Thiourea Reverses Cross-Links and Restores Biological Activity in DNA Treated with Dichlorodiaminoplatinum(II)

Abstract. Cis *and* trans *dichlorodiaminoplatinum(II) compounds bind to DNA and form DNA cross-links, which are usually considered to be irreversible. Thiourea can reverse these cross-links without any apparent breakdown of the DNA. In addition,* cis- *and* trans-*Pt(II) treatment of* λ *DNA decreases its transfectivity. After suitable incubation with thiourea, full transfectivity of Pt(II)-treated* λ *DNA can be restored.*

The *cis* and *trans* isomers of dichlorodiaminoplatinum(II) [*cis*-Pt(II) and *trans*-Pt(II)] form various complexes with DNA. In one kind of Pt-DNA complex, Pt bridges the complementary DNA strands to form interstrand cross-links, which are believed to be responsible for at least some aspects of the biological effects of *cis*- and *trans*-Pt(II), and supposedly for the antitumor activity of the former (*1*).

Complexes of Pt(II) with DNA, including cross-linked complexes, are stable to alkali, heat, and trichloroacetic acid. While DNA can be recovered intact from complexes with Ag^+ or Hg^{2+} after dialysis, such attempts with Pt(II)-DNA complexes have not been successful. Only a fraction of bound Pt was removed by prolonged dialysis in $10^{-3}M$ NaCN (*2*). Stone *et al.* (*3*) showed that *cis*-Pt(II) binding increased the isopycnic separation of mitochondrial and main-band (nuclear) DNA; however, they could not regenerate unmodified DNA from the complex.

The ability to recover intact DNA from Pt-DNA complexes would be useful in studies on DNA fractionation and in studies of the biological effects of Pt compounds. The ability to reverse the *cis*-Pt(II)–DNA complexes might also be applicable to chemotherapy. The binding constants of Pt(II) complexes with amino compounds are usually high, but frequently phosphorus or sulfur analogs show even higher affinity toward Pt. Therefore, we tested some sulfur-containing compounds for their ability to act as competing ligands for the removal of Pt from complexes with DNA. We report here the dissociation of Pt-DNA complexes with thiourea as determined by biochemical and biological methods.

The formation and removal of DNA interstrand cross-links during treatment of DNA with *cis*- or *trans*-Pt(II) and thiourea were determined by the method of Painter and Cleaver (*4*) and Roberts and Pascoe (*5*). To obtain DNA, we grew L1210 cells so that some portions of one strand of the DNA was labeled with 5-bromo-2′-deoxyuridine (BrdU) and [^{14}C]thymidine and therefore became both denser and radioactive. Nonlabeled DNA was monitored by its absorbancy (percentage transmission) at 253 nm. Figure 1a shows that in alkaline CsCl gradients the non-cross-linked DNA separates into two peaks of widely different densities. The radioactivity is found only in the heavy peak. After treatment with *cis*-Pt(II) (Fig. 1b) a new peak containing both radioactivity and absorbance appears at an intermediate density accompanied by a decrease in the amount present in the heavy and light peaks. In addition, the density of the remaining non-cross-linked material has increased, presumably because of the binding of Pt. The cross-linking shown in Fig. 1b was present immediately after treatment when the non-cross-linked DNA strands were separated by addition of alkaline CsCl.

When such material is treated with 1*M* thiourea for 2 hours at 50°C (Fig. 1c), most of the cross-linked material disappears and there is an increase in the amount of material in the non-cross-linked peaks. After being reacted for 24 hours with 1*M* thiourea at 37°C (Fig. 1d)

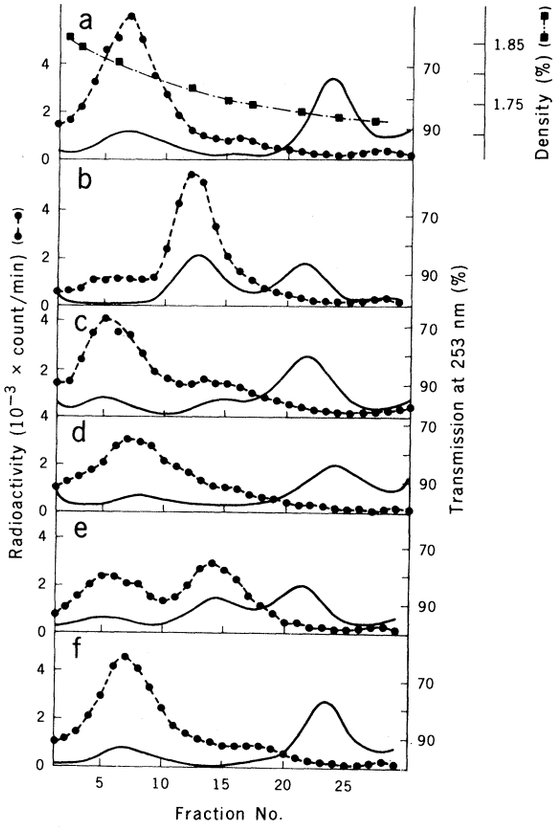

Fig. 1. Formation and reversal of interstrand cross-links. L1210 cells were grown for 2.5 hours in medium containing BrdU (5 μg/ml) and then for 3 hours with BrdU and [^{14}C]thymidine (0.1 μl/ml). The cells were washed, then grown for a further 2 hours in fresh medium with BrdU but no [^{14}C]thymidine. Cells were harvested and washed twice with 10 m*M* Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *p*H 7.5] containing 1 m*M* $CaCl_2$ and 0.32*M* sucrose. Nuclei were prepared with the same solution containing 0.3 percent Triton N-101, and were washed twice with this solution and then twice with 10 m*M* Hepes containing 1 m*M* $CaCl_2$. To prepare DNA, we lysed the nuclei in 10 m*M* Hepes and 1 m*M* $CaCl_2$ by the addition of Na_2 EDTA to a concentration of 2 m*M* and sarcosyl to 0.09 percent. The preparation was incubated with ribonuclease (4 μg/ml; Sigma) for 15 minutes at room temperature, then incubated with Proteinase-K (0.5 mg/ml; EM Laboratories) at 50°C for 30 minutes. The solution was made 1*M* in NaCl, shaken gently three times with a mixture of chloroform and isoamyl alcohol (10:1 by volume), then mixed with two volumes of ethyl alcohol to precipitate the DNA. Both *cis*- and *trans*-$Pt(NH_3)_2Cl_2$ were kept as 1 m*M* stock solutions in distilled H_2O at 37°C. After 48 hours the complexes became aquated (*1*). The solutions were used for 1 month, during which time we noticed no significant change in reactivity. The DNA was dissolved in 20 m*M* potassium phosphate buffer, *p*H 7.0 (3.02 A_{260} units per millimeter), and 25 μl of this solution was incubated for 2 hours at 37°C with 18.2 μ*M* *cis*- or *trans*-Pt(II) (Pt to DNA base input ratio of 0.04). Depending on the experiment, the reaction was stopped either by mixing the solution immediately with alkaline CsCl or by dialyzing the solution against 1*M* thiourea in the same buffer. Thiourea was removed by dialysis at 4°C for 18 hours against the same buffer. Samples were prepared for centrifugation in alkaline CsCl by increasing their volume to 0.5 ml with 20 m*M* potassium phosphate buffer, *p*H 7.0. The sample was then added to 6.7 ml of a solution containing 10.9 g of CsCl in 0.1*M* potassium phosphate buffer titrated to *p*H 12.6 with KOH. After centrifugation for 42 hours at 40,000 rev/min (25°C) in a Beckman 65 rotor, the tubes were punctured and the contents passed through an LKB Uvicord into a fraction collector (0.3-ml fractions). Fractions were diluted with 3.5 ml of H_2O and added to vials with 10 ml of Aquasol for radioactive counting. (a) Control; (b) [*cis*-Pt(II)] to [DNA base] input ratio = 0.04; (c) as in (b), followed by 1*M* thiourea, 2 hours, 50°C; (d) as in (b), followed by 1*M* thiourea, 24 hours, 37°C; and (e) [*trans*-Pt(II)] to [DNA base] input ratio = 0.04; (f) as in (e), followed by 1*M* thiourea, 2 hours, 50°C.

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the Pt-treated DNA gives a distribution pattern that resembles the control (Fig. 1a) more closely than the pattern after 2 hours at 50°C (Fig. 1c) in that the trace for percentage transmission shows no hybrid component and the non-cross-linked peaks have returned to positions almost coincident with the non-cross-linked peaks of the control.

The *cis*-Pt(II)-treated DNA sample (Fig. 1b) was added to alkaline CsCl to terminate the incubation by separating and diluting the strands. When similar samples were dialyzed and, except for the omission of thiourea, were treated according to the procedure described in Fig. 1, c and d, the cross-linking pattern was the same as that in Fig. 1b. This result shows that thiourea and not just dialysis is necessary for the cleavage of these cross-links.

Trans-Pt(II) also forms interstrand cross-links in DNA (Fig. 1e), although it is not as effective as *cis*-Pt(II) at the same Pt to DNA base input ratio. The cross-linking is effectively reversed after 2 hours at 50°C in 1*M* thiourea (Fig. 1f).

Other sulfur-containing compounds were also tested for their ability to dissociate Pt-DNA complexes. 2-Mercaptoethanol caused extensive DNA degradation. Tetramethylthiourea, sodium thiooxalate, and sodium thiosulfate were also found to dissociate Pt(II)-DNA complexes, but none of these reagents was clearly better than thiourea.

Interstrand cross-links are not the only possible type of modification introduced into DNA by reaction with Pt(II) compounds. Pascoe and Roberts (*6*) showed that only a small fraction of Pt(II) molecules is engaged in interstrand cross-link formation. To check whether Pt-induced lethal lesions are removable by thiourea treatment, we used λ phage DNA in a transfectivity assay; this enabled us to follow the diminishing transfectivity of the λ DNA after Pt treatment and to test whether incubation with thiourea would regenerate transfectivity. Transfecting activity of λ phage DNA after 1 hour of incubation at 37°C with different amounts of *cis*-Pt(II) is shown in Fig. 2 (circles). The logarithm of λ DNA survival is linear with *cis*-Pt(II) concentration. After incubation with 1*M* thiourea at 37°C, the transfectivity of the Pt-treated λ DNA increases and is close to the control levels after 60 hours of incubation. Figure 3 shows the data plotted according to the incubation time.

The behavior of *trans*-Pt(II)-treated λ DNA toward thiourea was briefly investigated. After 3 hours of incubation at 37°C, *trans*-Pt(II) (Pt to DNA base input ratio of 3×10^{-2}) reduced λ DNA transfectivity to 0.4 percent of the control. After 1 hour of treatment with 1*M* thiourea, λ DNA transfectivity was increased to 86 percent of the control (open squares in Fig. 3). Therefore, *trans*-Pt(II)-induced lethal lesions seem to be more easily reversed than those induced by *cis*-Pt(II). This result agrees with that obtained in Fig. 1; namely, that 2 hours at 50°C in 1*M* thiourea more completely reverses *trans*-Pt(II)-induced interstrand cross-links than *cis*-Pt(II)-induced interstrand cross-links.

One factor that affects the quantitative interpretation of the results but not their qualitative validity is that the second reaction, which actually forms the cross-link or possibly some other lethal DNA lesion, is often significantly slower (*7*) than the first reaction which binds the molecule to DNA. Since the Pt inactivation curve for λ DNA was obtained without any incubation with thiourea, inactivation of λ DNA could have continued after removal of free Pt(II) from the environment, for example, during the growth of bacteria after transfection. It is possible, therefore, that part of the thiourea effect is not true reversal of Pt(II)-induced lethal lesions, but merely the prevention of their formation. We found, in fact, that Pt(II)-treated λ DNA did continue to be inactivated at 37°C after removal of the unbound Pt(II), although the rate of inactivation was slower than when unbound Pt(II) was present. In addition, we found that incubation of the Pt(II)-treated λ DNA with 0.1*M* thiourea for 1 hour at 37°C (followed by dialysis to remove the thiourea) effectively prevented further inactivation.

This last finding enabled us to estimate directly what fraction of the thiourea effect could be attributed to prevention of inactivation rather than reversal of inactivation. We treated λ DNA with *cis*-

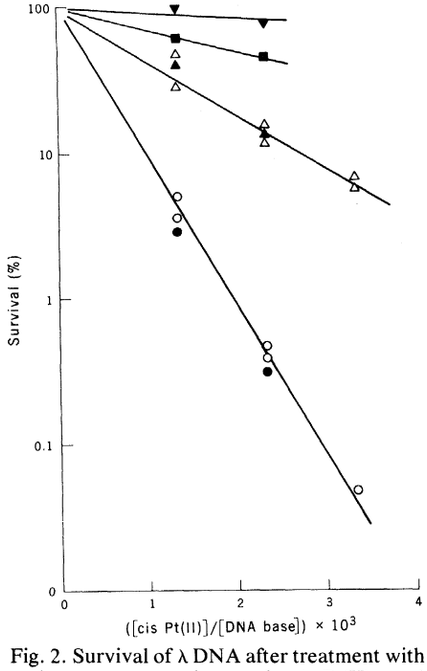

Fig. 2. Survival of λ DNA after treatment with various concentrations of *cis*-Pt(II) and thiourea. Ten micrograms of λ DNA were incubated with *cis*-Pt(II) in a volume of 23 μl for 1 hour at 37°C, then dialyzed against 0.1*M* tris-HCl, *p*H 7.2, at 4°C or incubated with 1*M* thiourea at 37°C for various times. Then all samples were dialyzed against 0.1*M* tris-HCl, *p*H 7.2, at 4°C for 3 hours. The DNA samples were diluted to 1 ml with 0.1*M* tris-HCl, *p*H 7.2, and 0.1 ml was saved for transfection and the other 0.9 ml was used to determine the DNA concentration [absorbancy at 260 nm (A_{260}), with it being assumed that 1.0 mg of DNA per milliliter yields 20.0 A_{260} units]. Depending on the A_{260} reading, the 0.1-ml portion was diluted serially with 0.1*M* tris-HCl, *p*H 7.2, so that 0.1-ml portions contained 100, 10, or 1 ng DNA. Transfection was performed according to Mandel and Higa (*10*) as modified by Cameron *et al.* (*11*). λ DNA from strain C1857 Sam 7 (Bethesda Research Laboratories) and *Escherichia coli* ED 8654 [*Sup E, Sup F, hsd* $R^-M^+S^+$, *met*$^-$ *trpR* (*12*)] were used. After transfection, cells were poured onto TB plates (2.5 g of NaCl, 10 g of Difco Tryptone, 11 g of Difco agar, and 1 ml of 0.01 percent thiamine hydrochloride per liter). Plaques were counted after overnight growth at 37°C without thiourea (○ and ●); and after 13 hours (△ and ▲), 42 hours (■), and 60 hours (▼) in the presence of thiourea. Open and closed symbols represent two different experiments.

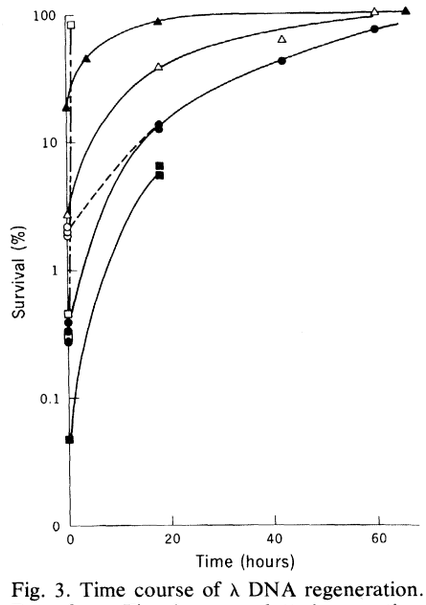

Fig. 3. Time course of λ DNA regeneration. Data from Fig. 2 are replotted as a time course. The [*cis*-Pt(II)] to [DNA base] input ratios are: 3.3×10^{-3} (■), 2.3×10^{-3} (●), and 1.0×10^{-3} (△). The value obtained when the *cis*-Pt(II) reaction was stopped with 0.1*M* thiourea for 1 hour at 37°C (see text) is shown by (○). The [*trans*-Pt(II)] to [DNA base] input ratio of 3×10^{-2} obtained after 3 hours of incubation at 37°C followed by 1 hour of incubation with 1*M* thiourea at 37°C is shown by (□).

Pt(II) (Pt to DNA base input ratio of 2.3×10^{-3}) for 1 hour at 37°C as shown in Fig. 2 and then with 0.1*M* thiourea for 1 hour at 37°C. Instead of 99.6 percent inactivation (0.4 percent survival; closed circles in Fig. 3) inactivation was decreased to 98.0 percent (2 percent survival; open circles in Fig. 3). Since 2 percent survival is still considerably less than the 12 percent survival found after 18 hours in 1*M* thiourea, this result shows that the predominant effect of prolonged incubation with 1*M* thiourea is to reverse the lethal lesions present in the λ DNA, and not merely to prevent their formation.

This study shows that thiourea reverses Pt(II)-induced DNA cross-links and lethal lesions in isolated DNA. Since *cis*-Pt(II) is a useful anticancer agent, it is a question of considerable interest whether thiourea or a related compound can reverse cytotoxic lesions of the drug in vivo. Burchenal *et al.* (*8*) have recently reported that thiourea can block the action of *cis*-Pt(II) complexes on mouse leukemia cells. It remains to be determined whether toxic lesions produced by Pt complexes in mammalian cells can actually be reversed. This question can be approached in the context of recent studies of the kinetics of formation and removal of DNA-related cross-links in mammalian cells treated with pharmacologically reasonable doses of Pt complexes (*9*).

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Melatonin Synthesis by the Retina

Abstract. *Melatonin fulfills many of the criteria for classification as a hormone. Until recently it was considered to be elaborated exclusively by pineal organs. Melatonin synthesis by other tissues has been indicated but not demonstrated unequivocally. Trout retinas in a whole-organ culture system in vitro synthesized tritiated melatonin from a substrate containing tritiated serotonin. This raises the possibility that the trout retina is an endocrine organ.*

Several sources of evidence indicate that tissues other than the pineal gland are capable of melatonin formation. However, an exacting demonstration of nonpineal melatonin synthesis has not, to our knowledge, been accomplished. Three tissues, lateral eye retina (*1–3*), enterochromaffin cells (*4, 5*), and Harderian gland parenchyma (*1, 2*) contain melatonin, but it has not been shown that the melatonin is synthesized by these tissues rather than obtained by uptake from a circulating pool of melatonin originating from the pineal. Hydroxyindole-*O*-methyltransferase (HIOMT), the enzyme that catalyzes the *O*-methylation of *N*-acetylserotonin, which results in the formation of melatonin, has been demonstrated in the retina (*6–8*) and Harderian gland (*9*). Nevertheless, HIOMT activity, although being indicative of melatonin formation, does not irrefutably imply this molecule's synthesis, because it can catalyze the formation of several compounds in addition to melatonin (*10*).

Attempts have been made to establish the rat retina as a melatonin-synthesizing tissue both in vivo and in vitro by demonstrating the conversion of isotopically labeled precursor to melatonin (*11, 12*). However, with the techniques then available, it was not possible to demonstrate conclusively that melatonin synthesis occurs in this tissue.

By using whole-organ cultures of rainbow trout (*Salmo gairdneri*) retinas, we have shown that the retina is capable of synthesizing melatonin from a serotonin substrate.

Individual, whole retinas, stripped of all pigmented epithelium, were incubated in a medium of Hanks buffered saline solution, containing glucose, $1 \times 10^{-6}M$ norepinephrine, and [^{3}H]serotonin hydrochloride (70,000 count/min; 2.39 ng) under an atmosphere of 95 percent O_2 and 5 percent CO_2 for 4 hours at 21°C. After incubation, each retina was homogenized in its incubation medium and the resulting homogenate extracted (*13*). Each retinal extract (50 μl) was spotted separately onto a thin-layer chromatography (TLC) plate that was subsequently developed in a solution of chloroform and methanol (9:1). Upon development, the ascent lanes were cut into 20 segments (0.05 R_F each) and the synthesized ^{3}H-labeled molecules deposited along the ascent lanes were quantified by liquid scintillation spectrometry with toluene, PPO-POPOP (*14*), and Triton X as the fluors.

A ^{3}H-labeled molecule with a mobility identical to melatonin (R_F 0.50 to 0.65) (*15*) was formed by the incubated retinas (Fig. 1). No ^{3}H-labeled products were found on the TLC plates on which extracted medium blanks containing no tissue were spotted. However, melatonin and 5-methoxytryptamine cannot be separated easily by TLC procedures (*16*) and 5-methoxytryptamine can be formed by *O*-methylating serotonin (HIOMT catalyzed) (*10*); therefore, melatonin synthesis, although suggested, was not conclusively demonstrated.

Individual retinas were cultured as before and extracts of each retina and medium were subjected to the same TLC separation procedure. This time, however, the entire area of the ascent lane where melatonin was suspected to reside, after development of the chromatogram, was removed and the tritiated products were eluted off the lanes with chloroform. The combined eluates were dried under N_2 and then resuspended in 200 μl of phosphate-buffered saline and 0.1 percent gelatin (PBS-gel). After allowing 24 hours for resuspension, we reextracted this suspension in 2.5 ml of petroleum ether (*17*). Melatonin-specific rabbit antiserum in EDTA-PBS (2.5 μl of a 1:400 initial dilution) was added to a 100-μl portion of the petroleum ether–extracted, resuspended retinal extract, resulting in a final dilution of 1:16,000 of the specific antibody. Another 100-μl portion was removed and 2.5 μl of normal rabbit serum in EDTA-PBS (1:400 initial dilution, 1:16,000 final dilution) was added. These suspensions were incubated at 4°C for 24 hours in a shaker bath. The petroleum ether eluate was taken to a volume of 200 μl under N_2, after which four 50-μl portions were removed and placed into scintillation vials. The amount of ^{3}H-labeled product removed by this extraction was then quantified.

After the 24-hour incubation period, the ^{3}H-labeled products bound to normal rabbit serum and the [^{3}H]melatonin bound to the specific antibody were as-

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