MOLECULAR EFFECTS OF TUMOR-INHIBITING PLATINUM COMPOUNDS IN MAMMALIAN CELLS IN VITRO, INVESTIGATIONS INTO THEIR POSSIBLE MECHANISMS OF ACTION

> Thests for the Degree of Ph. D. MICHIGAN STATE UNIVERSITY Harold Cecil Harder 1970



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MOLECULAR EFFECTS OF TUMOR-INHIBITING PLATINUM COMPOUNDS IN MAMMALIAN CELLS IN VITRO: INVESTIGATIONS INTO THEIR POSSIBLE MECHANISMS OF ACTION

presented by

HAROLD CECIL HARDER

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ABSTRACT

MOLECULAR EFFECTS OF TUMOR-INHIBITING PLATINUM COMPOUNDS IN MAMMALIAN CELLS IN VITRO: INVESTIGATIONS INTO THEIR POSSIBLE MECHANISM OF ACTION

By

Harold Cecil Harder

Tumor-inhibiting, bacterial filament-inducing Cis-Platinum(II) diamminodichloride (Cis-Pt(II)) was first shown to inhibit cell division and also to induce giant cell formation in human amnion AV₃ cells, grown in culture.

Cis-Pt(II) was then shown to selectively inhibit DNA synthesis at 5μ M, measured by the incorporation of ³H-thymidine into an acid insoluble material. At 25 μ M the same compound only preferentially inhibited DNA synthesis, for RNA and then protein syntheses as measured by the incorporation of ³H-uridine and ³H-leucine respectively, were also inhibited but more slowly than DNA synthesis. Two other tumor-inhibiting platinum compounds, Cis-Platinum(IV) diamminotetrachloride (Cis-Pt(IV)) and platinum(II) ethylenediaminedichloride (Pt(II)en) were also demonstrated to preferentially inhibit DNA synthesis at 25 μ M. The relative ordering of inhibitory effects on DNA synthesis was the same as the relative effectiveness in sarcoma 180 tumor-growth inhibition.

On the other hand, Trans-Platinum(IV) diamminotetrachloride (Trans-Pt(IV)) and platinum(II) tetramminodichloride ([Pt(II)]⁺²),

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neither of which inhibits Sarcoma 180 growth in mice nor induces bacterial filaments, were shown to have no effect on DNA, RNA or protein synthesis at 25 μ M. The possibility these inhibitory effects on DNA synthesis were a result of a platinum induced limitation of passage of the labeled ³H-thymidine into the cell was eliminated by showing that there was no effect on the uptake of the radioactive precursor into the cell's acid soluble pools with 5 μ M Cis-Pt(II). Hence these experiments support the hypothesis that DNA synthesis is the primary target of tumor inhibiting platinum compounds.

In order to determine how DNA synthesis is inhibited, experiments were performed to determine whether Cis-Pt(II) acts as an antimetabolite. as an inhibitor of enzymes directly involved with the replication of and maintenance of DNA, or by reacting directly with DNA itself. The effects of Cis-Pt(II) on the incorporation of ³H-deoxyuridine, ³H-cytidine, ³H-deoxycytidine, ³H-deoxyadenosine and ¹⁴C-methyl from ¹⁴C-formate into DNA and RNA were shown to be similar to the inhibitory effects on ³H-thymidine and ³H-uridine uptake. However, the incorporation of ³H-thymidine was more efficiently inhibited than any other precursor. This may indicate that the thymidine kinases are inhibited. More significant was the finding that 14C-methyl from 14C-formate incorporation into RNA was inhibited to the same extent as its incorporation into DNA at 5 uM. This was interpreted as evidence for a platinuminduced inhibition of de novo purine synthesis. Nevertheless, the extent of the reduction of purine synthesis was too small to account for the magnitude of DNA synthesis inhibition. Furthermore, when unlabeled deoxyribonucleosides were present in a sufficient quantity, no reversal of the DNA synthesis-inhibitory effects were observed. Hence it was concluded that platinum compounds do not act primarily as antimetabolites.

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To determine whether platinum compounds affect enzymes involved in the maintenance and replication of DNA, studies on the DNase and DNA polymerase were performed. With both enzymes, a concentration-dependent enhancement of activity was observed in extracts of AV3 cells treated with between 50 and 500 µM Cis-Pt(II), Cis-Pt(IV) and Pt(II)en during growth, except for Cis-Pt(II), which had no effect on the DNase activity. However, all 3 of the above were shown to inhibit the DNase and DNA polymerase activities when added to assays containing untreated cell extracts. Hence platinum compounds cannot be directly activating these enzymes by an allosteric interaction with them. $[Pt(II)]^{+2}$ did not inhibit DNA polymerase up to 240 uM. Also Cis-Pt(II) had no effect on the purified enzyme, DNA dependent RNA polymerase from Pseudomonas putida. The latter was taken as evidence that the effectiveness of a given platinum compound strongly depends on its having the proper configuration of ligands attached to it rather than simply being a heavy metal poison effect. To account for both the enhanced DNase and DNA polymerase activities in extracts of treated cells and the inhibition of the same enzymes when assays containing untreated cell extract were incubated with platinum compounds, it was suggested that a DNA repair system was being activated by reactions of the DNA with platinum in treated AV₃ cells.

In summary, the molecular effects of tumor-inhibiting platinum compounds were shown to mimic closely those of bifunctional alkylating agents, especially HN2, in terms of preferential inhibition of DNA synthesis, giant cell formation, inhibition of *de novo* purine syntheses, and enhanced DNase and DNA polymerase activities in extracts of treated cells. Also paralleling the effects of bifunctional alkylating agents is the ability of platinum compounds to induce lysogenized prophage



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(Reslova, 1970), and lower the melting temperature of DNA (Horacek and Drobnik, 1970).

In contrast to bifunctional alkylating agents, tumor-inhibiting platinum compounds inactivate both single and double-stranded DNA containing bacteriophage with equal and high efficiency (Drobnik et al., 1970); hence platinum compounds probably do not act by reating interstrand cross-links. On the basis of the size of the platinum molecule, a hypothesis was proposed that the mechanism of action of the tumorinhibiting platinum emopounds involves the creation of platinum coordinated intrastrand cross-links between 2 adjacent purines. Coordination could occur at either 1 or 2 sites of each adjacent purine. Depending upon the strength of the ligand bonds, it is predicted that the effects of such platinum coordinated purine dimers would mimic the effects of UV induced pyrimidine dimers. On the basis of this hypothesis, it would be predicted that Trans-platinum compounds could not give rise to such dimers for steric reasons and that bulky groups such as in bridged platinum compounds would make the reaction more difficult to occur; however. Cis-Pt(IV) should be about as effective as Cis-Pt(II). Furthermore there would be no interstrand cross-link type mutagenic effects such as dominant lethal mutations. There would probably also be a repair mechanism for such platinum induced DNA lesions. All of the presently known molecular effects of tumor-inhibiting platinum compounds are consistent with the above predictions of this hypothesis.

Horacek, P., and J. Drobnik (1970). Personal communication. Reslova, S. (1970). Induction of lysogenic strains of *Escherichia* coli by Cis-platinum(II) diamminodichloride. (submitted for publication)

Drobnik, J., A. Krekulova, and A. Kubelkov (1970). Inactivation of bacteriophages with Cis-dichlorodiamine-Platinum II. (manuscript in preparation)



MOLECULAR EFFECTS OF TUMOR-INHIBITING PLATINUM COMPOUNDS IN MAMMALIAN CELLS *IN VITRO*: INVESTIGATIONS INTO THEIR POSSIBLE MECHANISMS OF ACTION

By

Harold Cecil Harder

A THESIS

Submitted to Michigan State University in partial fulfillment of the requirements for the degree of

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Dedicated to my wife, son,

and to all those who have long hoped for better anti-cancer drugs



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I wish to express my sincere gratitude to Linda, who patiently endured 5 years as a graduate student's wife and who, along with my son David, was always a source of encouragment in times of discouragement.

I would also like to acknowledge indebtedness to Dr. John Boezi, whose stimulating lectures on biochemistry and molecular genetics were responsible for my own interest in this field; to James Johnson for the gift of DNA dependent RNA polymerase; and to Dr. James E. Trosko, without whose generosity in allowing me the use of his equipment and without whose valuable assistance this research could not have been performed. Finally, I wish to express my sincere thanks to my thesis director, Dr. Barnett Rosenberg, for granting me a very wide decree of freedom in pursuing this research project.

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I. INTRODUCTION

A. History of Biological Effects of Platinum Compounds

From its inception the study of the biological effects of platinum compounds has been, to paraphrase Rosenberg, "a story in serendipity." It started about 6 years ago when Rosenberg designed an experiment to study the effects of alternating electric fields on bacterial cell division. The very first time the current was turned on at 1000 cps in the presence of a continuous culture of E. coli B, cell division was inhibited. Microscopic examination revealed that the bacteria continued to grow, forming very long filaments. Instead of being caused directly by the electric field, however, these effects were found to be attributable to various platinum species generated by the electrolysis of the presumably-inert platinum electrodes (Rosenberg et al., 1965). At the conclusion of this initial publication a number of questions were posed: "Can these metal ions inhibit cell division in other bacteria and cells? Where is the locus of action in the bacterial cell? How does the effect of these metal ions relate to the actions of other causative agents of filamentous growth--is there a weak link that all operate on? Finally, what is the mechanism of action of these metal ions?"

In later studies the neutral platinum compound $\text{Cis-Pt(IV)(NH}_3)_2\text{Cl}_4$ was identified as being a most effective inhibitor of cell division and inducer of filaments (Rosenberg *et al.*, 1967a). This was in sharp contrast with the much less efficient trans isomer and the almost complete lack of cell division inhibition by the single and double negative



species, $[Pt(IV)NH_3CI_5]^-$ and $[Pt(IV)CI_6]^=$, although the latter was found to be bactericidal at very low concentrations. Of the many other group VIII-B transition metal complexes tested, only high concentrations of some rhodium and ruthenium compounds were able to induce elongation in *E. coli* B significantly, but not to the extent nor to the uniformity of the analogous platinum complexes (Rosenberg *et al.*, 1967b). The questions as to whether cell division and elongation are produced in other bacteria by platinum compounds was found to be affirmative with all tested gram negative bacilli; however, grampositive bacilli showed only slight elongation at near-toxic levels (Rosenberg *et al.*, 1967b).

By following the distribution of ¹⁹¹Pt labeled platinum compounds among various bacterial cellular components, Renshaw and Thomson (1967) attempted to establish the locus of action of the active platinum compounds. Their results, summarized in Table 1, showed that the Cis-Pt(IV)(NH3)2Cl4 in elongated E. coli B was associated with metabolic intermediates, nucleic acids and cytoplasmic proteins. On the other hand, in (Pt(IV)Cl₂)⁼ growth inhibited E. coli B, the platinum was bound only to cytoplasmic proteins. Since inhibition of cell division did not occur when nearly all the platinum was bound to cytoplasmic protein, it may be concluded that binding to cellular proteins per se is not responsible for inhibition of cell division. Conversely, growth inhibition was directly correlated with the binding of the platinum cytoplasmic proteins. In similar experiments with 191Pt(IV)(NH3)2C14 treated Bacillus cereus and Staphylococcus aureus, in which neither growth nor cell division were inhibited, the metal complex was bound predominantly to metabolic intermediates. Significant binding still



	Percentage of Radioactivity ^b											
Treatment	Cis-191	191Pt(IV)C1										
Bacterium	B. cereus	S. aureus	E. coli B	E. coli B								
Inhibitory effect on	none	none	division	growth								
Fraction												
Metabolic intermedi- ates	74	60	19	1								
Lipids	2	1	6	3								
Nucleic acids	19	20	30	1								
Cytoplasmic proteins	5	19	45	96								

Table 1. Average distribution of Cis- $^{191}\rm{Pt}(IV)(\rm{NH}_3)_2\rm{Cl}_4$ and $[191\rm{Pt}(IV)\rm{Cl}_6]^{-2}$ among various bacterial cellular components

 a Made by UV irradiation of $[^{191}\text{Pt}(IV)\text{Cl}_6]^=$, of which photoproducts, Cis-191Pt(IV)(NH3)_2Cl4 was the most predominant, Trans-191Pt(IV)(NH3)_2Cl4 being only a minor component.

^bData from Renshaw and Thomson (1967).


occurred with the nucleic acids, but to only two thirds of the extent in elongated E. ∞ii . The lack of a high degree of platinum binding to cytoplasmic proteins and the lack of growth inhibition in E. *cereus* and S. *cureus* is consistent with the conclusion drawn above from results with E. ∞ii . In fact, the high degree of binding to metabolic intermediates is indicative that this is a possible detoxification mechanism, because the concentration of the platinum compound is far too small to tie up a high enough fraction of most metabolites to be responsible for either of these inhibitory effects. Therefore, the results of Renshaw and Thomson provide some evidence that the binding of platinum to nucleic acids plays a role in the induction of elongation.

In ultraviolet light induced *E. coli* filaments, cytokinesis (cross septation) can be initiated by pantoyl lactone treatment or by incubation at 42° C. (Adler and Hardigree, 1965b). Similarly in UV, penicillin and D-amino acid induced *Erwinia* filaments, cytokinesis can be induced by pantoyl lactone or divalent cation treatment (Grula and Grula, 1962). However, Rosenberg *et al.* (1967b) found that cytokinesis or cell division in platinum induced *E. coli* B filaments could not be induced by any of these treatments. Reversal of the inhibition of cell division could be achieved only by removal or decrease in the platinum concentration. Therefore, in relating the effects of platinum treatment with other agents that cause filamentous growth, they concluded from these observations that platinum-induced inhibition of cell division in *E. coli* B differs from the inhibition by other agents mentioned above.

At this point, research had been published on all but the last question stated in the first paragraph. Then it was discovered that



3 bacterial elongating compounds, Cis-Pt(II)(NH₃)₂Cl₂ (abbreviated hereafter by Cis-Pt(II), Cis-Pt(IV)(NH₃)₂Cl₄ (abbreviated Cis-Pt(IV)), and Pt(II)(NH₂)₂(CH₂)₂Cl₂ (abbreviated Pt(II)en) (see Figure 1) were potent inhibitors of Sarcoma 180 tumors in mice (Rosenberg *et al.*, 1969). More recently it has been shown that large, well-developed, solid S-180 tumors in mice (Rosenberg and VanCamp, 1970), Dunning Ascites Leukemia and intramuscular Walker 256 carcinosarcoma (Kociba *et al.*, 1970) in rats can be successfully regressed by Cis-Pt(II). Rapidly accumulating experimental results now indicate that the platinum compounds are a new class of broad spectrum antineoplastic drugs (Rosenberg, 1970; Haddow, 1970; Drobnik, 1970). The anti-tumor activity and the filamentousinducing activity of these platinum compounds now urgently require an answer to the last question of the opening paragraph: "What is the mechanism of action?" This is the question to which the research in this thesis is addressed.

B. Approach to the Problem: Clues to the Possible Mechanism of Action

Since the effective platinum compounds are not structurally related to other anti-neoplastic agents, this presented a problem as to where to begin the investigation. Therefore, clues to possible mechanisms of action of the tumor inhibiting platinum compounds were taken from the mechanisms of action of other anti-tumor compounds which also induce bacterial filamentous formation. These include ultraviolet light (and ionizing radiation), nitrogen mustard, mitomycin C, hydroxyurea, and azaserine (see Table 2).

Ultraviolet light in low doses affects cells primarily by the induction of pyrimidine (primarily thymine-thymine) dimers (Setlow *et al.*, 1963). In this case the net effect is to stop DNA synthesis until the DNA is repaired. The nitrogen mustard, methylbis(2-chloro-





NH3

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Tetraamminodichloride

Platinum (肛)

Diamminotetrachloride

Trans-Platinum (12)

ω

°HN

ັວ

NH3

NH3

ā

2CI-

ā



A bibliography of tumor inhibiting agents that inhibit cell division and induce filament formation in bacteria Table 2.

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	Ant	i-tumor Activity	Fils	ament Induction	Inhihi	tion of DNA Sunthococ
	Effect	References	Effect	References	Effect	References
(1) Ultraviolet light and ionizing radi-	+	Knock, 1967; Cole, 1970	+	Adler and Hardigree, 1964, 1965a, 1965b	+	Setlow <i>et al.</i> , 1963; Creighton and Birnie, 1970
(2) Nitrogen mustard (HN2)	+	Connors, 1969; Ochoa and Hírschberg, 1967; Bosen and Davís, 1969	+	Maxwell and Nickel, 1954; Harold and Ziporin, 1958	+	Ochoa and Hirschberg, 1967; Wheeler, 1962; Harold and Ziporin, 1958
(3) Mitomycin C	+	Sugiura, 1961; Evans, 1961	+	Reich <i>et al.</i> , 1961; Grula and Grula, 1962	+	Shiba $et \alpha l.$, 1959; Magee and Miller, 1962
(4) Hydroxyurea	+	Stearns et al., 1963;	+	Rosenkranz, 1966	+	Young $et \alpha l.$, 1967; Young and Hodas, 1964
(5) Azaserine	+	Stock, 1966	+	Maxwell and Nickel, 1954	+	Skipper et al., 1954; Hartman et al., 1955
(6) Platinum compounds	+	Rosenberg <i>et al.</i> , 1969; Rosenberg and Van Camp, 1970	+	Rosenberg <i>et al.</i> , 1964; 1967a, 1967b	5	

"+" means that the effect is observed.



ethyl)amine (NH2) and all other bifunctional alkylating agents, react with DNA, RNA and protein as well as with many metabolites of the cell. Due to the lack of binding specificity, the only alkylation reaction which could reasonably lead to cell death is that with DNA itself. In that bifunctional alkylating agents are 50 to 100 times more effective than the corresponding monofunctional agents, it has been postulated that the polyfunctional alkylating agents are interstrand cross-linking agents; that is, they react at one point on each strand (Ross, 1962). The net effect of cross-linking is to prevent the separation of the 2 strands which is necessary in semiconservative DNA replication. Nevertheless, regardless of the specific site of interaction, all bifunctional alkylating agents result in the inhibition of DNA synthesis (Ochoa and Hirschberg, 1967; Wheeler, 1962).

The antibiotic mitomycins are another class of tumor inhibitors that also induce elongation in *E. coli*. Mitomycin C must be classified as a bifunctional alkylating agent for it efficiently creates DNA cross-links (Szybalski and Iyer, 1964) and causes radiomimetic type damage in cells. Thus, as with the nitrogen mustards, Mitomycin C inhibits DNA synthesis (Reich *et al.*, 1961; Grula and Grula, 1962).

Azaserine is another tumor inhibiting, bacterial elongating antibiotic; however, in contrast to Mitomycin C, it is classed as an antimetabolite. By non-competitive binding to enzymatic sites normally occupied by glutamine, azaserine primarily inhibits the amination of N-formylglycine-amide ribonucleotide by the enzyme, L-glutamine-amido



ligase (ADP) in the de novo synthesis of purines (Hartman et al., 1955; Skipper et al., 1954). Inhibition of purine biosynthesis by azaserine eventually leads to inhibition of DNA synthesis (Hartman et al., 1955; Krakoff and Karnofsky, 1958).

Finally, the bacterial elongating, tumor inhibiting antimetbolite known as hydroxyurea has been shown to inhibit the production of deoxyribonucleotides by inhibiting the enzyme ribonucleoside diphosphate reductase (Krakoff *et al.*, 1968). This chemotherapeutic agent, therefore, also results in the inhibition of DNA synthesis (Young and Hodas, 1964; Young *et al.*, 1967; Krakoff *et al.*, 1968).

All of the above 5 bacterial elongating, antineoplastic agents have one ultimate effect in common, the inhibition of DNA synthesis, although by different mechanisms. Therefore, it is logical to hypothesize that the antitumor platinum compounds also inhibit DNA synthesis regardless of the specific mechanism of action (see Table 2 for a summary). Accordingly, this hypothesis was employed as the starting point for this dissertation research.

C. Scope of the Thesis

The overall purpose of this research is to elucidate the mechanism(s) of action of tumor-inhibiting platinum compounds.

The first major objective was to determine the primary target of the platinum compounds in terms of general cellular function. This was accomplished by measuring the effects on overall DNA, RNA and protein syntheses. Hence this part of the research will prove whether or not the hypothesis of inhibition of DNA synthesis is correct. Because initial results showed that DNA synthesis is indeed inhibited (Harder and Rosenberg, 1969), the second major question to which this research



is addressed is, "How do the platinum compounds inhibit DNA synthesis?" That is, is the tumor inhibiting activity of platinum compounds caused by a platinum induced inhibition of the production of the deoxyribonucleotide triphosphates as antimetabolites, or is it caused by a direct interaction of the platinum compound with the cellular DNA (as is the case for alkylating agents and certain antibiotics)?

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II. EXPERIMENTAL PROCEDURE

A. Materials

1. <u>Platinum Compounds</u>. All platinum compounds were prepared in our laboratory by Mr. Thomas Krigas as described elsewhere (Lededinskii and Korabel'nik, 1947;Kauffman, 1963), except for $Pt(II)(NH_2)_2(CH_2)_2Cl_2$, which was purchased from Alpha Inorganics (Beverly, Mass.) and recrystallized before use. Crystalline platinum compounds were purified by repeated recrystallization and sent to Galbraith Laboratories, Inc. (Knoxville, Tenn.) for analytic tests. The sum of the deviations from the predicted values was less than 0.5%. Stored in the dark in the crystalline state, platinum compounds were freshly dissolved into media immediately before use to avoid unnecessary photochemical and hydrolytic decomposition of these compounds.

 <u>Other Drugs</u>. Hydroxyurea was purchased from Nutritional Biochemicals Corporation.

3. <u>Growth Media</u>. Human Amnion, type AV_3 , tissue culture cells were obtained from American Type Culture Collection (Rockville, Md.) and grown in glass bottles at 37° C. in a humidified atmosphere supplemented with 5% CO₂. The cells were cultured in Eagle's minimal essential medium (MEM) with Earl's salts and supplemented with 10% calf serum, all purchased from either Grand Island Biological Company, Grand Island, N.Y., or Flow Laboratories, Rockville, Md. The growth



medium also contained 100 units/ml penicillin G plus 100 μ g/ml streptomycin sulfate obtained from Eli Lilly, Indianapolis, Ind. Trypsin (0.25%) from Difco Laboratories, Detroit, Mich., in Hank's Balanced Salt Solution (HBSS) was used to transfer the cells.

 <u>Radioisotopes</u>. All radioisotopes were purchased from Swartz Bioresearch, Inc., Orangeburg, N.J.

B. Methods

1. Measurements of DNA, RNA and Protein Syntheses. Human Amnion AV2 cells from several growth bottles were harvested by trypsin treatment, pooled, and then inoculated in equal numbers into 5 cm diameter Falcon disposable tissue culture petri dishes. The plates, containing 2-3x10⁶ cells as determined by a standard hemocytometer count at the beginning of the experiment, were incubated overnight at 37° C. to allow the cells to attach. DNA synthesis was specifically measured by the incorporation of [methyl-3H]-thymidine (0.5 to 1.0 u C/ml, specific activity 1.9 C/mmole) and by [6-3H]-deoxyuridine (2 uC/ml, specific activity 24 C/mmole). RNA was specifically measured by the uptake of [5-3H]-uridine (2 µC/m1, specific activity 20 C/mmole). DNA and RNA syntheses together were followed by the incorporation of [5-3H]-cytidine (2 µC/ml, 25.9 C/mmole): [5-³H]-deoxycytidine (2 µC/ml, 27 C/mmole): [8-3H]-deoxyadenosine (uC/m1, 20.3 C/mmole); and [¹⁴C]-sodium formate (1.0 µC/m1, 52.4 mC/mmole). Protein synthesis was measured by the incorporation of L-[4.5-3H]-leucine (2 µC/ml, 2.5 mC/mmole). All of the above concentrations and specific activities represent the concentration and specific activity in MEM at the time it is applied to the cells in MEM.



In earlier experiments, both the labeled precursor and the platinum compound freshly dissolved in MEM and filter sterilized were applied to the plates at the beginning of the experiment (3 ml/plate). To assess more accurately the effects of treatment over a 24-hour interval, later experiments were performed by applying media containing only platinum (2.9 ml/plate) at time zero. Then 2 hours prior to analysis after a specific exposure period, the appropriate radioactive precursor (0.1 ml) was added. By this method, all plates were tested for incorporation activity over an identical pulse period and isotopic dilution was minimized. In all experiments incorporation was determined at various times up to 24 hours of treatment by setting the plates on ice to stop the uptake of the labeled precursor, sonicating the monolayered cells for 10 sec. in 3 ml Puck's saline D with a Branson 140C sonifier, followed by applying 0.10 ml of well agitated sonicate onto each of 2 Whatman 3 MM, 2.3 cm diameter filter discs. After being washed 3 X in iced 5% trichloroacetic acid (TCA), 2 X in absolute ethanol and once in acetone, the discs were dried and counted in 5 ml 2,5-bis-2-(5-tert-Butylbenzoazolyl)-Thiophene (BBOT) in toluene (4 g/1) with Packard Tri Carb Liquid Scintillation Spectrometer. The effective background in all experiments was determined by applying the radioactive precursors to prechilled plates which were then analyzed as described above. The counting efficiency was 35 to 40% for ³H and 75 to 80% for ¹⁴C determined by the channel's ratio method. Each data point consists of the average of the counts from duplicate plates and duplicate samples from each plate less the background. This is a modification of procedures described elsewhere (Bollum, 1966; Regan and Chu. 1966).



In experiments in which RNA and DNA were simultaneously labeled, the RNA was separated from the DNA by the following procedure: The radioactive media from 2 sets of plates was decanted and the plates were placed on ice. After 3 iced saline rinses, the plates were incubated 1 hour with 3 ml of 0.2 N $HCIO_4$ on ice to fix the cells. Following 2 additional rinses with HBSS to neutralize the acid, 2 ml of 40 μ g/ml Worthington bovine pancreas RNase A (heat activated by incubation at 80 C. for 15 minutes) in HBSS was added to one set of plates. To another set of plates, 2 ml of HBSS was added. After both sets were incubated 1 hour at 37 C, all plates were set on ice and an additional 1 ml of saline was added to each plate to give a total of 3 ml/plate. Finally the plates were sonicated and analyzed as described above.

2. <u>Measurement of the Reversibility of Platinum Treatment</u>. The reversibility of the effects of treatment with platinum compounds was measured by the recovery of DNA synthesis from inhibition. The procedure used was to preincubate all plates 4 hours in MEM containing a given platinum compound followed by washing 2 X in Puck's saline D and adding 2.5 ml fresh MEM. Two hours prior to the termination of incubation 0.5 ml of ³H-thymidine (³H-TdR) in MEM was added to yield a total of 0.5 μ C/ml. The zero hour points, i.e., 4 hours after application of the test media, were obtained by adding the ³H-TdR to the plates 2 hours after application of the test media, and stopping the incorporation at time zero. The remaining procedure was as described above.

3. <u>Measurement of Membrane Effects</u>. To determine the amount of 3 H-TdR penetrating treated cells, AV₃ cells on 10 cm Falcon plates were pretreated at 37° C. with 9.5 ml MEM containing Cis-Pt(II)(NH₃)₂Cl₂ for



6 or 22 hours and then 0.50 ml of 10 $\mu\,\text{C/ml}^{-3}\text{H-TdR}$ was added. After a 2-hour labeling period at 37° C, the culture dishes were chilled on ice, rinsed 3 X with ice-cold 0.10 mM thymidine in Hank's balanced saline solution (HBSS) and 2 X in iced HBSS. After the dishes were drained thoroughly, 5 ml of ice-cold 0.2 N HC10, was placed onto each. After 15 minutes the HClO4 solution was collected and the dishes were rinsed with an additional 2 ml 0.2 N HClO4. The combined HClO4 solutions from each dish were centrifuged 5 minutes at about 1500 X g in a clinical centrifuge. The 0.D.260 of the supernatant was measured and the radioactivity in two 0.5 ml fractions/plate was determined by liquid scintillation spectrometry using 10 ml of the dioxane fluor described above. The acid fixed cells were dissolved in 5 ml of 0.5 N KOH by heating 30 minutes at 50° C. Using Worthington calf thymus DNA as a standard, the concentration of DNA was measured by the method of Ceriotti (1952). The acid insoluble radioactivity was determined by neutralizing 0.5 ml of the alkaline extract with .25 ml 1N HCl, placing 0.10 on each of 2 2.3 cm filter papers and following the same procedure as described above.

C. Enzyme Assays

1. <u>Preparation of Enzyme Extracts</u>. For all deoxyribonuclease (DNase) measurements, enzyme extracts of AV_3 cells served as the source of enzymes to be studied. After incubation with a given concentration of a platinum compound for up to 24 hours, about 10⁷ AV_3 cells were collected by EDTA treatment, centrifuged, washed 2 X with saline D and resuspended in 0.5 ml saline D. The extracts were prepared by the quick freeze-thaw method, using an acetone dry ice slurry repeated 4 times. Finally the lysate was centrifuged at 20,000 X g for 15 minutes and



the supernatant transferred to another test tube. This extract was stored at -20° C. and diluted before use with saline D.

A different procedure was found to be necessary for preparation of the enzyme extract for DNA polymerase measurements. After treatment, approximately 5 X 107 AV3 cells were collected by scraping the plates with a rubber policeman in saline D. The cells were spun down and the supernatant decanted. Following a very quick rinse in 2 ml iced 10 mM Tris-HCl, pH 8.1 containing 1 mM MgCl2, which caused the cells to swell to about 2-3 X their initial size, the cell pellet was resuspended and incubated in the same iced buffer. The hypotonic and osmotic shock treatment resulted in a very efficient breakage of the cell walls. The cell nuclei remain intact in this buffered solution for up to about 2 hours and are free of cytoplasmic debris. Hence this is an excellent way to isolate nuclei. However, for this purpose, the nuclei and cell debris were spun down before they lysed after about 1 hour of incubation at 0° C. Although the integrity of the nuclear membrane is temporarily maintained, the DNA polymerase apparently passes through it into the supernatant because extracts of these nuclei had no DNA polymerase activity. The supernatant was centrifuged a second time at 30,000 X g for 1 hour at 4° C. and stored in the refrigerator.

 Protein Assay. The protein content in all extracts was measured by the method of Lowry et al. (1951).

3. <u>Acid Soluble DNase (Exonuclease) Assay</u>. The acid soluble DNase assay used here was adapted from the *E. coli* exonuclease II assay of Lehman and Richardson (1964). The assay measures the conversion of DNA with a ³H-TdR label to trichloracetic acid (TCA) soluble fragments.



The incubation mixture (0.25 ml) contained 20 umole of sodium acetate buffer, pH 4.80, 1 u mole MgCl_, 10 ug ³H-DNA (prepared by phenol extraction from E. coli 15 T cultured in $4 \mu g/m1$ ³H-TdR) 2 to 20 μg enzyme extract and water to yield a total volume of 0.25 ml. The reaction was started by adding the enzyme extract to the rest of the assay mixture (chilled) and mixing by gently swirling the reaction test tube. After removing a zero time aliquot, the reaction was quickly started by swirling the assay tube in a 37° water bath. Thereafter at regular time intervals 25 µl aliquots were removed and placed in iced test tubes containing 0.2 ml 100 ug/ml heat denatured salmon sperm DNA, followed by the addition of 0.5 ml iced 5% TCA and vigorous shaking. After at least 5 minutes additional incubation in ice, each assay aliquot was centrifuged 5 minutes at 20,000 X g and the supernatant was transferred to another test tube. Following 4 ethyl ether extractions, 0.60 ml of the aqueous fraction was counted in a polyethylene scintillation vial with dioxane fluor containing 3 g BBOT plus 150 G naphthalene per liter.

4. Endonuclease Assay. The nitrocellulose disc method of measuring single stranded DNA as described by Geiduschek and Daniels (1965) was used for determining endonuclease activity. Although this method is most sensitive to endonucleases, it might also be sensitive to induced excision repair enzymes, if they are present in sufficient amounts or nonspecific. The incubation mixture contained 20 μ moles of sodium acetate buffer at pH 4.8, 1 μ mole MgCl₂, 10 μ g native *E. coli* ³H-DNA (see previous paragraph), 3 to 10 μ g enzyme extract, and water to make a total volume of 0.25 ml. The reaction was initiated in the same way as described above. At various times thereafter, 25 μ l aliguots



were removed from the reaction tube and placed into another test tube containing 1 ml of iced solution of 4 mM NaH₂PO₄, 7 mM Na₂HPO₄, 1 mM disodium ethylenediaminetetraacetate, pH 7.06, to stop the reaction. Next the remaining DNA was denatured by heating 6 minutes in boiling water followed by quick cooling in an ice bath. After further diluting the mixture with 15 ml of a 0.5 M KCl, 10 mM tris-HCl, pH 7.5, solution, and vigorous mixing on a vortex stirrer, the remaining DNA was collected on a nitrocellulose filter (24 mm, Scheicher and Schuell, Keene, N.H., membrane filter, type B-6) that had been presoaked in the same tris-KCl solution at least 15 minutes. Then the assay tube and filter (in a millipore apparatus) were washed with a total of 75 ml of the same solvent and finally by 5 ml of 100% ethanol. After drying, the filters were counted in 5 ml of BBOT-Toluene.

5. <u>DNA Polymerase Assay</u>. The DNA polymerase assay was essentially the same as that described for measuring the DNA polymerase from *Micrococcus lysodeikticus* as described by Zimmerman (1966). The assay mixture contained 25 µmoles Tris-HCl buffer, pH 7.6; 0.5 µmoles MgCl₂; 0.4 µmoles 2-mercaptoethanol; 50 µg heat-denatured calf thymus DNA (Worthington) as primer; 10 mµmoles each of deoxyribonucleotide triphosphate (dCTP, dGTP, dTTP, dATP) including either ¹⁴C-dATP (10 mC/ mmole) or ³H-dATP (25 mC/mmole), 30 to 300 µg of enzyme extract, and water, with or without platinum compounds, to yield a total volume of 0.25 ml. The reaction was initiated by adding the enzyme extract (see above extraction procedure) to prewarmed assay tubes containing the assay mixture. Following 1 to 2 hours incubation at 37° C, the label incorporated into DNA was measured by adding 0.5 ml iced 7% HClO₄ to stop the reaction. After incubation 10 minutes in ice, the acid precipitated samples were



diluted with 2 ml of cold 0.0 lM $Na_4P_2O_7$ followed by collection of the DNA on moistened 2.4 cm glass filter paper (Whatman GF/C). After the reaction tube and filter were washed with 10 ml of cold 0.7% HClO₄ in 3 portions, the disc was rinsed with 1 ml of 100% ethanol, dried, and counted in a glass vial with 5 ml of BBOT-Toluene.

6. DNA Dependent RNA Polymerase from Pseudomonas putida. Several attempts were made to establish the conditions necessary to measure DNA dependent RNA polymerase activity in extracts of AV3 cells, without success. Since the purified enzyme from *Pseudomonas putida* was readily available (due to a generous gift from J. Johnson of the Department of Microbiology and Public Health at Michigan State University), this purified bacterial DNA dependent RNA polymerase was used instead. The assay mixture contained 5 µ moles Tris acetate buffer, pH 8.0; 0.5 µ mole Mg(CH₃COO)₂; 0.125 µmole Mn(CH₃COO)₂; 0.10 µmole each of ATP, GTP, UTP, and ³H-CTP (2500 cpm/mµmole); 25 µg heat denatured calf thymus DNA; 4 to 17 µg peak II RNA polymerase (purified about 1000 X, with about 1000 units activity/ml, 1 unit being defined as the incorporation of 1 mumole CTP/mg/hour), and glass distilled water with or without freshly dissolved platinum compounds to yield a total assay volume of 0.165 ml (Johnson et al., 1971). The reaction was initiated by adding the enzyme to prewarmed test tubes with the rest of the assay mixture. After incubation for 30 minutes at 30° C, the incorporation was terminated by adding 3 ml of iced 10% TCA, placing the reaction mixture in ice 10 minutes and adding a few drops of carrier DNA (denatured salmon sperm). Each sample was collected on a nitrocellulose filter, presoaked in water and prewashed with 10 ml iced 10% TCA immediately before use. Then



the filter was rinsed with 15 ml of iced 10% TCA in 3 portions, dried and counted in a glass vial with 5 ml BBOT-Toluene.



III. RESULTS

A. Effects of Cis-Pt(II)(NH₃)₂Cl₂ on AV₃ Cell Growth, Division, and Survival

The effect of Cis-Pt(II)(NH₃)₂Cl₂ on cell division and survival was measured simply by counting the number of cells remaining attached on plates as a function of time after treatment. Figure 2 shows the effects of a 4 and 24 hour treatment of AV₃ cells with 0, 1, 5, and $25 \,\mu\text{M}$ Cis-Pt(II)(NH₃)₂Cl₂. Initially all plates had the same number of cells. After the 4 or 24 hour treatment period, the platinum containing media was decanted, the plates were rinsed twice with Puck's saline D and 3 ml fresh MEM added to the plates. Each point represents the average of the number of cells remaining on duplicate plates determined by duplicate hemocytometer counts (2X2 slides) of each plate. A concentration dependent, duration of treatment dependent, inhibition of cell division was shown to occur in Figure 2. Furthermore, the inhibition of cell division persists up to at least 4 days after treatment at 1 or 5 μ M without a marked apparent loss of cell survival.

In contrast to this inhibitory effect on cell division, it was noted that 3 to 4 days after AV_3 cells were initially treated, a majority of the surviving population had grown into giant cells many times the diameter of untreated cells. Figure 3 shows the treatment duration dependence of giant cell formation. Only 2 days after AV_3 cells were treated four hours with 5 μ M Cis-Pt(II), the cells appeared larger (Figure 3b) than untreated cells (Figure 3a). By 86 hours after




Figure 2. Effect of Gis-Pt(II)(NH_3)_2Cl_ on cell survival and cell division in ${\rm AV}_3$ cells.





Figure 3

Giant cell formation in AV3 cells treated with 5 µM Cis-Pt(II)(NH3)2Cl2.

- 3a: Untreated AV, cells at t = 98 hrs.
- 3b: AV₃ cells treated 4 hrs at t = 48 hrs; cells slightly larger than untreated cells.
- 3c: AV₃ cells treated 24 hrs at t = 98 hrs; giant cells up to 200 µM in size.
- 3d: AV₃ cells treated 4 hrs at t = 84 hrs; cells larger than in 3b and micronucleated.
- 3e: AV₃ cells treated 24 hrs at t = 98 hrs; many normal size nuclei in one cell.
- 3f: AV₃ cells treated 4 hrs at t = 98 hrs; possible crossseptation of giant cells.

Time t is the time after the Cis-Pt(II)(NH₂)₂Cl₂ containing medium was initially applied to the cells. All plates initially had the same cell density. After 4 or 24 hours of treatment with $5 \perp M$ Cis-Pt(II)(NH₂)₂Cl₂, the platinum-containing medium was decanted and the plates were rinsed 2 X with Puck's saline D. Then fresh HEM was applied and the plates were incubated at 37° C. Pictures were taken with an inverted phase Nikon microscope, and the pictures were taken of live cells in MEM through a dark green filter. The total magnification is 300 X in all pictures. Pt(II)(NH3)2C12.

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treatment initiation, the cells appeared even larger (Figure 3d). Cells that were incubated 24 hours of 5 µM Cis-PT(II) grew up to 200 μ M in diameter (Figure 3c) on the plates in contrast to an average $10-25 \ \mu$ M in diameter for untreated cells (Figure 3e). Some giant cells appeared perfectly normal (Figure 3c); some contained from 2 to 6 normal sized nuclei (Figure 3e); while others had several micronuclei (Figure 3d). The ability of these giant cells to divide to normal sized cells was not quantitatively determined, although preliminary studies indicated that 7 to 10 days after treatment a large fraction of the surviving population had returned to normal size. Figure 3f shows what appears to be cytokinesis occurring in giant cells containing several normal sized nuclei. Note also that the cells in Figure 3f, at 98 hours postinitiation of treatment, are smaller than the cells in Figure 3d at 86 hours after a 4-hour treatment. Hence Cis-Pt(II) (NH₃)Cl₂ inhibits AV₃ cell division over a long period of time while not apparently affecting total cell growth.

B. Effects of Cis-Pt(II)(NH₃)₂Cl₂ on DNA, RNA and Protein Syntheses.

Figure 4 shows the concentration dependent effect of Cis-Pt(II) $(NH_3)_2Cl_2$ on DNA synthesis as measured by the incorporation of ³H-TdR in human amnion AV₃ cells over a 24-hour period. DNA synthesis was inhibited to a small degree at concentrations as low as 0.50 µM. At 25 µM, which is approximately equivalent to a single injection of 8 mg/ ⁻ kg, the best therapeutic dose in Swiss white mice for treatment of S-180, assuming a uniform distribution of the compound over the entire body, there is almost complete inhibition after 6 to 10 hours of treatment. The decrease in total ³H-TdR incorporated between 10 and 24 hours at 125 µM is probably the result of the degradation of previously





Figure 4. Effect of Cis-Pt(II)(NH_3)_Cl_2 on DNA synthesis in NV_3 cells as measured by the uptake of $^{3}\mathrm{H-thymdine}$. Both the platinum compound and $^{3}\mathrm{H-thymdine}$ were applied to cells at time zero.



synthesized, labeled DNA caused by release of degradative enzymes in dead or dying cells.

Figures 5 and 6 show the effect of Cis-Pt(II) on RNA and protein synthesis as measured by the uptake of ³H-uridine (³H-UR) and ³H-leucine respectively. In contrast to the inhibition of the incorporation of ³H-TdR, there was very little inhibition of ³H-UR and ³H-leucine incorporation at 5 μ M.

In order to facilitate the comparison of these effects on DNA. RNA and protein syntheses, the data from charts 4, 5, and 6 were replotted as the incorporation activity relative to the control, i.e., [cpmt2-cpmt1]exp./[cpmt2-cpmt1]control. From this transformation Figures 7 and 8 are obtained showing the relative effects of Cis-Pt(II) on the uptake of isotopically labeled precursors into DNA, RNA and protein at 25 and 5.0 µM. At 25 µM. Figure 7 shows that all processes were completely inhibited after 24 hours: however, DNA synthesis was inhibited much more quickly. For example, after 6 hours the relative incorporation rates are 0.17, 0.49 and 0.67 of the control for the uptake of 3H-TdR, 3H-UR, and 3H-leucine respectively. Figure 8 shows that the incorporation of ³H-TdR at 5 µM is almost selectively inhibited, there being little effect on RNA and protein synthesis. The 24-hour point in Figure 8 for DNA synthesis is higher than the 10-hour point due to isotopic dilution in the control plates after the first 10 hours of incubation. In later experiments with 5 uM Cis-Pt(II), in which plates were pulse labeled for just 2 hours prior to sonication, no such increase in the relative incorporation occurred between the 10th and the 24th hour of treatment.





Figure 5. Effect of Cis-Pt(II)(NH3)2Cl2 on RNA synthesis in AV3 cells as measured by the uptake of 3H-uridine. Both the platinum compound and 3H-uridine were added at time zero.





<code>Figure 6. Effect of Cis-Pt(II)(NH3)2Cl2</code> on protein synthesis in AV3 cells as measured by uptake of ³H-leucine. Both the platinum compound and ³H-leuce were applied at time zero.</code>





Figure 7. Relative effect of 25 μ M Cis-Pt(II)(NH3)_2Cl₂ on DNA, RNA and protein synthesis. The data from figures2, 3, and 4 at 5 μ M was transformed using the expression [(cPmt_2^-cPmt_1)_exp/(cPmt_2^-cPmt_1)_control] where cpmt_2 and cpmt_1 represent the counting rates for the incorporation of the lafeled precursors at times t_2 and t_1.





Incubation Time in 5.0 \muM Cis-Pt(II)(NH₂)₂Cl₂ Media Figure 8. Relative effect of 5 \muM Cis-Pt(II)(NH₂)₂Cl₂ on DNA, RNA and protein synthesis. The data from figures 2, 3, and 4 at 25 \muM was transformed using the expression [(^{Cpm}t_2^{-Cpm}t_1)_{exp}/(^{cpm}t_2^{-Cpm}t_1)_{control}] where cpmt_2 and cpmt_1 represent the counting rates for the incorporation of the labeled precursors at times t₂ and t₁.



C. Comparison of the Effects of Three Effective Tumor-Inhibiting, Bacterial Filament-Forming Platinum Compounds with Two Ineffective Platinum Compounds

In this section, the effects of the tumor inhibiting, bacterial filament-forming platinum compounds on DNA, RNA and protein syntheses are compared with the effects of 2 non-tumor inhibiting, non-bacterial filament forming platinum compounds in an attempt to establish effects common to all platinum, anti-tumor compounds. Table 3 gives the Test/Control ratios for S-180 tumor inhibition and the ability of 5 platinum compounds to induce filaments in E. coli, Clearly, the first 3 compounds, Cis-Pt(II). Cis-Pt(IV), and Pt(II)en, exhibit some degree of anti-tumor and bacterial filament forming activity, whereas Trans-Pt(IV) and [Pt(II)(NH3)/]+2 exhibit very little or no effect on tumor growth and bacterial elongation. Figure 9 shows the effects of these compounds on the incorporation of ³H-TdR into DNA at 25 µM, expressed relative to untreated cells. In this and all of the remaining experiments cells were pulse labeled with the appropriate isotopically labeled precursor for only the last 2 hours of treatment. Thereby the results could be directly compared at each time period without the problem of isotopic dilution. Cis-Pt(II). Cis-Pt(IV), Pt(II)en inhibit DNA synthesis at 25 uM almost completely after 10 hours of treatment and in the order of the anti-tumor activity shown in Table 3. However, 25 µM Trans-Pt(IV) and [Pt(II)(NH2),]+2 show very little inhibition of DNA synthesis. In fact, in earlier studies, no effect of 375 µM [Pt(IV)(NH3)/Cl2]Cl2 could be found on DNA synthesis (Harder and Rosenberg, 1969).

Figures 10 and 11 show the effects of these compounds on incorporation of 3 H-uridine and 3 H-leucine into RNA and protein respectively. Once again, both figures show that Cis-Pt(II), Cis-Pt(IV), and Pt(II)en at 25 μ M inhibit RNA and protein synthesis progressively over a 24-hour



Comparison of anti-tumor activity and bacterial filament forming abilities of 5 platinum compounds Table 3.

Compound	Best T/C ^b	Dose	Best Elongation	Concentration which inhibits 0.D. to 50% of control 0.D. after 6 hours of treatment
$Cis-Pt(II)(NH_3)_2Cl_2$	ę	8 mg/kg (1 sh	ot) 45 μM, 95-100%, 2-20x	100 µМ
Cis-Pt(IV)(NH3)2Cl4	18	8 mg/kg (1 sh	ot) 20 μM, 100%, 2-20x	25 µM
Pt(II)(NH2)2(CH2)2C12	30	8 mg/kg (1 sh	ot) 45 μM, 90%, 2-20x	100 µM
Trans-Pt(IV)(NH3)2C14	119	40 mg/kg (1 sh	ot) 75 μM, 10%, 2-8x	100 µM
[Pt(II)(NH ₃)4]C1 ₂	96	200 mg/kg (day	1-9) no elongation	2 13.5 mM

 $^{\rm a}{\rm Swiss}$ white mice were treated with either one shot 1.P. on day 1 only or several shots over a 9-day test period after S-180 tumor implantation. On day 10, T/C ratios were determined by the treated to the control tumor weight.

 $^{\mathrm{b}\mathrm{The}}$ best T/C using criteria that no more than one of six animals died at the specified dose within the test period.











Incubation Time in Platinum Media

Figure 10. The relative effects of $Cis-Pt(II)(NH3)_2Cl_2$, $Cis-Pt(IV)(H3)_2Cl_4$, $Pt(II)(NH2)_2(Cl_2)_2Cl_2$, Trans- $Pt(IV)(H3)_2Cl_4$ and $[Pt(II)(NH3)_2)_2(H3)_2Cl_2$ on RIA synthesis in AV3 cells measured by the uptake of H-uridine. Flatinum compounds were applied at time zero and cells were pulse labeled for only the last 2 hours of treatment at each time period. Results are expressed relative to the control.









period, whereas Trans-Pt(IV) and $[Pt(II)(NH_3)_4]^{+2}$ at 25 µM do not inhibit RNA synthesis at all. Higher concentrations of the latter compounds were used to determine if any inhibitory effect could be established at a higher concentration. Trans-Pt(IV) at 100 µM inhibits protein synthesis to 50% of control.

Comparing the effects of Cis-Pt(II), Cis-Pt(IV), and Pt(II)en at 25 μ M in Figures 9, 10 and 11, one will observe that DNA synthesis is most rapidly inhibited, followed by RNA and protein syntheses, as in Figures 7 and 8. Therefore, there appears to be a good correlation between the effectiveness of a platinum compound's oncostatic activity toward Sarcoma-180 tumor growth (see Table 3) and the inhibitory effect on DNA, RNA and protein syntheses as measured by the uptake of labeled thymidine, uridine and leucine. On the other hand, Trans-Pt(IV) and $[Pt(II)(NH_3)_4]^{+2}$, platinum compounds known to be ineffective against S-180 growth, do not inhibit DNA, RNA and protein syntheses at 25 μ M. Only 100 μ M Trans-Pt(IV) shows a significant inhibition of protein synthesis, but not of RNA synthesis.

D. Irreversibility of DNA Synthesis Inhibition After Platinum Treatment

It is desirable to establish whether or not the effects of the platinum compounds are reversible in terms of the recovery of DNA synthesis following 4 hours of treatment. As before, the cells were pulse labeled with ³H-thymidine for only the last 2 hours of treatment prior to sonication. Figure 12 displays the effects of a 4-hour pretreatment with from 0.10 to $25 \,\mu$ M Cis-Pt(II)(NH₃)₂Cl₂ on DNA synthesis. There is no recovery of DNA synthesis over a 24-hour pretreatment with as low as $0.10 \,\mu$ M. Similarly, it is shown in Figure 13 that all 3 tumor inhibiting platinum compounds irreversibly inhibited





Incubation Time after Removal of Pt Media Figure 12. The relative effect of a 4 hour pretreatment of AV3, cells with Cis-Pt(II)(NH₃)₂Cl₂ on DNA synthesis measured by the uptake of 3 SH-thymidine. Platinum media was applied at time -4 hours, and decanted at time zero. After the cells were washed 2 X with Puck's saline D, fresh MEM was added and cells were pulse labeled for only the last 2 hours of each period. Results are expressed relative to the control.



DNA synthesis when the cells were incubated only 4 hours at $5 \,\mu$ M.

To compare the irreversibility of inhibition of DNA synthesis by these platinum compounds with a reversible type of inhibition, the effect of a 4-hour pretreatment with 1 mM hydroxyurea, which is known to reversibly inhibit DNA synthesis (Kim et al., 1967; Young and Hodas, 1964) is included in Figure 13. The recovery of DNA synthesis from hydroxyurea treatment starts immediately after the removal of hydroxyurea and is complete 6 hours later. DNA synthesis continues to be inhibited to a greater extent in the platinum pretreated cells even up to 6 hours after the platinum media is removed. This comparison further illustrates the irreversibility of the inhibition of DNA synthesis over a 24-hour period in human amnion AV, cells pretreated 4 hours with Cis-Pt(II), Cis-Pt(IV), and Pt(II)en at 5 µM. Since the DNA synthesis inhibitory effects of these 3 tumor inhibiting platinum compounds are all irreversible, there can be no correlation of the relative effectiveness with irreversibility of inhibition: only the extent of inhibition can be correlated with anti-tumor activity.

E. <u>Effect of Cis-Pt(II)(NH₃)₂Cl₂ on the Uptake of</u> ³H-Thymidine into The Acid Soluble Pool.

Table 4 shows that there was no inhibition of ${}^{3}\text{H}$ -TdR uptake into the acid soluble pool after 6 hours of treatment at 25 μ M. On the contrary, there appears to be a small degree of accumulation of ${}^{3}\text{H}$ -TdR in the acid soluble pool. At the same time, the average specific activity of the DNA measured by the incorporation of thymidine during the last 2 hours of treatment dropped to 40 and 14% of the control for a 6-hour treatment with 5 and 25 μ M Cis-Pt(II) respectively. However, after 24 hours of treatment when the average specific activity was 11 and 3%




Incubation Time after Removal of Experimental Media Figure 13. The relative effect of a 4 hour pretreatment of M_3 cells with 5 μ M Gis-Pr(11)(Maj)2cl₂, Cis-Pt(11)(Maj)2cl₂, Cis-Pt(11)(Maj)2cl₂, Cis-Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Cis-Pt(11)(Maj)2cl₂, Pt(11)(Maj)2cl₂, Pt(11)(Ma



	6 hr treatm	ent ^a	24 hr treatm	ent ^a
	CPM/OD ₂₆₀ (X10 ⁻³) in acid soluble extract	CPM ^b /µg of DNA	CPM/OD ₂₆₀ (X10 ⁻³) in acid soluble extract	CPM ^b /µg of DNA
Control	182	4312	217	4501
Control	184	5021	218	4304
5 µ M	227	2025	168	510
5μΜ	219	1729	161	487
25 µM	193	658	132	116
25 µM	184	615	121	132

Table 4. The effect of Cis-Pt(II)(NH₃)₂Cl₂ on the uptake of 3 H-thymidine into the acid soluble pool in AV₃ cells

 $^{a\,3}_{\rm H-thymidine}$ was present during only the last 2 hours of treatment. The data for the 6 and 24 hour treatments are from 2 separate experiments.

 $^{\rm b}{\rm The}$ counts per minute (cpm) were based on the amount of DNA applied to the filters.



of the control, the average amount of ${}^{3}H$ -thymidine in the acid soluble pool was reduced to 73 and 58% of the control respectively. Nevertheless the amount of this decrease in the uptake of ${}^{3}H$ -thymidine into the cell's acid soluble pool is insufficient to account for the extent of the decrease in the incorporation of ${}^{3}H$ -tdR into DNA.

F. Stability of Cis-Pt(II)(NH3)2Cl2 in MEM

The stability of the DNA synthesis inhibiting effects of Cis-Pt(II) (NH₃)₂Cl₂ was measured by incubating a 25 μ M solution of Cis-Pt(II) dissolved in MEM 21 hours at 37° C. before applying it to the AV₃ cells. The effects of the day-old Cis-Pt(II) solution were contrasted with the inhibitory effects of a 25 μ M Cis-Pt(II) solution freshly dissolved in MEM that was also preincubated 21 hours at 37° C. AV₃ cells were then tested every 2 hours up to 6 hours for DNA synthesis by the incorporation of ³H-TdR during the last 2 hours of treatment in the usual manner. The results which are given in Table 5 were expressed relative to untreated cells having 21-hour preincubation MEM on them. Although the 21-hour preincubation at 37° C. in MEM reduces the extent of the DNA synthesis inhibition, the difference in the amount of inhibition is small enough that the stability of Cis-Pt(II) (or the active species) in MEM would not be a problem before freshly prepared platinum containing media is added to the cultures or during early hours of treatment.

G. <u>Comparison of the Inhibitory Effects of Cis-Pt(II)(NH3)2Cl2 on</u> the Incorporation of Labeled Precursors into RNA and DNA

It has now been established that tumor inhibiting platinum compounds result in the inhibition of cell division but not of growth *in vitro* in human amnion AV_3 cells. It has also been demonstrated that a primary target of these compounds is DNA synthesis and that this



	Durati	on of AV3 Treatme	nt (hrs)
	2	4	6
Control	1.00	1.00	1.00
25 μM Cis-Pt(II)-MEM incubated 21 hrs	.86	.44	.17
Fresh 25 µM Cis-Pt(II)-MEM	.84	.30	.10

Table 5. The effect of a 21 hr pre-incubation of Cis-Pt(II)(NH₃)₂Cl₂ in MEM on its DNA synthesis-inhibitory activity in AV₃ cells measured by ³H-thymidine incorporation



inhibitory phenomenon is not caused by a platinum induced limitation of the uptake of labeled thymidine into the acid soluble pools of the cells. Therefore, the next problem to be investigated is the mechanism of inhibition of DNA synthesis. Among other possibilities, three general ways in which DNA synthesis can be interrupted will be considered: First, by inhibiting the production of the precursor deoxynucleoside triphosphates as with the antimetabolites; second, by reacting with enzymes directly responsible for replication and maintenance of DNA; third, by reacting directly with the DNA itself, as with the alkylating agents, such that the DNA can no longer serve as a primer for replication.

One way to determine if a DNA synthesis-inhibiting drug acts as an antimetabolite is to examine its effects on the incorporation of a variety of different labeled precursors into DNA. The results of a series of experiments on the incorporation of 6 nucleosides. ³H-thymidine (³H-Tdr), ³H-cytidine (³H-CR), ³H-deoxycytidine (³H-CdR), ³H-uridine (³H-UR), ³H-deoxyuridine (³H-UdR), ³H-deoxyadenosine (³H-AdR), and ¹⁴Cmethyl from ¹⁴C-sodium formate into both DNA and RNA following 6 and 24 hours of treatment with 5 and 25 µM Cis-Pt(II)(NH3)2Cl2 are shown in Table 6. These results were obtained by incubating the AV3 cells with the labeled precursor for only the last 2 hours treatment. One set of plates labeled with 3H-CR, 3H-CdR, 3H-AdR, and 14C-formate were treated with RNase to degrade labeled RNA as described under experimental methods. The difference between the counts incorporated in the RNase treated plates and those not treated with RNase was taken for the amount of the label incorporated into DNA. The RNase treatment removes all but 4% of the counts from ³H-UR incorporation but leaves 95% of the counts due to 3H-TdR incorporation.

The data in Table 6 are best summarized in Table 7, in which the range of the relative inhibitory effects of Cis-Pt(II)(NH3)2Cl2 on the



A comparison of the inhibitory effects of Cis-Pt(II) (NH3)_2Cl2 on the relative incorporation of $^{\rm 2H}_{\rm -}$ -thymidine, $^{\rm 2H-urridine}$, $^{\rm 2H-urridine}$, $^{\rm 2H-urridine}$, $^{\rm 2H-deoxycridine}$, $^{\rm 2H-deoxycridine}$, and $^{\rm 14}$ -c-methyl from 14c-formate into NAA and DNA Table 6.

		3 _H - thymid 6 hr	line 24 hr	3 _H - uridir 6 hr	ne 24 hr	3 _H - deoxyı 6 hr	irídine 24 hr	3 _H - cytidi 6 hr	ne 24 hr	3 _H - deoxyc 6 hr	ytidine 24 hr	3 _H - deoxys 6 hr	adenosine 24 hr	14C- form 6 hr	ate 24 hr
DNA															
(T)	Control	1.00	1.00	Х	Х	1.00	x	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(2)	5 µM	0.30	0.11	х	х	0.62	x	0.55	0.66	0.74	0.77	0.55	0.70	0.59	0.55
(3)	25 µM	0.13	00.00	х	×	0.39	Х	0.28	0.05	0.33	0.01	0.31	0.01	0.33	0.01
RNA															
(1)	Contro1	Х	Х	1.00	1.00	х	Х	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
(2)	5 иМ	х	x	0.88	0.87	Х	Х	0.96	0.82	1.21	0.62	0.88	0.77	0.52	0.48
(3)	25 µ M	х	Х	0.48	10.01	Х	Х	0.58	0.02	0.66	0.00	0.44	0.01	0.06	0.00

X = not tested



labie /.	A summary of lable 6. The range of the fullbitory effects
	of Cis-Pt(II)(NH3)2Cl2 on the relative incorporation of
	labeled precursors into DNA and RNA compared with
	³ H-thymidine and ³ H-uridine respectively

	DNA	1		RM	IA ¹
	6 hr	24 hr		6 hr	24 hr
3 _{H-TdR}			3 _{H-UR}		
5 M	0.30	0.11	5 M	0.88	0.87
25 M	0.13	0.00	25 M	0.48	0.01
Other Precursors ²			Other Precursors ³		
5 M	0.55-0.74	0.55-0.77	5 M	0.88-1.21 (0.54) ⁴	0.62-0.88 (0.48) ⁴
25 M	0.28-0.39	0.11-0.05	25 M	0.44-0.66 (0.06) ⁴	0.00-0.02

¹All results are expressed relative to untreated cells.

²Including ³H-UdR, ³H-CR, ³H-Cdr, ³H-AdR and ¹⁴C-formate.

³Same as 2 excluding ³H-UdR.

 $^{4}\mathrm{The}$ figures in parentheses represent values for $^{14}\mathrm{C}\textsc{-formate}$ which were considered outside of the range of the results achieved with the nucleosides.



incorporation of the labeled precursors into DNA are compared with ³H-TdR incorporation, and the range of the relative inhibitory effects on the precursor incorporation into RNA are compared with ³H-UR incorporation. There are a number of points to be noted: First, there is a dosage dependent inhibition of incorporation of all the other labeled precursors into both DNA and RNA, as with ³H-TdR and ³H-UR. Second, the nucleoside incorporation into DNA is more efficiently inhibited than nucleoside incorporation into RNA, as occurs when these measurements are made using ³H-TdR and ³H-UR. However, there are a number of differences: First, the degree of inhibition of DNA synthesis as measured by the uptake of precursors other than ³H-TdR is less than the inhibition of the uptake of ³H-TdR at both 5 and 25 uM. Second. the inhibition of DNA synthesis at 5 µM appears to have either leveled off after 6 hours of treatment or to have recovered to the 6 hour rate of incorporation. On the other hand, the inhibition of ³H-TdR incorporation into DNA progressively increases with time at 5 uM. Third. the extent of the inhibition of the ¹⁴C methyl from ¹⁴C-formate into RNA is as great as the inhibition of its incorporation into DNA. Nevertheless, these results generally demonstrate that Cis-Pt(II) inhibits the incorporation of all tested nucleosides into DNA and RNA, the only major difference being the greater inhibition of ³H-TdR incorporation over that of the other precursors.

H. Effect of Unlabeled Deoxynucleosides on the Inhibition of DNA Synthesis by Cis-Pt(II)(NH₃)₂Cl₂

There is another way to determine if the mechanism of action of a DNA synthesis-inhibiting drug involves the inhibition of the production of the precursors of DNA synthesis. That is to look for a recovery of



DNA synthesis when a sufficient supply of the necessary precursors is applied to treated cultures. Accordingly, an experiment was performed in which, after AV₃ cells were preincubated 4 hours with 5 μ M Cis-Pt(II) at 37° then rinsed 2 X with Puck's saline D and MEM containing 10 μ M each of unlabeled deoxyadenosine, deoxycytidine and deoxyguanosine was added to one set of treated plates while just MEM was added to the other set of treated plates. Then the effects of the Cis-Pt(II) pretreatment were measured by the incorporation of ³H-TdR during the last 2 hours of treatment up to 6 hours after the removal of Cis-Pt(II) and the addition of the deoxynucleosides. The results in Table 8 provide good evidence that the addition of the deoxynucleosides even after the platinum is removed does not reverse the inhibition of DNA synthesis. Therefore, it may be concluded that the Cis-Pt(II) induced inhibition of DNA synthesis is not brought about by limiting deoxyribonucleoside production at 5 μ M.

I. Effects of Platinum Compounds on the Acid Soluble DNase and Endonuclease Activities in Cell Free Preparations of AV₃ Cells

The previous results strongly indicate that the mechanism of the platinum compounds, and specifically Cis-Pt(II)(NH₃)₂Cl₂, does not primarily involve the inhibition of deoxyribonucleoside production. Therefore, the following experiments were performed to examine the possible effects on those enzymes which use DNA as a primer or substrate. First, the effects of the platinum compounds on the acid soluble deoxyribonuclease (DNase) and endonuclease activity were studied. AV₃ cells were incubated for a given period of time in MEM containing a given platinum compound at a certain concentration. Then cell free extracts of both treated and untreated cells were prepared as described under Methods.



	Time Af	ter Removal of	5 µM Cis-Pt(I	I)-MEM ^a
	0	2 hrs	4 hrs	6 hrs
Control	1.00	1.00	1.00	1.00
4 hr pretreated ^b	0.59	0.30	0.16	0.11
4 hr pretreated and deoxynucleosides ^C	0.59	0.31	0.22	0.16

Table 8. Effect of the presence of unlabeled deoxynucleosides on the relative inhibition of DNA synthesis by Cis-Pt(II)(NH₃)₂Cl₂ by ³H-TAK incorporation

 $^{\rm d}{\rm AV}_3$ cells were preincubated 4 hours at 37° C. in 5 $\mu{\rm M}$ Cis-Pt(II) (NH_3)_2Cl_2, then rinsed twice with Puck's saline D and fresh MEM added. To half of the plates, MEM having 10 $\mu{\rm M}$ each of deoxyadenosine, deoxy-guanosine, and deoxycytidine was added. H-thymidine was present during the 2 hours of incubation.

 $^{\rm b}{\rm Relative}$ to untreated cells without deoxynucleosides.

^CRelative to untreated cells with deoxynucleosides.



1. Characteristics of the DNase Activity. The optimum assay conditions for the acid soluble DNase activity in AV, cell extracts were first determined. Table 9 shows that with an extract from untreated cells, pH 4.80 (acetate buffer) was the optimum pH for releasing acid soluble deoxyribonucleotides. A pH 9.00 assay (Tris buffer) also showed a small amount of activity, whereas essentially no activity was observed in a pH 7.54 phosphate buffer. The table also shows that a native labeled DNA substrate was preferred over a heat denatured labeled DNA by a factor of 3 to 4. Also included in Table 9 are the results of a parallel study using extracts of AV3 cells which had been treated with 50 and 500 µM Cis-Pt(IV) for 24 and 12 hours respectively; the data show that the enzyme extract from treated cells was also most active at pH 4.80 with a native DNA substrate. However, at pH 9.00, denatured DNA was the preferred substrate. A similar study demonstrated that the endonuclease activity in AV3 cell extracts was also most active with a native DNA substrate at pH 4.80.

No additional efforts were attempted to further characterize the acid soluble DNase and endonuclease activities, as, for example, to determine the nature of the products by chromatographic separation or to determine the molecular weight of the products of the endonuclease activity. Nevertheless, the general kinetics of the activity, the pH optimum and high preference for native DNA parallel the characteristics of mammalian DNase II, a spleen endonuclease which can yield mononucleotides (Sugar and Sierakowski, 1967).



	pH	= 4.80	pH	= 7.54	pH	= 9.00
Extract (3.1 µg/assay)	Native DNA	Denatured DNA	Native DNA	Denatured DNA	Native DNA	Denatured DNA
Control	5,465	1,460	88	107	179	196
50 μM Cis-Pt(IV) 24 hr treatment	7,262	1,867	34	66	142	662
500 μM Cis-Pt(IV) 12 hr treatment	12,009	3,795	96	50	172	1,202

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Table 9. The effect of pH and the state of the DNA substrate on the acid soluble DNase activity in CPM in extracts of AV₃ cells grown in Cis-Pt(TV)(NH₃)₂CL₄ using a 2-hour assay



Acid Soluble DNase and Endonuclease Activity in Extracts of AV₃ Cells Treated with Cis-Pt(I1)(NH₃)₂Cl₂, Cis-Pt(IV)(NH₃)₂Cl₄ and Pt(II)(NH₃)₂(CH₂)₂Cl₂ During Growth.

The acid-soluble DNase activity was measured in extracts of AV, cells treated with 50 and 500 µM Cis-Pt(IV)(NH3)2Cl4 for 24 and 2 hours respectively as a function of assay incubation time at 37° C. 18.6 µg protein-enzyme extract was added to each reaction tube to start the assay. The results, shown in Figure 14, demonstrate a marked concentration-dependent enhancement of the DNase activity. The acid soluble counts released into the assay medium by the extract from 500 uM treated cells was higher than that by the control extract by a factor of approximately 3 after 120 minutes of incubation. Similarly when the same extracts were used for measuring endonuclease activity, using only 3.1 ug enzyme extract per reaction tube, an enhancement of the endonuclease activity was also observed (Figure 15). In this assay, activity is measured by the rate of loss of acid precipitable 3H-DNA. The maximum enhancement was a factor of 2 with the extract from the 500 µM treated AV2 cells. However, this enhancement was limited to the early minutes of the reaction, for after 60 minutes of incubation, the extent of the enhanced endonuclease activity was greatly reduced. Perhaps this apparent rapid decline in the endonuclease activity was due to substrate limitation.

The results of similar studies using the enzyme extract from AV₃ cells treated 5 hours with 0, 50, 150, and 500 μ M Cis-Pt(II)(NH₃)₂Cl₂ (5 μ g protein reaction tube) are shown in Figures 16 and 17. Clearly Cis-Pt(II)(NH₃)₂Cl₂ does not induce an enhancement of either acid soluble DNase or endonuclease activity. On the other hand, in a parallel experiment using extracts of AV₃ cells treated 6 hours with 0, 50, 150 and 500 μ M Pt(II)(NH₃)₂Cl₂(CH₂)₂Cl₂ (3.35 μ g protein per assay tube) a dosage











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Figure 16. Acid soluble DNase activity in CPM in extracts of AV₃ cells treated 5 hours with Cis-Pt(II)(NH₃)₂Cl₂. $5 \mu g$ protein/assay.





Figure 17. Endonuclease activity in CPM in extracts of AV₃ cells treated 5 hours with Cis-Pt(II)(NH₃)₂Cl₂. 5μ g protein/assay.



dependent enhancement of acid soluble DNase was measured (Figure 18). However, no enhancement of endonuclease activity was observed as with extracts from cells treated as with Cis-Pt(II) (NH_3)₂Cl₂.

3. Effects of the Presence of Cis-Pt(II)(NH₃)₂Cl₂, Cis-Pt(IV) (NH₃)₂Cl₄ or Pt(II)(NH₂)₂(CH₂)₂Cl₂ in the Acid Soluble DNase and Endonuclease Assays Using Enzyme Extracts from Untreated Cells

The previous experiments established a dosage dependent enhancement of nuclease activities in extracts of cells that were treated during growth with Cis-Pt(IV). This raises the question as to whether this enhancement is caused by a direct platinum induced activation of the nucleases or due to a platinum induced activation of nuclease production such that a higher concentration of such enzymes would be present. The following experiments were performed to test the former possibility. The three tumor inhibiting platinum compounds Cis-Pt(II), Cis-Pt(IV) and Pt(II)en were added directly to the assay mixtures before adding the enzyme extract from untreated AV₃ cells. Figure 19 shows a rather steep, concentration-dependent inhibition of acid soluble DNase activity. Pt(II)en was the most effective inhibitor followed by Cis-Pt(IV) and then Cis-Pt(II). Similarly towards endonuclease activity, Pt(II)en was the most potent inhibitor (Figure 20) followed by Cis-Pt(II); however, Cis-Pt(IV) showed almost no effect on the endonuclease activity from untreated cells. These 2 experiments provide sufficient evidence to conclude that the platinum compounds do not directly activate the nuclease activities by an allosteric interaction with these enzymes because the platinum compounds inhibit their activity.










Figure 19. Effect of Cis-Pt(II)(NH₃)₂Cl₂, Cis-Pt(IV)(NH₃)₂Cl₄ and Pt(II)(NH₂)₂Cl₂(Cl₂)₂Cl₂ on the acid soluble DNase activity in CPM in extracts from untreated AV₃ cells. 2.5 µg protein/assay; 2 hours incubation at 37° C.





Figure 20. Effect of Cis-Pt(II)(NH3)_2Cl₂, Cis-Pt(IV)(NH3)_2Cl₄ and Pt(II)(NH₂)_2(CH2)_2Cl₂ on the endonuclease activity in CPM in extracts of untreated AV3 cells. 2.5 μ g protein/assay; 2 hours incubation at 37° C; 19,100 CPM retained in assay lacking enzyme.



J. DNA Polymerase Studies

1. Enzyme Characterization. The DNA polymerase obtained from extracts of AV3 cells prepared as described under Methods was characterized as summarized in Table 10. First it was noted that the DNA polymerase had considerable preference for a heat denatured DNA primer over a native one. It was also shown that a phosphate buffer of the same concentration and pH greatly inhibited the activity. To demonstrate that the incorporated or that the measured radioactivity was being incorporated into DNA, various controls were performed. For example, it was shown that enzymatic activity was required for the incorporation of 3 H-dATP because when the incubation was done at 0° C, essentially no counts were incorporated. Likewise, the DNA primer was an absolute requirement, as was the presence of both pyrimidine and purine deoxynucleotide triphosphates for maximum incorporation. The dependence of the activity on the protein content, or on the amount of enzyme extract added to the assay, was found to be linear up to more than 300 μ g. Finally, the DNA polymerase activity was found to be linear with time up to and including 2 hours of incubation at 37° C. Therefore, assay incubation periods of 1 to 2 hours were normally employed to maximize the incorporation. Hence even though this DNA polymerase was only in the form of a crude extract, it behaved quite as well as a DNA polymerase preparation for these studies.

DNA Polymerase in Extracts of AV₃ Cells Treated with Tumor Inhibiting Platinum Compounds.

In extracts of AV₃ cells preincubated 6 hours at 37° C. in MEM containing Cis-Pt(IV) and Pt(II)en the DNA polymerase activity as a function of protein content per 1 hour assay is shown in Figure 21.



		³ H-dATP incorporated into DNA (CPM)
1.	Complete assay plus 62.5 µg enzyme ext incubated 2 hours at 37° C.	ract, 2745
2.	Complete but incubation 2 hours at 0° (. 127
3.	Complete, minus DNA, 2 hours at 37° C.	147
4.	Complete, minus dCTP	2431
5.	Complete, minus dCTP and dTTP	917
5.	Complete, minus dCTP, dTTP, and dGTP	389
7.	Complete, but native instead of heat denatured DNA 1584	
3.	Complete, but phosphate buffer instead HC1, same pH	of tris- 154

Table 10. Characteristics of DNA polymerase in ${\rm AV}_3$ cell extracts





Figure 21. DNA polymerase activity in CPM in extracts of AV₃ cells treated 6 hours with Cis-Pt(IV)(NH₃)₂Cl₄ and Pt(II)(NH₂)₂(CH₂)₂Cl₂.



Except for the 50 μ M Pt(II)en extract, there was a concentrationdependent enhancement of the DNA polymerase, which was especially large in the extract prepared from 500 μ M Cis-Pt(IV) treated cells. In addition, this figure shows that the effect is greater in Cis-Pt(IV) than in Pt(II)en extracts. In a parallel study using extracts prepared from AV₃ cells preincubated 5 hours with Cis-Pt(II), the DNA polymerase activity was also greater (Figure 22). By comparing Figures 21 and 22, one will see that the enhancement by Cis-Pt(II) was about the same as that by Pt(II)en.

Effect of the Addition of Platinum Compounds to the DNA Polymerase Assay Using Untreated AV, Cell Extract.

Once again this platinum enhanced DNA polymerase activity might be considered to arise by a direct activation of the enzyme or by the stimulation of a higher production rate of DNA polymerase. Accordingly, experiments were performed in which platinum compounds were added directly to the assay mixture to which an untreated enzyme extract was added. The results in Figure 23 show that there was a concentration dependent inhibition of the DNA polymerase activity by Pt(II)en, Cis-Pt(IV) and Cis-Pt(II) in that order. Therefore, platinum compounds cannot directly activate the DNA polymerase activity by binding to the polymerase molecules. However, absolutely no inhibition of the DNA polymerase activity was observed by [Pt(II)(NH3)4]Cl2 up to 250 µM. the highest concentration tested. This latter result is consistent with the lack of inhibition of DNA synthesis by [Pt(II)(NH₃)₄]Cl₂. The possible implications of this finding may be of great importance, as discussed later. On the other hand, the relative ordering of the inhibition of DNA polymerase by the 3 active compounds is opposite to the















ordering of the relative effectiveness of both the inhibition of DNA synthesis and S-180 tumor growth.

K. <u>Effect of Cis-Pt(II)(NH₃)₂Cl₂ on Purified DNA Dependent RNA</u> Polymerase from *Pseudomonas putida*

A similar set of experiments dealing with the effects of platinum compounds on DNA dependent RNA polymerase from AV₃ cells had been planned. However, when I was unable to establish the proper assay conditions and extraction technique, I decided to try to use the purified DNA dependent RNA polymerase instead. Thus when Cis-Pt(II) was added to the assay mixture in the presence of native DNA and the assay mixture was allowed to incubate 30 minutes at 30° C, the results in Figure 24 were obtained. Absolutely no inhibition of the RNA polymerase activity occurred up to 160 μ M, the highest concentration tested. Hence, Cis-Pt(II) has no effect on a purified bacterial DNA dependent RNA polymerase, whereas it inhibits both AV₃ DNA synthesis and DNA polymerase when added to extracts of AV₃ cells.

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Figure 24. Effect of Cis-Pt(II)(NH3)2Cl2 on the purified DNA dependent RNA polymerase from *Pseudomonas putida*.



IV. DISCUSSION

A. Platinum Induced Inhibition of Cell Division

Cis-Pt(II)(NH₃)₂Cl₂ induced inhibition of cell division was demonstrated to occur in Human Amnion AV₃ cells, as shown in Figure 2. The finding that Cis-Pt(II) also induces giant cell formation 3 to 4 days after treatment (Figure 3), in conjunction with the observed inhibition of cell division, closely parallels its effects in *E. coli* B, i.e., cell division inhibition leading to filament formation (Rosenberg *et al.*, 1967a). Following exposure to Cis-Pt(II) or other tumor inhibiting platinum compounds, cytokinesis was observed to occur uniformly throughout the filamentous *E. coli* B after removal of the Cis-Pt(II). The resulting daughter cells appeared to be normal in every respect. On the other hand, it is not yet known whether giant AV₃ cells regain the ability to complete mitotic division successfully and give rise to competent progeny cells. However, preliminary observations indicate that at least a given fraction of giant cells begin to divide 4 to 10 days after an initial 4 hour, $5 \mu M$ Cis-Pt(II) treatment.

Other anti-neoplastic drugs also induce giant mammalian cells. These include the nitrogen mustards, as HN2 (Wheeler, 1962; Levis *et al.*, 1963) and Mitomycin C (Shatkin *et al.*, 1962). Perhaps the most important conclusion which may be inferred from giant cell formation is that RNA and protein synthesis is not being inhibited. This conclusion must be qualified somewhat to state that at least those proteins and their respective messenger RNA which are required for cell growth are



not being inhibited a few days after treatment. Hence the above does not necessarily apply to those proteins employed for mitosis.

B. Inhibition of DNA Synthesis: A Primary Target of Tumor Inhibiting Platinum Compounds

The results shown in Figure 4 clearly demonstrate a dosagedependent inhibition of DNA synthesis in AV₃ cells. Furthermore, Figure 9 shows that all 3 tested platinum anti-neoplastic compounds, Cis-Pt(II), Cis-Pt(IV) and Pt(II)en inhibit DNA synthesis in these cells, whereas Trans-Pt(IV)(NH₃)₂Cl₄ and [Pt(II)(NH₃)₄]Cl₂, which have no antineoplastic activity against S-180 growth, did not inhibit DNA synthesis at 25 μ M. In 1969, Harder and Rosenberg reported that Pt(II)(NH₃)₄Cl₂ had no effect on DNA synthesis up to 375 μ M.

DNA synthesis was examined extensively by measuring the inhibition of 3 H-TdR incorporation into a TCA insoluble product. Table 7 shows that Cis-Pt(II) also inhibits the incorporation of the nucleosides, 3 H-UdR, 3 H-CdR, 3 H-CR, 3 H-AdR as well as the 14 C-methyl from 14 C-formate into DNA.

The possibility that this inhibitory effect was caused by a platinum induced reduction of the permeability of the cell membrane to the nucleosides was eliminated by the findings that there was essentially no effect at 5μ M on the uptake of ³H-TdR into the acid soluble pool. Although there was a measurable reduction in the uptake of ³H-TdR into the cell pool by a 24-hour treatment with 25μ M Cis-Pt(II), the extent of this reduction was far too small to account for the degree to which its incorporation into DNA was reduced. The relative effect of Cis-Pt(II) on the specific activity of ³H-TdR incorporation in this same experiment agrees very well with the results on the inhibition of DNA synthesis obtained in the usual manner. The validity of measuring DNA synthesis



in the way most frequently employed was therefore substantiated.

Figures 5 and 6 show the Cis-Pt(II) concentration-dependent inhibition of RNA and protein syntheses as measured by the incorporation of ³H-UR and ³H-L-leucine respectively. However, it was shown that at 5 μ M, and lower concentrations, DNA synthesis was selectively inhibited (Figure 8). Further, DNA synthesis was inhibited more rapidly than RNA synthesis, which in turn was inhibited more rapidly than protein synthesis at the higher concentration of 25 μ M (Figure 7). The same effects on RNA and protein synthesis in relation to DNA synthesis occurred in AV₃ cells treated with Cis-Pt(IV) and Pt(II)en (Figures 10 and 11). Similarly 25 μ M Cis-Pt(II) inhibited the incorporation of the nucleosides ³H-CR, ³H-CdR, ³H-AdR into RNA but not as rapidly as the same nucleosides were inhibited from incorporation into DNA (Table 7). With all precursors except 14 C-formate and 3 H-TdR the extent of inhibition of incorporation into DNA and RNA was very similar. Thus all of these studies on the inhibitory effects of platinum compounds toward the incorporation of nucleosides into DNA and RNA provide evidence in support of the hypothesis that DNA synthesis is the primary target of the tumor-inhibiting platinum compounds.

In further support of this hypothesis are the results of Howle and Gale (1970), who found that DNA synthesis was selectively and persistently inhibited in Ehrlich Ascites tumor cells removed periodically from rats up to 4 days after treatment with a single injection of 10 mg/kg of Cis-Pt(II). RNA and protein synthesis were also inhibited initially, but these processes recovered after 4 days. When Ehrlich ascites tumor cells were treated *in vitro* with high concentrations of

Cis-Pt(II), (100 μ M), DNA, RNA and protein syntheses were inhibited in that order, in agreement with the results reported here on AV₃ cells. Unfortunately, the effects of Cis-Pt(II) at lower concentrations were not reported so that a more thorough comparison of the results with the Ehrlich ascites cells with the effects in human amnion cells is not possible.

Additional support for the above conclusion is given by the finding that sufficient platinum is deposited or sequestered in S-180 tumors in mice to inhibit DNA synthesis in AV₃ cells. When mice having well developed S-180 tumors were given a single injection of 8 mg/kg Cis-Pt(II), the optimum therapeutic dose, a maximum of about 1.5μ g/mg of platinum was found in the tumor within 24 hours of the injection (Toth-Allen, 1970). This concentration is roughly equivalent to 5μ M, assuming a gram of tissue is equivalent to a gram of water. Actually because only about 70% of the body is water (Hirshaut, *et al.*, 1969), the above figure is obviously an underestimate, but it will serve as a conservative estimate. Since at concentrations of 5μ M and below, DNA synthesis was selectively inhibited in AV₃ cells, one may infer from this that the mechanism of action of these platinum compounds may ultimately involve the inhibition of DNA synthesis *in vivo* as well as *in vitro*.

C. <u>Possible Evidence for a Metabolically Transformed, Effective</u> <u>Platinum Moiety or an Irreversible Reaction of Platinum with the</u> <u>Target Molecules</u>

In contrast to hydroxyurea, which is also a tumor-inhibitor and induces filaments in *E. coli*, platinum compounds inhibit DNA synthesis at a relatively slow rate in the therapeutic dose range. For example, at a 1 mM concentration of hydroxyurea, DNA synthesis in HeLa S-3 cells

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is reduced to about 10% of the control in 2 hours (Kim *et al.*, 1967; Pfeiffer and Tolmack, 1967; Young *et al.*, 1967), whereas in our studies DNA synthesis is still about 82% of the control in human amnion AV_3 cells treated 2 hours with 25 μ M Cis-Pt(II). Further, hydroxyurea does not inhibit RNA or protein synthesis *in vitro* at 1 mM as do the platinum compounds at 25 μ M.

One possible explanation for the slow rate of inhibition by platinum compounds is that the applied platinum compound is itself inactive and must be converted intracellularly to an active species, by means of a one or a many step reaction. Howle and Gale, 1970, suggest a 2 stage transformation, the first yielding a product inhibitory in general to DNA, RNA and protein syntheses, and the second inhibiting only DNA synthesis. The results given here support a different interpretation of this posulate: $Pt_0 \rightarrow Pt_1^{2}Pt_2$. Here Pt_0 represents the initial platinum species and Pt₁ is a slowly increasing metabolically changed platinum species to which DNA synthesis is more sensitive than RNA and protein synthesis. Another possibility is that Pt, inhibits only DNA synthesis and a second cellularly transformed platinum species, Pt₂, is responsible for RNA and protein synthesis inhibition. The latter suggestion would account for the slow rate of DNA synthesis inhibition, assuming a slow rate of Pt₁ formation. Furthermore a low equilibrium constant for Pt₂ production would account for the selective DNA synthesis inhibition at low concentrations and the slower rate of RNA and protein synthesis inhibition at higher concentrations. This proposal also accounts for the results of Howle and Gale (1970). Incidentally, Mitomycin C (Szybalski and Iyer, 1964) and cyclophosphamide (Bosen and Davis, 1969) are 2 examples of other tumor-inhibitors that must be enzymatically activated.



However, a far better explanation of the sequential inhibition of DNA, RNA and protein synthesis is that the active platinum species binds directly to DNA such that at low concentrations, only DNA synthesis is affected. Then at high concentrations, a higher frequency of platinum induced lesions would lead to a measurable inhibition of messenger RNA production which in turn would eventually result in a reduction of protein synthesis. Such a mechanism would lead to irreversible inhibitory effects unless and until such damage would be repaired. Figures 12 and 13 show that tumor inhibiting platinum compounds irreversibly inhibit DNA synthesis.

Cross-linking DNA is, in fact, the mechanism proposed for the bifunctional alkylating agents (Wheeler, 1962). Bifunctional alkylating agents in general, including HN2, have been shown to inhibit DNA synthesis without much effect on RNA and protein synthesis in both *E. coli* and mammalian cells *in vivo* and *in vitro* (Wheeler, 1962; Harold and Ziporin, 1959; Brewer *et al.*, 1961; Lawey and Brooks, 1965; Smith and Busch, 1964). Similarly, Mitomycin C selectively and irreversibly cross-links and inhibits DNA synthesis without concomitant effects on RNA or protein synthesis in mammalian and bacterial cells (Magee and Miller, 1962; Shiba *et al.*, 1959; for an excellent review, see Szybalski and Iyer, 1964). Therefore, the effects of the tumor inhibiting platinum compounds on DNA, RNA, and protein syntheses in mammalian cells very closely mimic the effect observed with bifunctional alkylating agents!

A third possible explanation for the slow rate of inhibition is that the diffusion or active uptake of platinum compounds is the rate limiting step. Included in this category is a possible time dependent interaction of a platinum compound with the lipids of the cell membrane



which may render it more or less permeable to either the drug or labeled precursor. The absence of inhibition of uptake of 3 H-TdR into the acid soluble pools after 6 hours of treatment with Cis-Pt(II) provides conclusive evidence against the possibility that a membrane effect is responsible for the observed inhibition of DNA synthesis initially. However, the dose dependent decrease in uptake after 24 hours indicates that there may be a very slow membrane effect which plays a role in long duration measurements of DNA synthesis inhibition. Thus the irreversible inhibition of DNA synthesis by Cis-Pt(II), Cis-Pt(IV) and Pt(II)en might be explained by the gradual limitation of the passage of 3 H-TdR into cells and a slow diffusion of the original or cellularly transformed platinum compounds out of the cell, such that 2 rinses would not efficiently remove them.

D. Are Tumor Inhibiting Platinum Compounds Antimetabolites?

Now that the inhibition of DNA synthesis has been shown to be a primary target of tumor inhibiting platinum compounds, the next question to which this thesis is addressed is whether or not the mechanism of this DNA synthesis inhibition is by the inhibition of the production of the precursors of DNA synthesis. This possibility was investigated by measuring the inhibitory effects of the platinum compounds towards the incorporation of various nucleosides and the ^{14}C -methyl from ^{14}C -formate into DNA and RNA. The results are given in Table 6 and summarized in Table 7 for the effects of Cis-Pt(II).

Interference with enzymes catalyzing the reduction of ribonucleosides to deoxyribonucleosides appears to be excluded because the incorporation into DNA of labeled 3 H-deoxycytidine and 3 H-cytidine were inhibited to almost the same degree. Further evidence that ribonucleoside



diphosphate reductase is not inhibited was provided by the finding that the addition of sufficient unlabeled deoxynucleosides did not reverse the inhibition of DNA synthesis (Table 8). In contrast to this, the addition of deoxyribonucleoside to HeLa cells being treated with hydroxyurea, which is known to inhibit this enzyme, results in the partial protection from the inhibition of ³H-TdR incorporation into DNA (Young *et al.*, 1967).

By a similar argument, the interference with the one carbon transfer in the pathway from deoxyuridilic acid to thymidylic acid seems unlikely because the incorporation into DNA of the labeled 3 H-UdR was inhibited to no greater extent than the labeled 3 H-TdR incorporation. The same conclusion is supported by the absence of a greater degree of inhibition of 14 C-methyl from 14 C-formate into DNA.

However, the finding that 3 H-TdR incorporation into DNA is most effectively inhibited compared to the other tested deoxyribonucleosides is evidence that the thymidine kinases may be inhibited. Likewise the finding that the incorporation of the label from 14 C-formate into RNA is inhibited to a far greater extent than the inhibition of the incorporation of the label from the other precursors (Table 7) is indicative of a platinum-induced inhibition of *de novo* ribonucleoside synthesis. It is most interesting that HN2, and other alkalating agents, have also been shown to inhibit the *de novo* synthesis of ribonucleosides by following the incorporation of 14 C from 14 C formate into the purines of DNA both *in vivo* and *in vitro* (Wheeler and Alexander, 1964b). Furthermore, it was shown that this inhibitory effect was less in HN2 resistant tumors than in HN2 sensitive ones. Their conclusion was based on the inhibition of incorporation of 14 C from 14 C-formate into RNA in the absence of the inhibition of ribonucleoside incorporation into RNA.
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Therefore Cis-Pt(II) again very closely mimics the effects of HN2 in this respect.

Nevertheless, the finding that the incorporation of exogenously applied ribo- and deoxyribonucleosides into RNA and DNA is also inhibited provides evidence against a mechanism of action based primarily on the inhibition of *de novo* ribonucleoside synthesis. This conclusion applies to Cis-Pt(II) as well as to HN2. Thus with the possible exception of the deoxynucleoside kinases, these results suggest that the tumor inhibiting platinum compounds are not interfering with the metabolism of an individual base, but rather that they react with either DNA *per se* or enzymes for which DNA is a primer or a substrate.

E. Effects of Platinum Compounds on Enzymes Using DNA as a Template or Substrate

1. <u>Deoxyribonuclease Activity</u>. At this point it is quite difficult to draw any valuable conclusions regarding the studies of the effects of platinum compounds on the stimulation of DNase activity in platinum treated AV₃ cells because Cis-Pt(II) has no enhancing activity. However, it should be emphasized that the different results on the DNase activities in extracts of AV₃ cells treated with Cis-Pt(II) and Cis-Pt(IV) represent the only major difference in the molecular effects of Cis-Pt(II) and Cis-Pt(IV). This may be an interesting point to pursue in further research. The results from experiments using Cis-Pt(IV) on AV₃ cells are consistent with earlier bacterial studies. These established that both pure Cis-Pt(IV) and UV irridiated K_2 Pt(IV)Cl₂, the major photo-product being Cis-Pt(IV), induced a similar enhancement of endonuclease activity in *E. coli* B (Harder and Zimmerman, 1968).



In comparison with inhibitory effects on DNA synthesis, the results show that the significant enhancement of DNase activity occurred only when AV₃ cells were treated with extremely high concentrations (500 μ M) of Cis-Pt(IV). Therefore, enhanced DNase activities measured by these techniques would seem to be ruled out as being involved in the mechanism of action of tumor inhibiting platinum compounds. The lack of enhanced DNase activities in extracts from Cis-Pt(II) treated cells supports this conclusion.

The same argument would appear to apply to the results of experiments in which the inhibition of the DNase activities were measured by the addition of platinum compounds to assays containing untreated cell extracts. However, it must be recalled that the inhibition of DNA synthesis was a rather slow process. This fact suggests that a greater degree of inhibition might have been observed if the measurement were performed over a 4- to 6-hour incubation period instead of just 2 hours. On the other hand, it is questionable if these nucleases could remain active that long at 37° C. A completely different method of measuring the effects of platinum compounds on overall DNase activities is therefore suggested for future research. This would be to prelabel mammalian cells with ³H-TdR, chase with unlabeled TdR, and follow both the increase in acid soluble ³H-nucleotides and the decrease in the amount of ³H-label incorporated into the DNA after the beginning of treatment.

Other tumor inhibiting agents also cause an elevation in DNase activities in HeLa cells. These include Mitomycin C, HN2, hydroxyurea, and FUdR (5-fluorouricil-deoxyriboside) (Studsinski and Cohen, 1966a). Thus it would appear that these agents might function by depolymerizing cellular DNA. But Studzinski and Cohen (1966b) also demonstrated that the DNA content of Mitomycin C-inhibited cells was not decreased and



acid soluble deoxyribose compounds did not accumulate to any great extent. Consequently it was concluded that the enhanced DNase activity played a doubtful role in the mechanism of the inhibitory action of Mitomycin C. Nevertheless, this result does not eliminate the possibility that the elevated DNase levels have a function. One example of this would be in the repair of the Mitomycin C-alkylated DNA.

2. DNA and RNA Polymerase Activities. The same reasoning that was given for the enhanced DNase activities also applies for the enhanced DNA polymerase activities which were observed in extracts of cells treated with all 3 tumor inhibiting platinum compounds. In other words, a significant enhancement of DNA polymerase activity occurs at only very high concentrations (500 μ M) compared to concentrations required to inhibit DNA synthesis. Therefore, enhancement of DNA polymerase activity may be excluded as a possible mechanism of action of the tumor inhibiting platinum compounds. This conclusion must be made a priori because a DNA synthesis-inhibitory platinum effect on DNA polymerase could conceivably occur only by the inhibition of its activity. This, of course, is exactly what was observed when tumor inhibiting platinum compounds were added to the DNA polymerase assay in which untreated AV₃ cell extracts were used as the enzyme preparation (Figure 23). However, the degree of inhibition is very low compared to concentrations at which DNA synthesis inhibition is observed. It must be recalled, however, that inhibition of DNA synthesis is rather slow, and after 2 hours at 25 μ M it is still about 80% of the control (Figure 9). Therefore, it is suggested that DNA be incubated with platinum compounds for various time periods and reisolated before use in the DNA polymerase



assay. Measuring the effect on the template activity using DNA which has been isolated from platinum treated cells would be another valuable experiment to be performed in the future. Such experiments could provide valuable evidence for biologically important reactions of platinum compounds with DNA.

Other drugs also have an effect on DNA template activity using the DNA polymerase assay. DNA isolated from Lettré-Ehrlich ascites cells treated *in vitro* with HN2 served as a better primer for DNA polymerase than DNA from untreated cells; yet the opposite result was obtained when cells were treated *in vivo* (Goldstein and Putman, 1964).

Both Mitomycin C and Porforomycin induced enhanced DNA polymerase activities in extracts of treated HeLa cells (Bach and Magee, 1962; Magee and Miller, 1962). On the other hand, the primer activity of Mitomycin-treated DNA is reduced for DNA polymerase (Pricer and Weissbach, 1964) but is not reduced for DNA dependent RNA polymerase. Therefore, the antineoplastic platinum compounds closely mimic the effects of alkylating agents in these properties as well.

The finding that $[Pt(II)(NH_3)_4]Cl_2$ has no effect on either DNA, RNA, protein synthesis (Figures 9, 10, and 11) or on DNA polymerase (Figure 23) at very high concentrations is significant because it shows that the inhibitory effects of the platinum compounds cannot be attributed to a nebulous, heavy-metal, poison effect. On the contrary, this indicates that the inhibitory effects of the platinum compounds are very specific and strongly depends upon the ligands surrounding the platinum atom. Moreover, this conclusion is substantiated



by the lack of inhibition of DNA dependent RNA polymerase (Figure 24) by Cis-Pt(II)($\dot{N}H_3$)₂Cl₂.

3. <u>A Possible Involvement of DNA Repair Enzymes</u>. How, then, might the enhanced DNA polymerase activity in extracts of platinum treated cells be explained? There is one very probable explanation which could account for both the enhanced DNA polymerase and DNase activities--an activation of DNA repair systems induced by a platinum reaction with the cellular DNA. Assuming platinum reacts with DNA, and the cell recognizes this as an insult to its genetic apparatus, the normal response to this or any DNA damage would be to attempt to repair the damage. This would involve the excision of the defective bases and the repair of the gap in the DNA by replication using the opposite strand as a template. At this point, the order of the process is unimportant.

The relevance of the repair of chemically induced DNA damage to the effectiveness of a given tumor inhibiting agent is just now becoming apparent. For example, Lawley and Brooks (1965) demonstrated that incubation of HN2 resistant $E.\ coli$, after alkylation, resulted in the excision of bifunctionally alkylated guanine moieties from the DNA, whereas a sensitive strain of $E.\ coli$ was deficient in this ability. Yet the DNA from both resistant and sensitive strains were alkylated to the same extent initially. Therefore, Lawley and Brooks (1965) proposed that the cytotoxic action of nitrogen mustards is due to the creation of interstrand cross-links in DNA, which would interfere with DNA replication but not transcription.

Similarly, HeLa cells (Roberts $et \ al.$, 1968; Crathorn and Roberts, 1966) and mouse L cells (Reid and Walker, 1969) have been shown to



excise both mono- and bifunctionally alkylated DNA products after sulfur mustard alkylation. Wheeler and Alexander (1964b) showed both in vivo and in vitro that HN2 alkylates the DNA in sensitive and resistant plastocytomas equally. Therefore, they concluded that the extent of gross alkylation of DNA is not related to the anti-tumor activity. However, their conclusion is probably invalid because with both in vivo and in vitro experiments, no time was allowed for repair after the administration of HN2. This latter point is borne out by some of the very recent results in vivo. When analine mustard was administered to both a resistant and sensitive tumor, Connors and Double (1970) showed that resistant tumors were alkylated to a greater extent than DNA from the sensitive tumor 12 hours after injection. However, 2 days after administration, the amount of drug still bound to the resistant-cell DNA was greatly reduced in contrast to that still bound to sensitive-tumor DNA. Thus a repair mechanism was invoked to explain the difference.

The tumor inhibiting properties of bifunctional alkylating agents have been attributed to the cross-linking ability partially because monofunctional alkylating agents lack anti-tumor activity (Bosen and Davis, 1969; Ochoa and Hirschberg, 1967). Nevertheless it is interesting to note that bacteria can also repair alkylated DNA caused by methyl methanesulfonate, MMS, a monofunctional alkylating agent (Reiter and Strauss, 1965), although by a different method. Furthermore, there is good evidence that a repair mechanism exists for MMS-induced DNA damage in mouse spermatozoa at certain stages of development (Cumming and Walton, 1970).



F. Summary

In general the findings of this research show that platinum compounds mimic the effects of the alkylating agents. These include giant cell formation; the persistent inhibition of cell division; selective inhibition of DNA synthesis; inhibition of *de novo* purine synthesis; enhanced DNase and DNA polymerase activities in extracts of treated cells; and the inhibition of the same enzymes when added to assays with untreated cell extracts. Recently Reslova (1970) showed that Cis-Pt(II) but not Trans-Pt(II) has the ability to induce lysogenic strains of *E. coli*. Similarly HN2 (Endo *et al.*, 1963), UV (Endo *et al.*, 1963; Witkin, 1967), Mitomycin C (Heinemann and Howard, 1964), azaserine (Price *et al.*, 1964), but not hydroxyurea (Heinemann and Howard, 1964) induced lysogenized bacteria. This makes the parallel of the platinum effects with the effects of alkylating agents even more striking.



V. A HYPOTHESIS OF THE MECHANISM OF ACTION OF TUMOR-INHIBITING PLATINUM COMPOUNDS

If the mechanism of action of tumor-inhibiting platinum compounds is indeed shown to involve a direct interaction with DNA as proposed above, how and where do platinum compounds react with DNA? Cross-linking of DNA by platinum compounds would appear, at first, to be unlikely because the 2 chlorine ligands in Cis-Pt(II) are separated by only about 3.3 Å (Milburn and Truter, 1966). Yet for the most probable reaction which would occur between 2 guanine residues at the N-7 (imidizole nitrogen) position, a minimum of about 8 Å must separate the 2 functional groups in a bifunctional alkylating agent in order to bridge the interstrand gap. HN2 has been shown to decrease the melting temperature (T_m) of DNA (Wheeler and Stephen, 1965). However, the monofunctional alkylating agent, MMS, also causes a decrease in the ${\rm T}_{\rm m}$ of the synthetic, double-stranded DNA, polyA-polyU (Ludlum et al., 1964). In addition, copper (II) causes a large T_m decrease (Eickhorn and Clark, 1965). Both the MMS and Cu(II) effects have been interpreted as evidence for a direct reaction at those sites directly involved in hydrogen bonding. Specifically, the N-6 nitrogen of adenine in the MMS example, or C-6-keto oxygen of guanine. Preliminary results by Rahn (1970) and Horacek and Drobnik (1970) indicate that Cis-Pt(II) also lowers the T_m of calf thymus DNA.

Taking both this and the size of Cis-Pt(II) into consideration, one may hypothesize that the reaction of Cis-Pt(II) occurs by



substitution at both the N-6 and/or N-7 position of 2 adjacent adenines on the same strand of DNA. Substitution at the C-6-keto oxygen and N-7 position of guanine might also occur. Thus, depending upon whether 2 or all 4 ligands are displaced, the following intrastrand crosslinks might occur: [Pt(II)(AdR)₂], [Pt(II)(AdR)(GdR)], [Pt(II)(GdR)₂], $[Pt(II)(AdR)_2(NH_3)_2], [Pt(II)(AdR)(GdR)(NH_3)_2], and [Pt(II)(GdR)_2(NH_3)_2].$ On the other hand, one may hypothesize that Trans-Pt(II) can react with only one base, just as the monofunctional alkylating agents. This hypothesis is primarily based on the reactions of Cis- and Trans-Pt(II) $(NH_3)_2Cl_2$ with the adenine analogue, 6-mercaptopurine (H₂mp): at neutral pH, Cis-Pt(II) forms a Pt(II)(Hmp)₂ complex, whereas Trans-Pt(II) forms a $[Trans-Pt(II)(NH_3)_2(H_3mp)]^{+4}$ complex with 6 mercaptopurine (Grinberg et al., 1968). However, intrastrand purine cross-links should not be possible with Trans-Pt(II) because the 2 chlorine ligands are separated by 4.6 Å (Milburn and Truter, 1966), whereas the bases are separated by about 3.4 Å in DNA. It is further speculated that substitution or reaction with 2 bases is required in order to create a sufficiently rigid structure to sterically interfere with the replication process. This is indicated by the lack of anti-tumor activity and lack of DNA synthesisinhibition by monofunctional alkylating agents as MMS (Wheeler, 1962). The effects of such platinum-coordinated, purine dimers would be expected to be similar to the effects of UV induced pyrimidine dimers (Setlow et al., 1963), depending on the stability of the platinum bonding to the bases.

In addition to the effects on T_m , this hypothesis of the mechanism of action is also consistent with the following known biological effects of the platinum compounds. First, it predicts that the Transplatinum compounds would be ineffective, or far less effective



inhibitors of DNA synthesis and therefore less effective tumor inhibitors. Second, in experiments on ϕX 174, a bacteriophage having single stranded DNA, and therefore in which no interstrand crosslinking can occur, monofunctional alkylating agents have been found to be just as efficient as bifunctional agents in the inactivation of this virus. However, with bacteriophage containing double-stranded DNA, bifunctional alkylating agents were more effective than their monofunctional counterparts (Loveless, 1966). Recent results of Drobnik et al. (1970) show that Cis-Pt(II) inactivates both single and double stranded DNA containing bacteriophage with high and equal efficiency. Hence this is evidence against an interstrand cross-linkage mechanism but the intrastrand reaction mechanism proposed above is consistent with this finding. Moreover, if the reaction would occur predominantly with 2 adjacent adenines, one would predict a greater inhibition of thymidine incorporation into DNA than any of the other deoxynucleosides, which is in agreement with the results reported here.

Due to the similarity to UV induced pyrimidine dimers, a repair mechanism that is similar if not the same as that for UV dimers would be expected to exist for platinum-coordinated purine dimers. This prediction agrees well with the interpretation of the experiments reported here on the activation of the DNases and DNA polymerase. Furthermore, an activation of a repair mechanism stimulated by platinum coordinated DNA lesions could very well lead to the excision or the activation of the virus genome while repairing the DNA of the viral genome. Hence because HN2, Mitomycin C, and UV also cause DNA lesions and stimulate repair processes, a generalized theory of prophage induction could be developed on this basis.



From the type of DNA damage proposed above, one would predict absence of damage caused by interstrand cross-links. Dominant lethal mutations, which are caused by interstrand cross-links, in fact, do not occur in Cis-Pt(II)(NH₃)₂Cl₂ treated Drosophila (Wilson, 1970). However, genetic damage due to effects on individual bases, as point mutations, would be expected to occur.

Finally, the proposed mechanism of the platinum coordination in between 2 purines with Cis-Pt(II)(NH₃)₂Cl₂ is equally applicable to Cis-Pt(IV)(NH₃)₂Cl₄ because the axis between the extra 2 chlorines would also lie in between the 2 coordinated purines. On the other hand, this hypothesis would predict that $Pt(II)(CH_2)_2(NH_2)_2Cl_2$ and platinum compounds with more bulky bridges would not create a bipurine purine coordination complex as easily due possibly to steric hindrance of the ethylenediamine bridge with the bases. Once more, the data on the tumor-inhibiting and DNA synthesis-inhibiting properties of these platinum compounds confirm these predictions.

As far as the author knows, this hypothesis of the mechanism of action is consistent with all of the presently known effects of the tumor inhibiting platinum compounds *in vivo* and *in vitro*.



VI. EPILOGUE

Seven years ago Furst (1963) made the following predictions in discussing the design of new anti-cancer compounds:

Compounds without chelating groups, active against at least one experimental tumor should be modified, keeping the following spacing in mind: between two atoms of carbon, or between one atom of carbon and one of N, O, S, P, there should be the following functional groups: NH₂, =NH, C=N, C=O, C-OH, C=S, C-SH, N-OH. These will give rise to representative combinations like those in CX [X=halides]. Bifunctional molecules may be better than monofunctional ones. The groups should be close enough spatially to complex the same metal: N-C-C-N, N-C-C-S, O-C-C-O, S=C-C-O, S=C-C-N, O=C-C-N, etc.

A few random examples may be suggested. ... Perhaps platinum or palladium derivatives should be made. Since thioethers have a strong tendency to unite with these metals, sulfur mustards [HS2] and chelates may prove useful. A reaction between K_2PtCl_4 with sulfur mustard would yield three isomers [two of which are]: cis-Pt(II)(HS2)Cl₂ and trans-Pt(II)(HS2)₂Cl₂. (Furst, 1963)

It is most amusing that although Furst first predicted the antitumor activity of platinum compounds, the structurally simpler Cis-Pt(II)(NH₃)₂Cl₂ behaves like, and appears to be at least as effective as other bifunctional alkylating agents.



BIBLIOGRAPHY



BIBLIOGRAPHY

- Adler, H. I., and A. J. Hardigree (1964). Cell elongation in strains of Escherichia coli. J. Bacteriol. 87, 223-226.
- Adler, H. I., and A. J. Hardigree (1965a). Growth and division of filamentous forms of Escherichia coli. J. Bacteriol. 90, 223-226.
- Adler, H. I., and A. J. Hardigree (1965b). Postirridiation, growth, division and recovery in bacteria. Rad. Research 25, 92-102.
- Bach, M. K., and W. E. Magee (1962). Interrelationship between the synthesis of host- and vaccina-DNA. Federation Proc. 21, 547.
- Bollum, F. J. (1966). Filter paper disc techniques for assaying radioactive macromolecules. In: Procedures in Nucleic Acid Research, G. L. Cantone and D. R. Davis, Eds., Harper and Row: New York.
- Bosen, E., and W. Davis (1969). Cytotoxic Drugs in the Treatment of Cancer. Edward Arnold Publishers, Ltd.: London.
- Brewer, H. B., J. P. Comstock, and L. Aronow (1961). Effects of nitrogen mustard on protein and nucleic acid synthesis in mouse fibroblast cells grown *in vitro*. *Biochem Pharmacal* 8, 281-287.
- Ceriotti, G. (1952). A microchemical determination of deoxyribonucleic acid. J. Biol. Chem. 198, 297-303.
- Cole, W. H. (1970). Chemotherapy of Cancer. Lea and Febiger: Philadelphia.
- Connors, T. A. (1969). Anti-cancer agents. Their detection by screening tests and their mechanism of action. In: Scientific of Cancer Chemotherapy, Recent Results in Cancer Research. 21. G. Mathé, ed.) Springer-Verlag New York Inc.: New York, 1-17.
- Connors, T. A., and J. A. Double (1970). Alkylation of the DNA of sensitive and resistant tumours by a nitrogen mustard derivative. Int. J. Cancer 5, 375-383.
- Crathorn, A. R., and J. J. Roberts (1966). Mechanism of the cytotoxic action of alkylating agents in mammalian cells and evidence for the removal of alkylated groups from deoxyribonucleic acid. *Nature*, 211, 150-153.

·

- Creighton, A. M., and G. D. Birnie (1970). Biochemical studies on the growth-inhibitory bisdioxpiperezines. I. Effects on DNA, RNA, and protein synthesis in mouse-embryo fibroblasts. Int. J. Cancer 5, 47-54.
- Cumming, R. B., and M. F. Walton (1970). Fate and metabolism of some alkylating agents in the mouse. I. Ethyl methanesulfonate and methyl methanesulfonate at sublethal dose in hybrid males. *Mutation Res.* (in press).

Drobnek, J. (1970). Personal communication.

- Drobnik, J., A. Krekulova, and A. Kubelkov (1970). Inactivation of bacteriophages with Cis-dichlorodiamine-Platinum II. (manuscript in preparation)
- Eichorn, G. L., and P. Clark (1965). Interactions of metal ions with polynucleotides and related compounds. V. The unwinding and rewinding of DNA strands under the influence of copper (II) ions. *Proceedings Nat. Acad. of Sciences* 53, 586-593.
- Endo, H., M. Ishizawa, T. Karmiya and S. Sonoda (1963). Relation between tumoricidal and prophage-inducing action. *Nature 198*, 258-260.
- Evans, A. E. (1961). Mitomycin C. Cancer Chemother. Rept. 14, 1.
- Furst, A. (1963). Chemistry of Chelation in Cancer. Charles C. Thomas Publishers: Springfield, Ill.
- Geiduschek, E. B., and A. Daniels (1965). A simple assay for DNA endonucleases. Anal. Biochem. 11, 133-137.
- Goldstein, N. V., and R. J. Putman (1964). Experimental chemotherapy studies. VII. The effect of alkylation on the *in vitro* thymidine-incorporating system of Lettré-Ehrlich cells. *Cancer Res.* 24, 1363-1367.
- Grindberg, A. A., Yu. S. Varshavskii, M. I. Gel'fman, N. V. Kiselava and D. B. Smolenskaya (1968). Complexes of platinum (II) and palladium (II) with 6-mercaptopurine. Zh. Neorg. Khim. 13(3), 803-813 [c.a. 68: 118933 P].
- Grula, M. M., and E. A. Grula (1962). Reversal of mitomycin-C induced growth and division inhibition in a species of Erwinia. *Nature 195*, 1126-1127.

Haddow, A. (1970). Personal communication.

Harder, H. C., and B. Rosenberg (1969). Inhibition of DNA polymerase in vitro and DNA synthesis in vivo in human amnionic AV₃ cells treated with tumor inhibiting platinum compounds. Third International Biophysics Congress Abstracts, Cambridge, Mass., Aug. 29 to Sept. 3, p. 31.



- Harder, H. C., and B. K. Zimmerman (1968). Elevated deoxyribonuclease levels in platinum induced, filamentous *Escherichia coli*. Unpublished results.
- Harold, F. M., and Z. Z. Ziporin (1958). The relationship between the synthesis of DNA and protein in *Escherichia coli* treated with sulfur mustard. *Biochim.*, *Biophys. Acta* 28, 492-503.
- Hartman, S. C., B. Levenberg, and J. M. Buchanan (1955). Involvement of ATP, 5-Phosphoribosyl-pyrophosphate and L-serine in the enzymatic formation of glycinamide ribotide intermediates in inosinic acid biosynthesis. J. Am. Chem. Soc. 77, 501-503.
- Heinemann, B., and A. J. Howard (1964). Induction of lambda bacteriophage in E. coli as a screening test for potential antitumor agents. Applied Microbiol. 12, 234-239.
- Hirshout, Y., G. H. Weiss, and S. Perry (1969). The use of long term Human leukocyte cell cultures as models for the study of antileukemic agents. *Cancer Res.* 29, 1732-1740.
- Horacek, P., and J. Drobnik (1970). Personal communication.
- Howle, J. A., and G. R. Gale (1970). Cis Dichlorodiammine Platinum II: Persistent and selective inhibition of Deoxyribonucleic acid synthesis in vivo. Biochim. Pharm. (in press)
- Johnson, J. C., M. Debacker, and J. A. Boezi (1971). DNA-dependent RNA polymerase of *Pseudomonas putida*. J. Biol. Chem. (in press)
- Kauffman, G. B. (1963). Inorganic Synthesis, Vol. 7, (J. Kleinberg, ed.) McGraw-Hill Book Co., Inc.: New York.
- Kim, J. H., A. S. Gelbard, and A. G. Berez (1967). Action of Hydroxyurea on the nucleic acid metabolism and viability of HeLa cells. Cancer Res. 27, 1301-1305.
- Knock, F. E. (1967). Anticancer Agents, Charles C. Thomas Publishers: New York.
- Kociba, R. J., and S. D. Sleight, and B. Rosenberg (1970). Inhibition of Dunning ascitic leukemia and Walker 256 carcinosarcoma with Cis-platinum (II) diammino dichloride. Cancer Chem. Reports. (in press)
- Krakoff, I. H., and D. A. Karnofsky (1958). Inhibition of uric acid biosynthesis in binds by O-diazoacetyl-L serine (azaserine) and 6-diazo-5-oxo-L-morleucine (DON). Am. J. Physiol. 195, 244-250.
- Krakoff, I. H., N. C. Brown, and P. Reichard (1968). Inhibition of ribonucleoside diphosphate reductase by hydroxyurea. Cancer Res. 28, 1559-1565.

т. т.

-

- Lawley, P. D., and P. Brooks (1965). Molecular mechanism of the cytotoxic action of difunctional alkylating agents and of resistance to this action. *Nature 206*, 480-483.
- Lededinskii, V. V., and R. K. Korabel'nik (1947). Invest. sektova plating i drug glagovod metal., Inst. Abshshei i Neorg. Khim. Akad. Nauk. U.S.S.R. 20, 95 [c.a. 44: 5257g, 1950].
- Lehman, I. R., and C. C. Richardson (1964). The deoxyribonucleases of *Escherichia coli*. IV. An exonuclease activity present in purified preparations of deoxyribonucleic acid polymerase. *J. Biol. Chem.* 239, 233-241.
- Levis, A. G., L. Spanio, and A. De Nadai (1963). Radiometric effects of a nitrogen mustard on survival, growth, protein, and nucleic acid synthesis of mammalian cells *in vitro*. *Exp. Cell Res. 31*, 19-30.
- Loveless, A. (1966). Genetic and Allied Effects of Alkylating Agents. Butterworth, England.
- Lowrey, O. H., N. J. Rosenbrough, A. L. Farr, R. J. Randall (1951). Protein measurement with the Folin Phenol Reagent. J. Biol. Chem. 193, 265-275.
- Ludlum, D. B., R. C. Warner and A. J. Wahba (1964). Alkylation of synthetic polynucleotides. *Science* 145, 397-399.
- Magee, W. E., and O. V. Miller (1962). Dissociation of the synthesis of host and viral deoxyribonucleic acid. Biochim., Biophys. Acta 55,818-826.
- Maxwell, R. E., and V. S. Nichel (1954). Filament formation in Escherichia coli induced by azaserine and other antineoplastic agents. Science 120, 270-271.
- Milburn, G. H. W., and M. R. Truter (1966). The crystal structure of *Cis* and *Trans*-Dichlorodiammineplatinum II. J. Chem. Soc. (A), 1609-1616.
- Ochoa, M., Jr., and E. Hirschberg (1967). Alkylating Agents. In: Experimental Chemotherapy 5 (R. J. Schneitzer and F. Hawkings, eds.), Academic Press: New York, 1-132.
- Pfeifer, S. E., and L. J. Tolmack (1967). Inhibition of DNA synthesis by hydroxyurea. *Cancer Research* 27, 124-129.
- Price, K. E., R. E. Buck, and J. Lein (1964). System for detecting inducers of lysogenized *E. coli* W 1709 (λ) and its applicability for antineoplastic antibiotics. *Applied Microbiol.* 12, 428-435.
- Pricer, W. E., Jr., and A. Weissbach (1964). Effect of lysogenic induction with mitomycin C on DNA and RNA polymerase of Escherichia coli K 12 λ. Biochem. Biophys. Res. Commun. 14, 91-95.

Rahn, R. (1970). Personal communication.



Reich, E., A. J. Shadkin, and E. L. Tatum (1961). Bacteriocidal action of Mitomycin C. *Biochim.*, *Biophys. Acta* 53, 132-149.

- Reid, B. D., and I. G. Walker (1969). The response of mammalian cells to alkylating agents. II. On the mechanism of the removal of sulfur-mustard-induced cross-links. *Biochim.*, *Biophys. Acta 179*, 179-188.
- Reiter, H., and B. Strauss (1965). Repair of damage induced by a monofunctional alkylating agent in a Transformable, Ultravioletsensitive strain of *Bacillus subtilis*. J. Mol. Biol. 14, 179-194.
- Renshal, E., and A. J. Thomson (1967). Tracer studies to locate the site of platinum ions within filamentous and inhibited cells of *Escherichia coli*. J. Bacterial. 94, 1915-1918.
- Reslova, S. (1970). Induction of lysogenic strains of *Escherichia* coli by Cis-platinum(II) diammino dichloride. (submitted for publication)
- Roberts, J. J., A. R. Crathron, T. P. Brent (1968). Repair of alkylated DNA in mammalian cells. *Nature* 218, 970-972.
- Rosenberg, B., L. Van Camp, and T. Krigas (1965). Inhibition of cell division in *Escherichia coli* by electrolysis products from a platinum electrode. *Nature 205*, 698-699.
- Rosenberg, B., L. Van Camp, E. B. Grimley, and A. J. Thomson (1967a). Inhibition of growth or cell division in *Escherichia coli* by different ionic species of Platinum (IV) complexes. J. Biol. Chem. 242, 1347-1352.
- Rosenberg, B., E. Renshaw, L. Van Camp, J. Hartwick, and J. Drobnik (1967b). Platinum-induced filamentous growth in Escherichia coli. J. Bacteriol. 93, 716-721.
- Rosenberg, B., L. Van Camp, J. E. Trosko, and V. H. Mansour (1969). Platinum compounds: A new class of potent antitumor agents. *Nature* 222, 385-386.

Rosenberg, B. (1970). Personal communication.

- Rosenberg, B., and L. Van Camp (1970). The successful regression of large solid Sarcoma 180 tumors by platinum compounds. *Cancer Research*. (in press)
- Rosenkranz, H. S., A. J. Garro, J. A. Levy, and H. S. Carr (1966). Studies with hydroxyurea. The reversible inhibition of bacterial DNA synthesis and the effect of hydroxyurea on the bactericidal action of streptomycin. *Biochim.*, *Biophys. Acta* 114, 501-515.
- Ross, W. C. J. (1962). *Biological Alkylating Agents*. Butterworth, London and Washington, D.C.




- Setlow, R. B., P. A. Swenson, and W. L. Carrier (1963). Thymine dimers and inhibition of DNA synthesis by ultraviolet irridiation of cells. Science 142, 1464-1465.
- Setlow, R. B., and W. L. Carrier (1964). The disappearance of thymine dimers from DNA: An error-correcting mechanism. Proc. Natl. Acad. Sci. U.S. 51, 226-231.
- Shatkin, A. J., E. Reich, R. M. Franklin, and E. L. Tatum (1962). Effect of Mitomycin-C on mammalian cells in culture. *Biochim.*, *Biophys. Acta* 55, 277-289.
- Shiba, S., A. Terawaki, T. Taquchi, and J. Kawamata (1959). Selective inhibition of formation of deoxyribonucleic acid in *Escherichia* coli by Mitomycin C. Nature 183, 1056-1057.
- Skipper, H. E., L. L. Bennett, Jr., and F. M. Schabel, Jr. (1954). Mechanism of action of Azaserine. *Fed. Proc. 298*.
- Smith, S. J., and H. Busch (1961). Effects of uricil mustard on in vivo incorporation of precursors into nucleic acids of the Walker tumor. Texas Rept. Biol. Med. 22, 721-740.
- Stearns, B., K. A. Losee, and J. Berstein (1963). Hydroxyurea: A new type of potential antitumor agent. J. Med. Chem. 6, 201.
- Stock, J. A. (1966). Antitumor antibiotics. In: Experimental Chemotherapy 5, (R. J. Schnitzer and F. Hawkings, eds.), Academic Press: New York, 239-277.
- Studzinski, G. P., and L. S. Cohen (1966a). Elevation of deoxyribonuclease activities in HeLa cells treated with selective inhibitors of DNA synthesis. Biochim. Biophys. Res. Comm. 25, 313-319.
- Studzinski, G. P., and L. S. Cohen (1966b). Mitomycin C induced increases in the activities of the deoxyribonucleases of HeLa cells. Biochim. Biophys. Res. Comm. 23, 313-319.
- Sugar, D., and H. Sierakowski (1967). Mammalian nucleolytic enzymes and their localization. In: Progress in Nucleic Acid Research and Molecular Biology, Vol. 7, J. N. Davidson and W. E. Cohn, eds. Academic Press: New York, 369-427.
- Sugiura, K. (1961). Antitumor activity of Mitomycin C. Cancer Chemother. Rept. 13, 51.
- Szybalski, W., and V. N. Iyer (1964). Crosslinking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics. Fed. Proc. 23, 946-957.

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- Toth Allen, J. (1970). Distribution and histopathological effects of Cis-Platinum(II) diamminodichloride on non-tumored and tumorized (Sarcoma 180) Swiss White Mice. Ph.D. Thesis, Michigan State University.
- Wheeler, G. P. (1962). Studies related to the mechanism of action of cytotoxic alkylating agents: A review. *Cancer Res.* 22, 651-688.
- Wheeler, G. P., and J. A. Alexander (1964a). Studies with mustards. V. In vivo fixation of C¹⁴ of labeled alkylating agents by bilaterally grown sensitive and resistant tumors. Cancer Res., 24, 1331-1337.
- Wheeler, G. P., and J. A. Alexander (1964b). Studies with mustards. VI. Effects of alkylating agents upon nucleic acid synthesis in bilaterally grown sensitive and resistant tumors. Cancer Res. 24, 1338-1346.
- Wheeler, G. P., and Z. H. Stephens (1965). Studies with mustards. VII. Effects of alkylating agents *in vitro* and *in vivo* upon the thermal properties of deoxyribonucleic acids from sensitive and resistant plastocytomas. *Cancer Research* 25, 410-416.
- Wilkin, E. M. (1967). The radiation sensitivity of Escherichia coli
 B: A hypothesis relating filament formation with prophage induction. Proc. Nat. Acad. Sci. U.S. 57, 1275-1279.
- Wilson, C. (1970). Personal communication.
- Young, C. W., and S. Hodas (1964). Hydroxyurea inhibitory effect on DNA metabolism. *Science* 146, 1172-1174.
- Young, C. W., G. Schochetman, and D. A. Karnofsky (1967). Hydroxyurea induced inhibition of DNA synthesis. Studies in intact cells. *Cancer Res.* 27, 526-534.
- Zimmerman, B. K. (1966). Purification and properties of deoxyribonucleic acid polymerase from *Micrococcus lysodeikticus*. J. *Biol. Chem.* 241, 2035-2041.







