Biochemical Pharmacology, Vol. 23, pp. 1659–1662. Pergamon Press, 1974. Printed in Great Britain.

SHORT COMMUNICATION

The effects of platinum ethylenediamine dichloride on the template activity of DNA

(*Received* 31 *July* 1973; *accepted* 1 *November* 1973)

THE ABILITY of certain co-ordination complexes of platinum to inhibit tumour growth and macromolecular synthesis in mammalian cells is now well established.[1–5] A number of studies have shown that these compounds bind to RNA, DNA and protein,[5–7] but the effect of such binding on the function of these macromolecules is not yet understood.

We have studied the effects of treating both native and denatured calf thymus DNA with platinum ethylenediamine dichloride [Pt (en) Cl_2] on their ability to act as a template for nucleic acid synthesis by RNA- and DNA-polymerases *in vitro*. Both RNA and DNA synthesis was reduced by treatment of the template with Pt (en) Cl_2; however the greatest effect on RNA synthesis was observed using native DNA as the template whereas the depression of DNA synthesis was largest when denatured DNA was used.

Materials and methods

Treatment of DNA. Calf thymus DNA (Type 1 "Highly Polymerised", Sigma London Chemical Co. Ltd., Kingston-upon-Thames, England) was dissolved in 0·01 M Tris–HCl buffer at pH 7·5 to give a concentration of 1 mg DNA/ml. "Denatured" DNA was prepared by heating portions of this solution at 100° for 10 min and cooling rapidly in ice. Preliminary experiments showed that this treatment yielded a DNA solution which was 87 ± 5 per cent (standard deviation on 7 determinations) denatured. Equal volumes of DNA solution and Pt (en) Cl_2 solution, 2×10^{-6} to 2×10^{-4} M in 0·01 Tris-HCl pH 7·5, were mixed and incubated at 37° for up to 4 hr.

Measurement of DNA and RNA synthesis. DNA polymerase (DNA nucleotidyl-tranferase EC 2.7.7.7.), from *Micrococus lysodeikticus*, and RNA polymerase (RNA nucleotidyl transferase EC 2.7.7.6.), from *Escherischia Coli* Strain K-12, were obtained from Sigma London Chemical Co. Ltd.

DNA polymerase activity[8] was assayed in 0·3 ml reaction mixture containing 20 μmoles Na glycinate pH 9·2; 2 μmoles $MgCl_2$, 0·3 μmoles mercaptoethanol; 10 nmoles deoxyadenosine-5′-triphosphate, deoxycytidine-5′-triphosphate, deoxyguanosine-5′-triphosphate and thymidine (methyl ^{3}H)-5′-triphosphate (1–2 μCi, sp. act. 5 Ci/m-mole, Radiochemical Centre, Amersham, England); 30 μl DNA solution (or drug-treated DNA solution) 0·1 units DNA polymerase [in solution[9] in 50 mM Tris–HCl pH 7·5; 0·1 M ammonium sulphate, 10 mM mercaptoethanol] and 1 mg/ml Bovine serum albumin (Sigma London Chemical Co.). The mixture was incubated at 37° for 15 or 30 min.

RNA polymerase activity was assayed[10] in 0·25 ml reaction mixture containing 10 μmoles Tris–HCl pH 7·9; 2·5 μmoles $MgCl_2$; 25 μmoles ethylenediaminetetraacetic acid; 25 μmoles dithiothreitol; 37·5 μmoles KCl; 100 μmoles K_2HPO_4; 20 nmoles each adenosine 5′ triphosphate, cytidine 5′ triphosphate, guanosine 5′ triphosphate and uridine 5,6, [^{3}H]-5′-triphosphate (1–4 μCi, specific activity 40 Ci/m-mole, Radiochemical Centre); 30 μl 0·5 mg/ml bovine serum albumin; 30 μl DNA template solution and 0·6 units RNA polymerase (in 50 per cent glycerol, 50 per cent 0·01 M Tris–HCl pH 7·9, 0·01 M Mg Cl_2, 0·1 M KCl, 0·1 mM EDTA, 0·1 mM dithiothreitol). The mixture was incubated for 15 or 30 min at 37°.

At the end of the incubation period ^{3}H incorporation into acid soluble material in both assays was determined by the method of Bollum.[11] 100 μl reaction mixture was placed on a 17 × 17 mm square of Whatman 3MM Chromatographic paper and placed in ice cold 10 per cent (w/v) trichloroacetic acid containing 0·01 M sodium pyrophosphate for 30 min. The paper was then placed in a further volume of this solution for 15 min, then in ethanol–diethylether (1:1) for 30 min and in diethylether for 5 min. The dried paper squares were placed in vials containing 5 ml 6 per cent (w/v) Butyl PBD in toluene and assayed for ^{3}H in a liquid scintillation counter.

Blank samples containing all the reactants except the DNA template were run with each batch of analyses.

Measurement of drug binding to DNA. The amount of Pt (en) Cl_2 bound to DNA was measured using drug labelled with ^{14}C in the ethylene diamine moiety.[12] Calf thymus DNA was incubated with the labelled drug (sp. act. 2·4 μCi/μmole) under the conditions described above. At the end of the 4 hr period

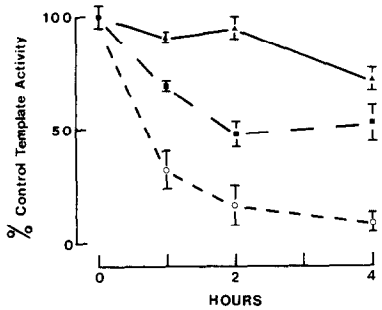

FIG. 1. The variation with time of treatment of the effect of Pt (en) Cl_2 on the ability of denatured calf thymus DNA to act as a template for DNA polymerase *in vitro* (15 min incubation with enzyme). (▲) 10^{-6} M, (■) 10^{-5} M, (○) 10^{-4} M. Errors bars represent standard deviations (3–7 values per point).

of incubation, the DNA was precipitated with an equal volume of 10 per cent trichloroacetic acid containing 0·01 M sodium pyrophosphate, allowed to stand at 2° for 30 min, centrifuged and washed 4 times with 4 vol. trichloroacetic acid solution and twice with 4 vol. ethanol. The washed precipitate was dissolved by heating in 1 vol. M $HClO_4$ at 65° for 1 hr and an aliquot of the resultant solution was assayed for ^{14}C by liquid scintillation counting.

Results and discussion

Preliminary experiments showed that the rate of incorporation of ^{3}H-TTP into DNA and ^{3}H-UTP into RNA was linear over a period of at least 30 min irrespective of whether drug-treated or non-drug treated native or denatured DNA was used as the template.

The effects on DNA polymerase activity of exposure of denatured DNA to different concentrations of Pt (en) Cl_2 for 1, 2 or 4 hr are illustrated in Fig. 1. Only a slight decrease in DNA polymerase activity is seen after exposure of the template to the drug at a concentration of 10^{-6} M, but marked depression is seen after exposure to the drug at 10^{-5} and 10^{-4} M. At these two higher concentrations the effect of the drug appears to be maximal after 2 hr.

The effects on both DNA and RNA synthesis of exposing native and denatured calf thymus DNA to three different concentrations of drug for 4 hr are shown in Fig. 2. The amounts of drug bound to template DNA during this period are listed in Table 1. These results show that at all three drug concentrations tested the depression of DNA synthesis is greatest with denatured DNA as template. However, the reverse effect is observed with RNA synthesis where the drug produces the largest effect when native DNA is used as the template.

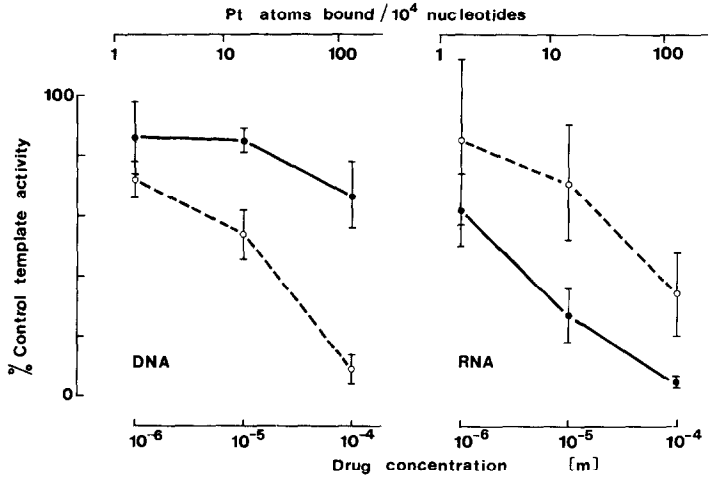

FIG. 2. The effect of Pt (en) Cl_2 on the template activity of native and denatured calf thymus DNA towards RNA and DNA polymerase. Template treated with drug for 4 hr prior to assay. Error bars represent standard deviation (6 values per point). (●) Native DNA; (○) denatured DNA.

TABLE 1. BINDING OF Pt (^{14}C-en) Cl_2 TO CALF THYMUS DNA (4 hr INCUBATION AT 37° AT pH 7·5)

Moles/l.	Drug concn Moles/10^4 nucleotides*	Pt atoms bound per 10^4 nucleotides Native DNA	Denatured DNA
10^{-4}	3200	133 ± 11†	151 ± 14
10^{-5}	320	15 ± 2	16 ± 1
10^{-6}	32	1·4 ± 1	1·4 ± 1

* Assuming average residue weight of 320.
† Mean ± standard deviation of three determinations.

Inhibition by platinum compounds of DNA polymerase activity has been reported by Harder and Rosenberg,[13] and of RNA polymerase activity by Srivastava *et al.*,[14] but neither group of authors have presented their findings in detail.

The effects of Pt (en) Cl_2 on DNA template activity which are reported here are similar to those observed by Ruddon and Johnson[15] following exposure to calf thymus DNA to nitrogen mustard, HN_2. These authors also observed that HN_2 depressed DNA polymerase activity to the greatest extent when denatured DNA was used as template but that the largest effects on RNA polymerase were seen with a native DNA templates. From their studies Ruddon and Johnson[15] calculated that about 25 molecules of HN_2 must be bound per 10^4 nucleotides to produce 50 per cent inhibition of RNA polymerase activity and that about 500 molecules $HN_2/10^4$ nucleotides were required for complete inactivation of the template. Similar calculations may be made for Pt (en) Cl_2 from the data in Table 1 and Fig. 2, these suggest that only 3·5 Pt atoms/10^4 nucleotides are required to reduce template activity towards RNA polymerase by 50 per cent and that about 200 Pt atoms/10^4 nucleotides are needed for complete inactivation. Ruddon and Johnson[15] also concluded that native DNA required six times more alkyations by HN_2 to produce a 50 per cent inhibition of DNA synthesis than were required to produce the same inhibition of RNA synthesis. For Pt (en) Cl_2 the corresponding figure for native DNA appears to be close to 300, with denatured DNA the figure is very much smaller, about 2·5.

These studies with a purified DNA template and purified enzymes suggest that RNA synthesis is more sensitive to Pt (en) Cl_2 binding to the template DNA than DNA synthesis. However, this appears to be in conflict with the studies of Harder and Rosenberg[6] in human amnion AV_3 cells which showed that *in vitro* DNA synthesis was inhibited to a greater extent than either RNA or protein synthesis by Pt (en) Cl_2 and related platinum compounds. The time dependence of the inhibiting action of Pt (en) Cl_2, Fig. 1, and the fact that addition of the drug to the enzyme reaction mixture at the start of the incubation period produces no significant inhibition of polymerase activity (93 ± 5 per cent of control) indicates that the effects observed result from the binding of the drug to the DNA template. The binding of platinum compounds to DNA has been studied by a number of workers[16–18] and Robins[16] has shown that Pt (en) Cl_2 reacts most readily with the deoxyguanosine residues. Srivastava *et al.*[14] have suggested, from studies of circular dichroism, that when Pt (en) Cl_2 reacts with native DNA the double helix remains essentially intact but that there is considerable conformational distortion, possibly due to chelation of the drug by two adjacent stacked bases. If the drug is bound predominantly to one strand of the double helix, with rare interstrand cross-linking, this might explain the greater effect on RNA polymerase activity as compared with that on DNA synthesis. The differences between the effects of Pt (en) Cl_2 on RNA and DNA synthesis *in vivo* and *in vitro* remain to be explained. The greater inhibition of DNA synthesis *in vivo* may well reflect the summation of the effects on RNA and protein synthesis as well as the direct effect of drug binding on the efficiency of the DNA as a template for DNA synthesis. Further studies on the template activity of chromatin and DNA extracted from cells treated with the drug *in vivo* might help to elucidate this point.

Acknowledgements—We wish to thank Mr. Andrew T. Buck for his help and the "Mario Negri Institute" for the award of a travelling fellowship to one of us (T.G.).

Biophysics Division,
Institute of Cancer Research,
Belmont, Sutton, Surrey, England.

TULLIO GIRALDI*
DAVID M. TAYLOR

* Permanent address: Instituto di Farmacologia, Universita degli Studi di Trieste, 34100, Trieste, Italy.

REFERENCES

1. G. R. GALE, J. A. HOWLE and E. M. WALKER, JR., *Cancer Res.* **31,** 950 (1970).
2. R. J. KOCIBA, S. D. SLEIGHT and B. ROSENBERG, *Cancer Chemother. Rep.* **54** (Pt 1) 325 (1970).
3. B. J. LEONARD, E. ECCLESTON, D. JONES, P. TODD and A. WALPOLE, *Nature, Lond.* **234,** 43 (1971).
4. B. ROSENBERG and L. VAN CAMP, *Cancer Res.* **30,** 1799 (1970).
5. J. A. HOWLE and G. R. GALE, *Biochem. Pharmac.* **19,** 2757 (1970).
6. H. C. HARDER and B. ROSENBERG, *Int. J. Cancer* **6,** 207 (1970).
7. J. D. JONES, D. M. TAYLOR and A. B. ROBINS, *Advan. Antimicrob. Antineoplastic Chemother.* **2,** 229 (1972).
8. P. T. ENGLUND in *Procedures in Nucleic Acid Research* (Eds. G. L. CANTONI and D. R. DAVIES), Vol. 2, p. 865. Harper & Row, New York (1972).
9. H. V. APOSHIAN and A. KORNBERG, *J. biol. Chem.* **237,** 519 (1962).
10. R. P. BURGESS and A. A. TREVERS in *Procedures in Nucleic Acid Research* (Eds. G. L. CANTONI and D. R. DAVIES) Vol. 2, p. 851. Harper & Row, New York (1972).
11. F. J. BOLLUM, in *Methods in Enzymology* (Eds. L. GROSSMAN and K. MOLDAVE) Vol. 12B, p. 169. Academic Press, New York (1968).
12. D. M. TAYLOR, J. D. JONES and A. B. ROBINS, *Biochem. Pharmac.* **22,** 833 (1973).
13. H. C. HARDER and B. ROSENBERG, *Abstr. 3rd Int. Biophys. Congr.*, Cambridge, Mass., p. 31 (1969).
14. R. C. SRIVASTAVA, J. FROEHLICH and G. L. EICHORN, in *Proc. 2nd Int. Symp. on Platinum Co-ordination Complexes in Cancer Chemotherapy*, Oxford 1973. Springer, Heidelberg, in press.
15. R. W. RUDDON and M. M. JOHNSON, *Mol. Pharmac.* **4,** 258 (1968).
16. A. B. ROBINS, *Chem.-Biol. Inter.* **7,** 11 (1973).
17. P. HORACEK and J. DROBNIK, *Biochim. biophys. Acta* **254,** 341 (1971).
18. S. MANSY, B. ROSENBERG and A. J. THOMSON, *J. Am. chem. Soc.* **95** 1633 (1973).