscopy reveal DNA chain distortions with radii as small as 30 Å. The extent of distortion increases with the drug/nucleotide ratio (r_b). These alterations of the secondary structure are responsible for the apparent shortening of the molecules. Measurements of the persistence lengths of the polymer as well as the end-to-end distances of elementary segments of various lengths, are obtained from digitized electron micrographs. The measurements are used to monitor and quantify the observed modifications of polymer structure upon cis-Pt binding at various r_b or incubation times. Poly(dG-m^5dC).poly(dG-m^5dC) in the B and Z forms have different persistence lengths. In the B form, this polymer is more altered by cis-Pt than in the Z one.

INTRODUCTION

Knowledge of the conformational changes induced by the binding of cis-diamminedichloroplatinum (II) (cis-Pt) to DNA can be useful to improve our understanding of the mechanism of its anti tumoral activity (1). Numerous physicochemical studies have already been performed on platinated DNA. For example, the binding of cis-Pt to DNA distorts the double helix as judged by circular dichroism (2, 3). Electron microscopy experiments indicate a shortening of platinated DNA (3-7). Because the **in vitro** reaction of cis-Pt with DNA forms several adducts, the analysis of physicochemical experiments is difficult. We have undertaken a systematic study of platinated synthetic polynucleotides in order to describe the conformational changes induced by each adduct. We have recently shown that in the reaction of cis-Pt and poly(dG-dC).poly(dG-dC) (abreviated poly(dG-dC)), or poly(dG-m^5dC).poly(dG-m^5dC) (abreviated poly(dG-m^5dC)), the nature of the adducts depends upon the conformation of the polynucleotide. When cis-Pt is added to the polymer in the B conformation the major adduct

is an intrastrand bidentate one and it occurs between two guanine residues (on the N7) separated by a cytosine (or a methyl^{5}cytosine) residue. A monodentate adduct is formed when cis-Pt is added to the polymer in the Z conformation and this monodentate adduct stabilizes it. Bidentate adducts induce a conformational change from the B form towards a new one. While this new conformation is recognized by antibodies to Z-DNA its ^{31}P NMR and circular dichroism spectra do not detect the canonical Z conformation (8-11).

Electron microscopy of nucleic acids has been improved by dark field observations of unshadowed, stained molecules, adsorbed onto a support without a basic protein film (12, 13). Since this technique permits more precise measurements on molecular size and shape than the more classical electron microscopic methods, we used it to reexamine the actual effects of cis-Pt on DNA and polynucleotides. To accurately account for the effects of cis-Pt binding and the resulting condensation and secondary structural alterations of poly(dG-dC), we digitally determined the coordinates of points taken along DNA filaments. From these coordinates, we computed two geometrical quantities from which one can determine the persistence length (a) : one is the average of the cosine of the angle θ between segments separated by a given contour length (s) according to the procedure of Frontali **et al.** (14), and the other is the average of the square of the end-to-end distances between two points separated by a given contour length (15). The persistence length of DNA molecules, as shown here, monitors the chemical modifications induced by cis-Pt on the helical conformation of the polymer. The procedure was applied to quantify alterations of the secondary structure of both platinated poly(dG-dC) and poly(dG-m^{5}dC), cis-Pt having been mixed with the polymer either in B or in Z conformation. Our results show that cis-Pt does not shorten the DNA.

MATERIAL AND METHODS

Electron microscopy of cis-Pt DNA complexes

Unplatinated and platinated samples at various r_b were prepared as previously described (8) (r_b is the molar ratio of cis-Pt per nucleotide). Platinated samples were designated poly(dG-dC)cis-Pt(r_b). The samples were diluted to a final concentration of 0.5 µg/ml in 10 mM Tris HCl pH 8,100 mM NaCl, 1 mM EDTA (TEN buffer). Five µl droplets of solution were deposited on positively charged carbon-coated grids, washed, stained with 2 % aqueous uranyl acetate and dried as described by Dubochet **et al.** (16). Grids were observed by tilted dark field using a Philips 300 electron

microscope. A 10 % error in the precise calculation of the magnification is inherent to the "Selected Area" mode used in dark field, because a manual adjustement of the diffraction lens current is required. To eliminate this error, in one experiment, ØX174 RF II or PM2 DNA molecules were added as internal size standards.

Conformational analysis of the molecules

The representation of DNA curvature was done by plotting log(cos θ) versus the contour length (s), as described by Frontali **et al.** (14). From a series of micrographs taken at the same magnification, 10 fold enlargements of 100 unselected segments of poly(dG-dC) were measured with an ACT 11-1 digitizer (from Altek, Silver Spring MD 20904 USA) with a precision of about 0.10 mm. At 320,000 times magnification the elementary segments correspond to a DNA size of approximately 30-40 Å. From the digitalized molecules, a microcomputer (Kontron PSI-80) was used to calculate 1) s : contour lengths separating two points on the DNA molecules ; 2) d : the end-to-end between two points ; 3) the log of the cosine of the angle θ between segments separated by the distance s. The procedure used for this calculation involves the construction of two histograms : one of cosine and one of square distance, with 50 Å increment. This 50 Å value gives a good resolution with regard to the size of the elementary segments on the drawings and the high resolution pictures obtained by our electron microscope procedures.

If n is the integral part of s/ds we build the histograms N(n) for the number n of observations, C(n) for the cosines and D(n) for the squares of the end-to-end distances :

$$N(n) = N(n) + 1$$

$$C(n) = C(n) + \cos \theta$$

$$D(n) = D(n) + d^2$$

After doing this for 100 molecules we compute the averages :

$$\langle \cos \theta(s) \rangle = C(n) / N(n)$$

$$\langle d^2 \rangle = D(n) / N(n)$$

If the bending of the polymer is restricted to a plane, Landau and Lifshitz (17) show that :

$$\langle \cos \theta(s) \rangle = \exp(-s/2a)$$

In the last equation, a is the persistence length, a parameter characterizing the flexibility of the polymer. Therefore we plotted $\log\langle \cos \theta(s) \rangle$ versus s and determined a from the slope.

Squares of the end-to-end distances $\langle d^2 \rangle$ were also plotted versus s.

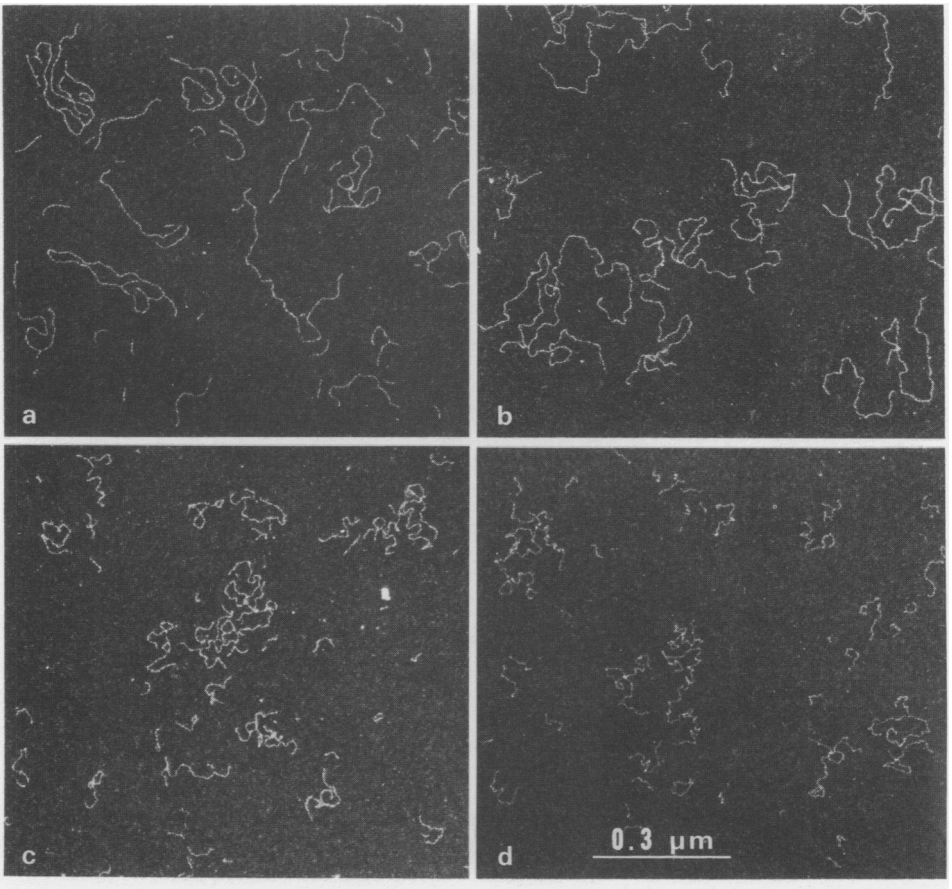

Figure 1 : Poly(dG-dC) at a final concentration of 0.5 µg/ml in TEN buffer was adsorbed to grids covered with a thin carbon film activated by a glow discharge in the presence of pentylamine. Pictures were taken in the dark field mode at 50,000 magnification. **a)** poly(dG-dC) ; **b), c), d)** poly(dG-dC)cis-Pt, r_b = 0.05 **(b)**, 0.10 **(c)**, 0.20 **(d)**.

RESULTS

poly(dG-dC)cis-Pt

Electron microscopy

Figure 1 shows electron micrographs of the unplatinated polymer (a), and of platinated polymers at r_b = 0.05 (b), 0.10 (c) and 0.20 (d). The pictures show the increasingly contorted shape of the molecules as r_b increases. The platinated molecules are more condensed than the control and thus seem to be shorter.

Examination at a higher magnification (fig. 2) shows only small

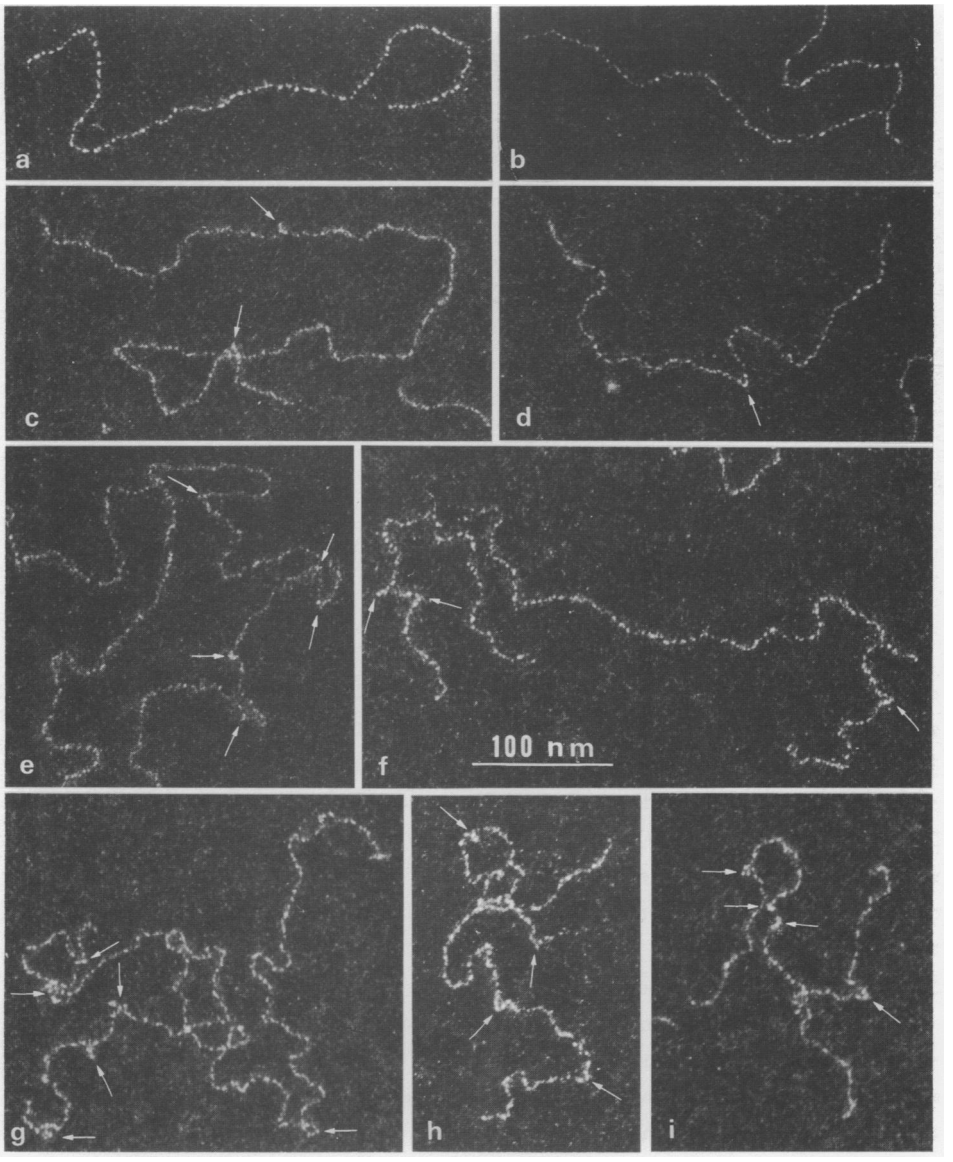

Figure 2 : High magnification of the specimens shown in figure 1. **a, b** : poly(dG-dC) ; **c, d** : poly(dG-dC)cis-Pt ar r_b = 0.05 ; **e, f** : at r_b = 0.10 ; **g, h, i** : at r_b = 0.20.

differences between the control (a, b) and the platinated polymer at r_b = 0.05 (c, d) ; the platinated sample presents only a few kinks and a somewhat wavy appearance. At r_b = 0.10 (e, f) and 0.20 (g, h, i), more

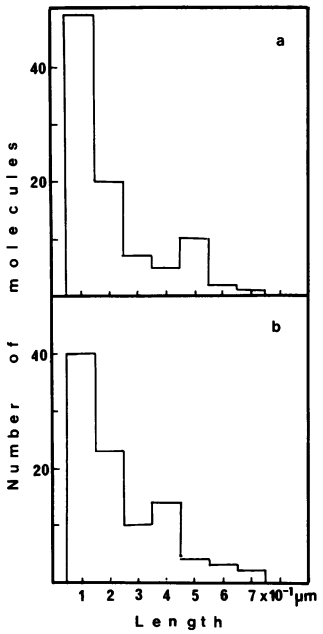

Figure 3 : Histograms of the distribution length of 100 linear molecules of poly(dG-dC) either without **(a)** or with **(b)** cis-Pt r_b = 0.20.

numerous and important perturbations of the polynucleotides are visible : small loops of 60 Å diameter, many short hairpins and curvatures with ca. 30 Å radii are seen. Some kinks, more thickened by the uranyl acetate staining could contain distorsions below the resolution possible with the DNA staining method used, i.e. in the order of 20 Å. These distorsions are very numerous at an r_b of 0.20 (g, h, i), and occur along with a smooth curvature of the filaments. This results in an apparent condensation of the molecules.

Size of the polymer molecules

The population of poly(dG-dC) used was very heterogeneous in size. Most linear segments were between 0.10 and 0.7 µm and some branched molecules were more than 4 µm. The histograms showing the distribution of the total contour length of 100 unbranched control molecules and of molecules complexed with cis-Pt at r_b = 0.20 (fig. 3) are not significantly different, and give mean lengths of 0.19 ± 0.03 µm and 0.21 ± 0.03 µm respectively.

Cos θ

Poly(dG-dC) was incubated for 24 h with cis-Pt at various r_b values. For each r_b and for the unplatinated polymer, 100 molecules were digitized to analyse their curvature by the graphic representation of the

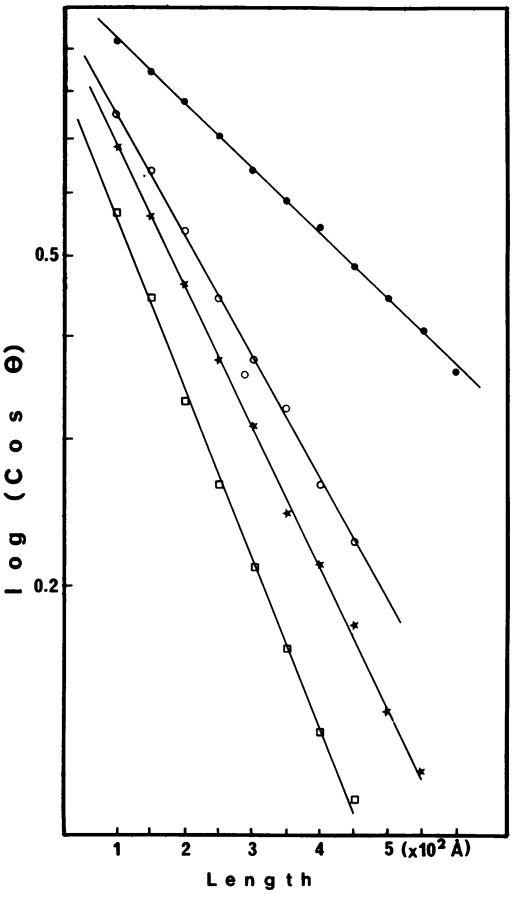

Figure 4 : Plot of the log(cos Θ) versus the length of elementary segments of poly(dG-dC) and poly(dG-dC)cis-Pt of increasing length : ●—● : r_b = 0 ; ○—○ : r_b = 0.05 ; ✱—✱ : r_b = 0.10 ; □—□ : r_b = 0.20.

log(cos Θ) versus s (Θ being the angle between two elementary segments, and s the curvilinear abscissa between them). As shown in figure 4, one obtains well defined straight lines for lengths between 100 and 500 Å. The values of the persistence lengths decrease as r_b increases : 315 Å for r_b = 0, 160 Å for r_b = 0.05, 130 Å for r_b = 0.10 and 90 Å for r_b = 0.20.

A kinetic experiment was also performed. Poly(dG-dC) and cis-Pt were mixed (input ratio r_b = 0.10) and aliquots were taken at various times. The results were analyzed by plotting log(cos Θ) versus s. No appreciable changes in persistence length occur during the first two hours (fig. 5). After 5 hours an incomplete binding of cis-Pt is indicated by

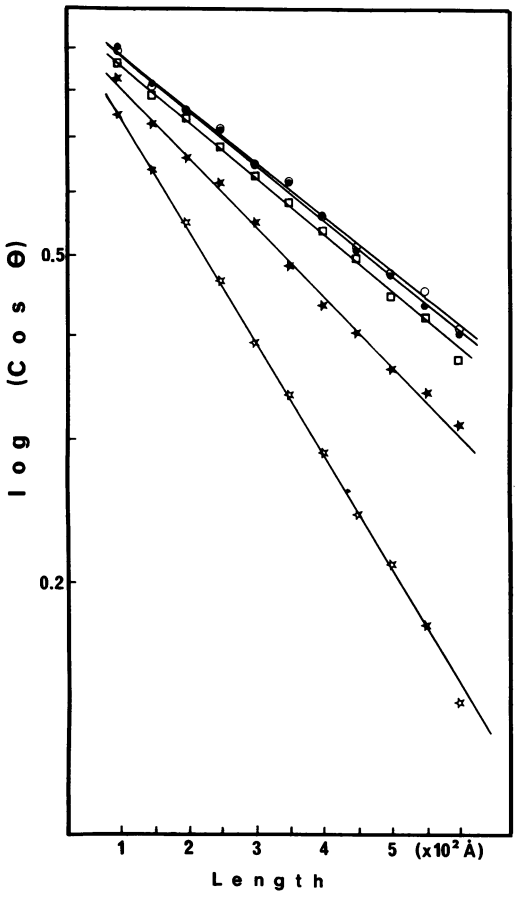

Figure 5 : A kinetic experiment of the binding of cis-Pt to poly(dG-dC) at r_b = 0.10. Plot of the log(cos Θ) versus the length of elementary segments (s). □ □ : 15 min. ; ●—● : 45 min. ; ○—○ : 2 h. ; ✶—✶ : 5 h. ; ✡—✡ : 7 h.

the intermediate curve. After 7 hours further binding is evidenced by the strong increase of the slope.

End-to-end distances

The same digitized molecules were used to establish a plot of the squares of the end-to-end distances versus the lengths (s) of elementary segments of increasing lengths (fig. 6). Unplatinated and platinated polymers at three r_b (0.05, 0.10, 0.20) are analysed. From 500 Å to 1600 Å the plots are linear and their slopes decrease as r_b

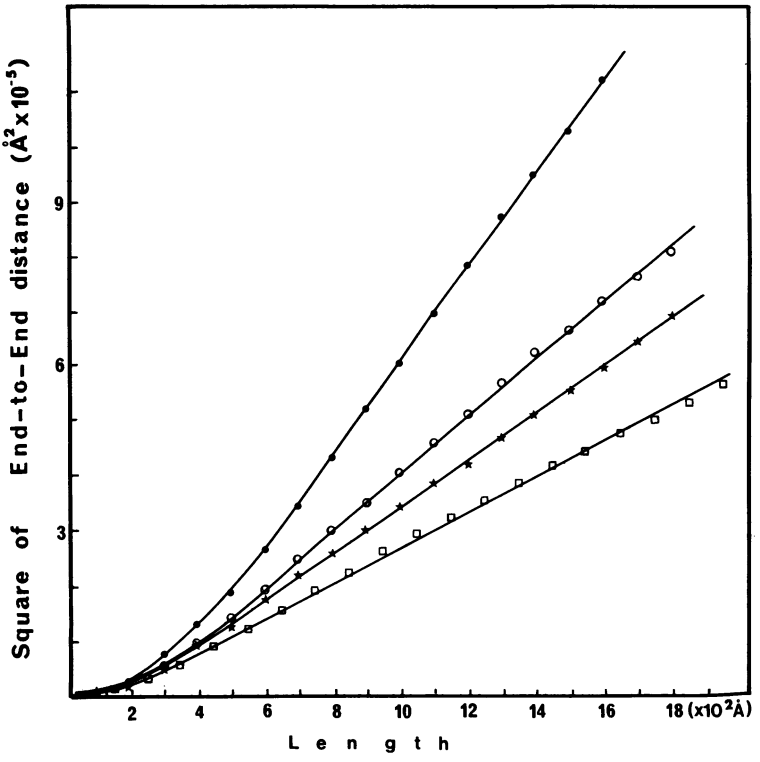

Figure 6 : Plot of the square of the end-to-end distances versus their length for elementary segments of poly(dG-dC)cis-Pt of increasing size. The labels are the same as for fig. 4. ●—● : r_b = 0 ; ○—○ : r_b = 0.05 ; ✱—✱ : r_b = 0.10 ; □—□ : r_b = 0.20.

increases. This complements the results obtained with the cos θ analysis and indicates no long-distance effect upon cis-Pt binding.

Poly(dG-m^{5}dC)cis-Pt

Experiments were performed with poly(dG-m^{5}dC). The cis-Pt was added to the polymer in either the B or Z conformation. The Z conformation of poly(dG-m^{5}dC) (fig. 7b) is stabilized by the addition of 10 mM $MgCl_2$ (18). Poly(dG-m^{5}dC) in the B conformation (fig. 7a) has a slightly different appearance from that of the non-methylated polymer and the samples studied here are shorter and more branched than poly(dG-dC).

The molecules in the B (fig. 7a) or in the Z (fig. 7b) conformation show the same aspects. However one can distinguish between the two conformations by the graphical representation of log(cos θ) versus s (fig. 8). The persistence lengths are 400 Å and 290 Å for the B and the Z

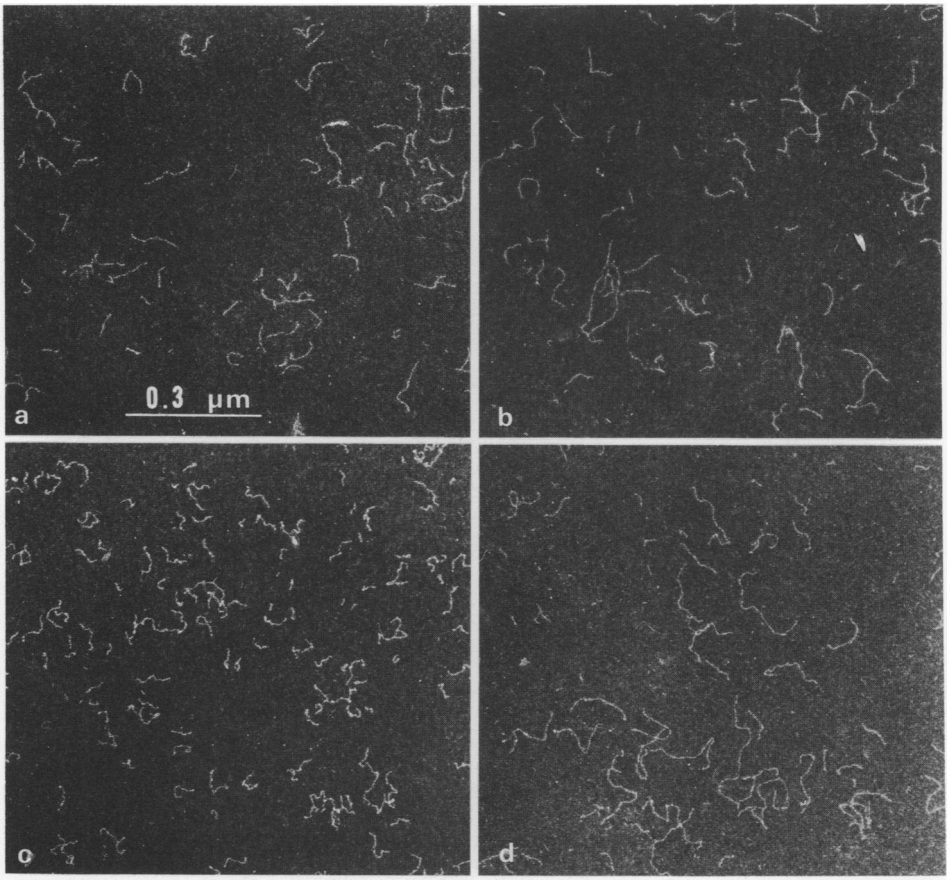

Figure 7 : Poly(dG.m^5dC) either in the B **(a, c)** or Z **(b, d)** conformation. Control molecules **(a, b)** and cis-Pt treated polymer at r_b = 0.10 **(c, d)**.

conformation, respectively. At r_b = 0.10, cis-Pt induces alterations in the poly(dG-m^5dC) in the B form (fig. 7c), while the Z form is not altered as much (fig. 7d, fig. 8). As shown in the plot of log(cos Θ) versus s, there is a large drop in the slope of the platinated B poly(dG-m^5dC) which leads to a very short value of the persistence length (100 Å) (fig. 8).

DISCUSSION

In the reaction between cis-Pt and poly(dG-dC) or poly(dG-m^5dC) in the B conformation, the main product is a bidentate adduct which arises from an intrastrand crosslink between two guanine residues separated by a

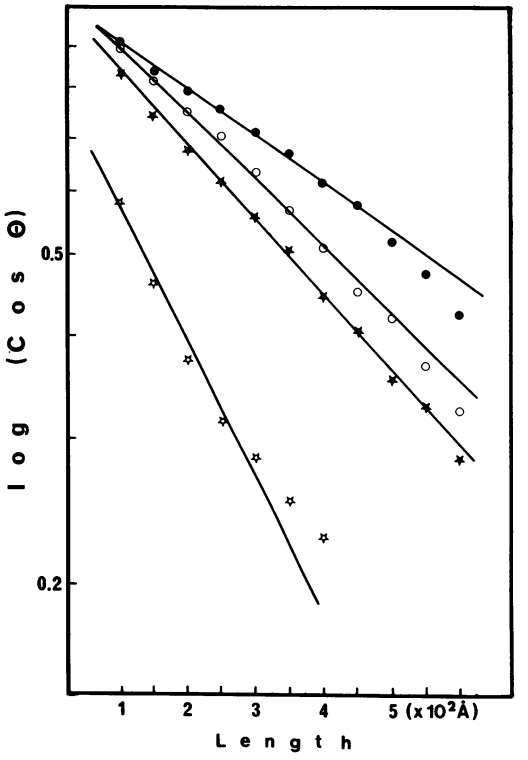

Figure 8 : Plot of the log(cos Θ) versus s of the platinated and unplatinated poly(dG-m^{5}dC) in the B and Z forms. ○—○ : B form at r_b : 0 ; ●—● : Z form at r_b = 0 ; ☆—☆ : B form at r_b = 0.10 ; ✶—✶ : B form at r_b= 0.10.

cytosine or a methyl^{5}cytosine residue. This crosslink causes a distorsion of the double helix which affects the phosphodiester backbone and the bases as demonstrated by spectroscopic data and the reaction with antibodies to Z-DNA (8, 9). The analysis by electron microscopy of the cis-Pt effects on these polynucleotides by measuring their persistence lengths shows that the shape of the platinated polymer is dramatically modified if it is in the B form. The monodentate adduct formed in the reaction of cis-Pt and poly(dG-m^{5}dC) in the Z conformation induces only small changes in the conformation of the macromolecules.

A theoretical problem arises when our values of persistence length for untreated polymers are compared with the values already published (14). The latter are usually about twice as large as ours. The main reason for this discrepancy could be simply related to the methodo-

logy used in this work.

The work of Frontali **et al.** assumes that : "the bidimensional pattern is **not** obtained by projection but by the allowed deformations of the flexible molecules. Under these conditions the adsorption process is equivalent to the blocking of one degree of freedom". This is reflected by the formula : $\langle \cos \theta(s) \rangle = \exp(-s/2a)$ in a two-dimensional system, whereas in a three-dimensional system, we have : $\langle \cos \theta(s) \rangle = \exp(-s/a)$ (18).

One must consider what actually happens when DNA molecules free in a solution are adsorbed to the surface of a liquid phase and trapped in a basic protein film. The molecules are allowed to exhibit their own stiffness in a plane, and this, consequently reflects their actual configuration in solution.

The adsorption method that we used consists of a rapid attachment of molecules free in solution to a supporting film which is positively charged (16). In the procedure used here, the binding of the DNA to the grid may not allow the DNA to reach equilibrium in the two dimensional state because it is so rapidly changed from a three dimensional state into a two dimensional one. Consequently, this may explain why our values of the persistence length differ from those obtained by Frontali **et al.** who used a different procedure for binding DNA molecules to a support film. We have observed for different polymers with various topologies (unpublished results), that spreading of a polymer on an aqueous surface or its adsorption onto a carbon film are not equivalent. For instance, molecules adsorbed onto an activated carbon film, as done here, have a more contorted aspect than those obtained by any procedure of spreading using a basic protein film or a detergent like BAC (19). Another procedure consisting in spraying thread-like molecules on mica has brought the authors to discuss this problem of bringing molecules from 3-D to 2-D (20).

An important question arises concerning the difference between the stiffness of Z-DNA compared with that of B-DNA. Attempts to measure this difference were done by using light scattering (21). These authors were able to show a large increase in the chain stiffness of Z-DNA as compared with the B form. Ultrastructural studies by us (13) and others (22) have confirmed previous observations (23) showing that Z-DNA, especially in the presence of divalent cations, has a strong tendency to build up parallel arrays of molecules stuck together. Poly(dG-dC) in 3 M NaCl actually presents this particular morphology of short thick segments (see fig. 3a in ref. 13). The persistence length measurements of polymers

showing three dimensional associations between molecules are likely to be overestimated values. In the present study persistence lengths of about 290 and 400 Å were calculated for poly(dG-m^5dC) in the B and Z forms (fig. 8), both molecular forms being studied as individual double-stranded segments.

Examination of high magnification pictures shows that the binding of cis-Pt to poly(dG-dC) and poly(dG-m^5dC) induces bends in molecules and a few loops and hairpins. As r_b increases, the molecules appear more compact. There is no change in the distribution of the total contour length but the persistence length decreases. The persistence length of poly(dG-dC)cis-Pt(0.20) is about three times smaller than that of poly(dG-dC). The effect of cis-Pt on poly(dG-m^5dC) seems to be more efficient in terms of reducing the persistence length, as compared to poly(dG-dC). For the poly(dG-m^5dC) in the Z conformation, the shapes of the platinated and unplatinated molecules look similar. For the Z form, as already shown (8), monodentate adducts prevail and consequently should not induce drastic changes in the shape of the molecule. However the persistence length of the platinated polymer is reduced as compared to the unplatinated one (260 and 400 Å respectively). This might reflect changes in hydration due to platinum residues and/or small distortions due to the interactions between cis-Pt and the neighbouring nucleotide residues.

The experiments reported here on platinated poly(dG-dC) show important changes in the secondary structure of molecules, making them more condensed, but without reducing their length. Moreover, the determination from high resolution electron micrographs of the persistence length of unplatinated and platinated polynucleotides allowed a precise monitoring of the alterations in DNA secondary structure induced by the different binding modes of cis-Pt.

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