

Toxicity of Platinum (IV) Salts for Cells of Pulmonary Origin

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The acute toxicity of tetravalent platinum was studied *in vitro* by use of rabbit alveolar macrophages and human lung fibroblasts (strain WI-38). Alveolar macrophages were exposed in tissue culture for 20 hr to platinum dioxide (PtO₂) or platinum tetrachloride (PtCl₄). There was no evidence of dissolution of PtO₂ and no decrease in viable cells at concentrations as high as 500 µg/ml. PtCl₄ was soluble in the macrophage system and after a 20-hr exposure, resulted in loss of viability in 50% of the cells originally present at a concentration of 0.30mM (59 µg Pt/ml). After a 20-hr exposure, rapidly growing human lung fibroblasts were rendered nonviable by PtCl₄ at comparable concentrations. A decrease in total cellular ATP was observed at lower concentrations in macrophages and fibroblasts along with a reduction in phagocytic activity of macrophages as compared to controls. With the fibroblasts, a 50% decrease in incorporation of ¹⁴C-thymidine was observed after a 22-hr exposure to PtCl₄ at a concentration of 0.007mM; higher concentrations were required to inhibit the incorporation of ¹⁴C-uridine and ¹⁴C-leucine.

Time-course studies indicated that the inhibition of ¹⁴C-thymidine incorporation was nearly complete (90%) after 7 hr in the presence of 0.06mM PtCl₄. Under the same conditions, there was little inhibition (15%) of ¹⁴C-leucine incorporation and moderate inhibition (50%) of ¹⁴C-uridine incorporation. Higher concentrations of PtCl₄ were required to inhibit ¹⁴C-thymidine incorporation into the acid-soluble fraction than were required to inhibit incorporation into the acid-precipitable fraction. Hence, the preferential inhibition of DNA synthesis by PtCl₄ may result from an impairment of the incorporation process.

Introduction

The increasing usage of platinum and its compounds, particularly as the active component of the automotive catalytic converter, has stimulated renewed interest in the pulmonary toxicology of this metal. The principal clinical manifestation of inhalation of complex salts of platinum, mainly chloroplatinates, is an allergic disease termed platinosis. Parrot et al. (1) have suggested that the release of histamine with intense bronchospasm observed in guinea pigs exposed to sodium chloroplatinate may mimic the asthmatic response seen in platinum workers. The principal difference

between the response seen in man and that observed in the experimental animal is related to time. Exposure for 2-6 months in the refining workshop is required before the onset of asthma, eczema, or urticaria. Approximately one half or more of those individuals heavily exposed to the complex salts demonstrated such a delayed response (1). Hence, it has been proposed that salts of platinum may combine in the body with a protein to form a complex antigen or haptene (1). Other known interactions of platinum metals and their complexes in biological systems have been reviewed recently by LeRoy (2).

The present investigation was undertaken to further examine an additional aspect of platinum toxicology which has developed from studies with platinum antitumor agents. These studies have

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demonstrated a correlation between antitumor potency and the effectiveness of these compounds in inhibiting DNA synthesis in mammalian cells in culture (3). Therefore, it was of interest to examine the cytotoxic properties of relatively insoluble and soluble platinum compounds in both nondividing and rapidly dividing cells of pulmonary origin.

Materials and Methods

Cell Culture

The techniques associated with the procurement and maintenance of rabbit alveolar macrophages have been previously described (4). For the present studies, replicate cultures of macrophages were established in 25 cm² Falcon plastic flasks (4 ml at 5 × 10⁵ cells/ml) and allowed to attach in a humidified atmosphere at 96% air and 4% CO₂ at 37°C, 3 hr being allowed for attachment of cells in the stationary cultures. Unattached cells (approximately 15% of the inoculum) were then poured off and platinum compounds were added in fresh medium.

Platinum dioxide (K & K Laboratories, Inc., Plainview, N.Y.) was added directly to supplemented Medium 199 in Hanks' balanced salt solution at pH 7.2. Platinum tetrachloride (K & K Laboratories, Inc.) was added to supplemented Medium 199, adjusted to pH 7.2 with ultrapure NaOH (Alpha Inorganics, Ventron; Beverly, Mass.) and filtered through a 0.22 μm Millipore filter. An aliquot was removed for atomic absorption spectrophotometry, and appropriate dilutions were made with additional supplemented medium.

Human lung fibroblasts in passage 17 were obtained from the American Type Culture Collection, Rockville, Maryland and were maintained in 75 cm² Falcon flasks. Cultures were subcultivated twice weekly by use of 0.25% trypsin in Gibco solution A with a 1:2 split ratio. Cultures were seeded at 1.75 × 10⁵ cells/ml (4.0 ml total volume) in 25 cm² Falcon flasks and maintained in Basal Medium Eagle (BME) with Earles salts plus 10% fetal bovine serum (virus screened), 2 μmole/ml L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin-B. Growth curves were established for cells in passages 22 and 33 (see results). Experiments were performed during the period of rapid growth from 24 to 72 hr after subcultivation.

Platinum tetrachloride was added directly to BME, and the pH was adjusted to 7.2 prior to Millipore filtration as previously described for macrophage cultures.

All tissue culture media and supplements were obtained from Grand Island Biological Company, Grand Island, New York.

Metal Analysis

Analysis of actual platinum concentrations in stocks of test media was performed on a Perkin-Elmer model 306 atomic absorption spectrophotometer equipped with a model HGA-2100 graphite furnace. Nitrogen was used as the purge gas at a flow pressure of 50 psi. Automatic sample processing in the graphite furnace included drying at 100°C for 34 sec, charring at 1050°C for 30 sec, and atomization at 2700°C for 8 sec. Standards were prepared in supplemented tissue culture media from chloroplatinic acid, 1000 ppm (Alfa Inorganics, Ventron), immediately prior to sample analysis. Absorbance was linear with respect to platinum concentration at levels of 5 ppm or less. Samples were diluted so that absorbance was within the linear range observed with standards.

Viability Determinations

Viability determinations were performed as follows. The culture medium was poured off and retained separately in a culture tube. Cells were dissociated by using 0.25% trypsin in Gibco solution A. The suspended cells were recombined with the original culture medium and chilled. Appropriate dilutions, usually 4-fold, were made by using cold 0.85% saline to yield a suspension of no more than 2 × 10⁵ cells/ml. Trypan blue, freshly diluted with 0.85% saline to a final concentration of 0.01%, was added to an equal volume of cell suspension for determination of cell viability. Simultaneous determinations of cell viability and cell numbers per milliliter of cell suspension were performed by using the Cytograf Model 6301 (Biophysics Systems, Mohapac, N.Y.). Viability was expressed directly as a percentage. The viability of cells from control cultures was routinely 95% or greater. Cell numbers were expressed as a percentage of the numbers of cells in control cultures. Viability determinations were multiplied by total cell numbers as a fraction of control cell numbers to yield the viability index, or net number of viable cells as a per cent of control.

$$\text{Viability index} = \text{Viability (\%)} \times \frac{\text{No. cells exptl}}{\text{No. cells control}}$$

Measurement of Phagocytic Activity

Phagocytic activity following a 19-hr exposure to platinum tetrachloride was measured by addition of 1.1 μm polystyrene latex particles (Dow Diagnostics, Indianapolis, Indiana) to alveolar macrophages cultured in Lab-Tek (Miles Laboratories, Inc., Naperville, Ill.) four-chamber microslides (approximately 25 particles/cell in 1 ml of supplemented medium). Preparation and maintenance conditions were as previously described for flask cultures. One hour after the addition of latex particles, the slides were drained, air-dried, and exposed for 3 min to concentrated Wright stain. The slides were then exposed for an additional 5–6 min with a 1:1 aqueous dilution of Wright's stain. After air drying, the slides were placed in xylene for 1 hr to dissolve extracellular particles according to the procedure of Gardner et al. (5). Following an additional drying step, the slides were mounted in permount. Phagocytic activity was determined under oil immersion by scoring a minimum of 200 cells. Each cell which contained at least one particle was considered phagocytically active. Typically, 80–90% of the cells in control cultures ingested one or more particles.

Adenosine Triphosphate Determination

ATP was determined according to a procedure supplied with the DuPont model 760 Luminescence Biometer. Dimethyl sulfoxide (0.9 ml) was used to extract ATP from a 0.1-ml aliquot of trypsinized cell suspension containing $0.3\text{--}0.4 \times 10^5$ cells. After 2 min at room temperature, 5.0 ml of cold 0.01M morpholinopropane sulfonic acid (MOPS) at pH 7.4 was added to buffer the extracted sample. The tube containing the buffered sample was then placed in an ice bath. Aliquots of 10 μl were injected into the luminescence meter's reaction cuvette containing 0.7mM luciferin (crystalline), 100 units luciferase (purified and stabilized),* and 0.01M magnesium sulfate in a total volume of 100 μl of 0.01M MOPS buffer, pH 7.4 at 25°C. Light emitted from the reaction cuvette was measured photometrically in the luminescence meter and was proportional to the ATP concentration of the sample.

*Unit (1) luciferase = $\frac{\text{response to } 1.64 \mu\text{mole ATP}}{\text{response to } 20 \mu\text{Ci } ^{14}\text{C calibrated light source}}$

Protein Determinations

For determination of total culture protein, cells washed twice with 0.85% saline were lysed in 1.0% sodium deoxycholate (Schwarz-Mann, Orangeburg, New York) and 0.1 ml aliquots were assayed according to the method of Lowry et al. (6) by using a bovine serum albumin standard (Nutritional Biochemicals Corp., Columbus, Ohio).

Deoxyribonucleic Acid Assay

DNA was determined on a 0.2 ml aliquot of 1% sodium deoxycholate lysate according to a modification of the procedure of Kissane and Robins (7). In brief, the details of this modification are as follows. The sample is precipitated in a 10 \times 75 mm disposable glass tube by addition of 0.2 ml of 10% trichloroacetic acid without mixing. The precipitate is sedimented by centrifugation, and the supernatant fluid is carefully removed by aspiration. A volume of 0.2 ml of 0.1M potassium acetate in absolute ethanol is added to the precipitate and allowed to stand without mixing for 5–10 min. The precipitate is again centrifuged and the potassium acetate solution is carefully removed by aspiration. A 20- μl portion of 2.0M 3,5-diaminobenzoic acid in 4.0N HCl is added and vigorously mixed with the precipitated sample by vortexing. The tubes are stoppered and incubated in a water bath at 60°C for 30 min. After this time, the tubes are removed, the samples cooled to room temperature, and 0.75 ml of 0.6N perchloric acid is added to each sample with mixing. The tubes are centrifuged to remove floating particles and the supernatant liquid is transferred by means of a pasteur pipet to cuvettes for reading. Disposable glass cuvettes, 6 \times 50 mm (DuPont Instruments, Wilmington, Delaware), were used in the present study. A Perkin-Elmer model 204 fluorescence spectrophotometer fitted with microcuvette adaptors was employed with an excitation wave length of 415 nm and an emission wavelength of 495 nm.

Radiochemical Assays

Solutions of thymidine-2- ^{14}C , uridine-2- ^{14}C , and leucine-1- ^{14}C (New England Nuclear Corp., Boston Massachusetts), each having a specific radioactivity of 54–57 mCi/mmmole, were diluted in supplemented BME with Earle's salts and added at 0.5 $\mu\text{Ci}/0.1$ ml to a 4.0 ml volume of medium in 25 cm 2 Falcon flasks. In all incorporation studies, radiochemicals were present for 2 hr only. After

this time, cultures were placed on ice and washed twice with 4 ml of ice-cold 0.85% sodium chloride. Cultures were thoroughly drained, and 2.0 ml of ice-cold 1.0% sodium deoxycholate was added to lyse the cells. Aliquots (0.2 ml) of the lysate were removed and transferred to 15 ml of Aquasol (New England Nuclear Corp.) for liquid scintillation counting of total cellular uptake radioactivity. The remaining lysate was stored frozen at -20°C . Upon thawing, aliquots (0.2 ml) were removed to test tubes and precipitated with 3 ml of ice-cold 1.07*N* perchloric acid (PCA). The precipitates were poured onto 2.4-cm glass fiber filters (Reeve Angel, Clinton, New Jersey) under vacuum and washed twice out of the tube onto the filter with 3 ml of cold 1.0*N* PCA. The precipitates were then washed three times with 3 ml of ice-cold 70% ethanol, and the filters were left to dry under vacuum. Filters were then removed to scintillation vials. Aquasol (15 ml) was added, and the vials were counted in a Packard model 3380 Tricarb liquid scintillation spectrometer using standard instrument settings for carbon-14. Counting efficiency was 87–91%. Counts per minute were corrected to disintegrations per minute by use of the automatic external standard ratio and validated quenched standards.

Disintegrations per minute per milliliter were divided by total protein per milliliter to correct for differences in numbers of cells per culture.

Statistics

All of the above determinations were performed in duplicate or triplicate. Since cell viability could be considered a binomial response, the arc-sine transformation was employed in the regression analysis (8). This technique helped to linearize the data when viability, as a percentage, was plotted versus the natural logarithm of the molar concentration. Other cytofunctional and biochemical measurements were considered continuously variable. For both types of data, the millimolar concentration of platinum tetrachloride that yielded a 50% response for any test parameter was obtained through inverse prediction of the simple regression line. Analysis of variance was employed to determine the significance of differences between DNA and total protein determinations in standardizing uptake and incorporation data.

Results

The washed, unsized particles of platinum dioxide were actively phagocytized by rabbit alveolar macrophages over a 20-hr exposure period by

microscopic observation but did not cause cell lysis or alter cell viability as determined by trypan blue exclusion tests. Concentrations as high as 500 $\mu\text{g}/\text{ml}$ of culture medium were without effect on the cells (Table 1).

Table 1. Effect of platinum dioxide on viability and numbers of rabbit alveolar macrophages after 20 hr.

No. of cultures	PtO ₂ $\mu\text{g}/\text{ml}$	Viability, %	Cell number, % of control	Viability index ^a
7	0	96.6 (± 1.13) ^b	100 (± 13.5) ^b	97.3 (± 13.2) ^b
2	100	96.5	120.6	116.4
2	200	95.5	87.7	83.3
4	500	95.7 (± 2.21) ^b	89.6 (± 21.4) ^b	84.9 (± 19.3) ^b

^aSee text.

^b \pm S.D. given in parentheses

Atomic absorption spectrophotometry indicated that very little platinum ($<0.2 \mu\text{g}/\text{ml}$) was present in the test medium after a 20-hr incubation at a concentration of platinum dioxide of 500 $\mu\text{g}/\text{ml}$. This determination was accomplished after particles of platinum dioxide were removed by passing the medium through a 0.22 μm porosity membrane filter. Platinum tetrachloride was relatively soluble in the test medium, so that 60–80% of the available platinum was released in soluble form (i.e., could not be removed by filtration) within 30 min to 1 hr.

Rabbit alveolar macrophages exposed to platinum tetrachloride for a period of 20 hr in three experiments exhibited a 50% reduction in cell viability at an estimated concentration of 0.40*mM* or 78 $\mu\text{g Pt}/\text{ml}$ (Fig. 1A).

Platinum tetrachloride was not markedly cytolytic for alveolar macrophages (Fig. 1B) as evidenced by the fact that cell numbers decreased only slightly over the concentration range that caused reduction in cell viability. Erratic determinations of cell numbers are believed to be due to clumping of macrophages which was noticeable at cytotoxic concentrations.

When estimates of cell viability in cultures exposed to platinum tetrachloride for 20 hr were corrected for the slight decrease in cell numbers over this period, the resultant figure was termed the viability index (cf. Materials and Methods). The curve for viability index as a function of concentration of platinum tetrachloride is shown in Figure 1C. A 50% reduction in viability index after 20 hr was observed at a platinum tetrachloride concentration of 0.30*mM* or 59 $\mu\text{g Pt}/\text{ml}$.

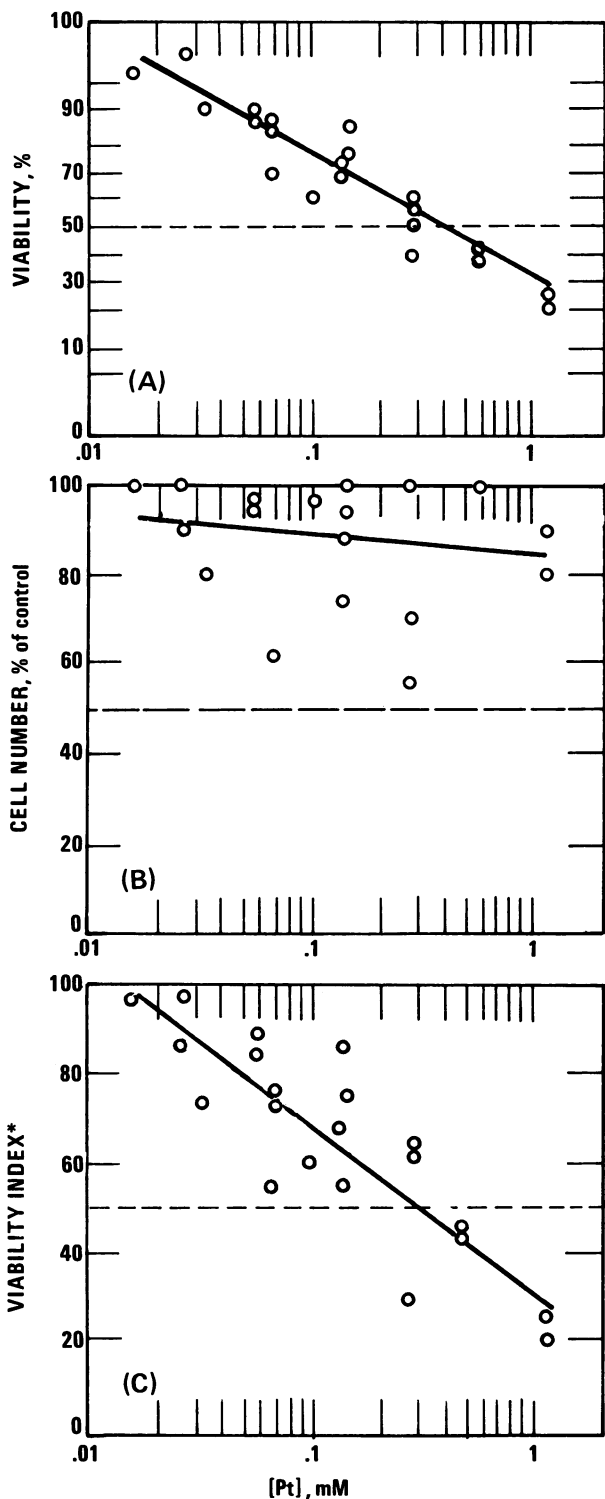


FIGURE 1. Effect of platinum tetrachloride on (A) viability, (B) cell numbers, and (C) viability index in rabbit alveolar macrophages after 20 hr. Each point represents one culture.

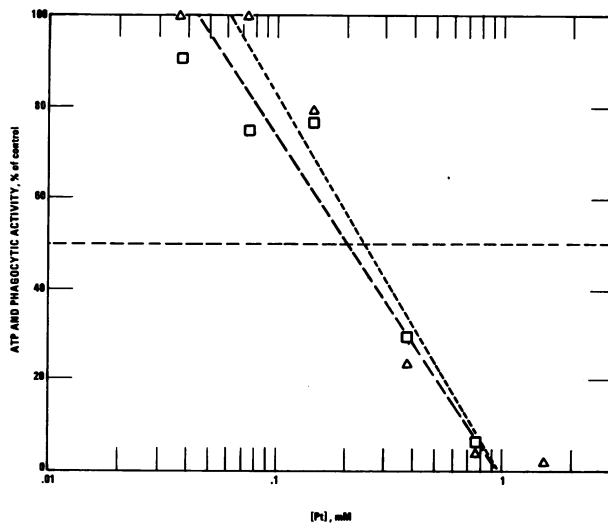


FIGURE 2. Platinum tetrachloride effect on (Δ) total ATP per 10^6 cells and (\square) phagocytic activity in rabbit alveolar macrophages after 20 hr. Each point represents the mean of two cultures.

A reduction in phagocytic activity and a decrease in total adenosine triphosphate (ATP) was observed at concentrations of platinum tetrachloride lower than those which caused loss of cell viability as illustrated in Figure 2. Concentrations of platinum tetrachloride which caused a reduction in ATP and phagocytic activity to 50% of control after 20 hr were $0.25mM$ ($48 \mu g$ Pt/ml) and $0.21mM$ ($41 \mu g$ Pt/ml), respectively.

Additional studies on platinum tetrachloride dealt with the strain WI-38 human lung fibroblast.

In the fibroblast system, the concentration which caused a reduction in cell viability to 50% after a 20-hr exposure was $0.79mM$ or $154 \mu g$ Pt/ml (Fig. 3A). After a 20-hr exposure the compound was determined to be lytic or, more likely, growth inhibitory for human lung fibroblasts. The effective concentration which resulted in a 50% reduction in cell number after 20 hr was $1.03mM$ or $201 \mu g$ Pt/ml (Fig. 3B). The net effect of loss in viability and lysis or growth inhibition in strain WI-38 human lung fibroblasts (Fig. 3C) was to reduce the net number of viable cells or viability index after 20 hr to 50% of control levels at a platinum concentration of $0.42mM$ ($82 \mu g$ Pt/ml). This concentration compares favorably with the figure of $0.30mM$ ($59 \mu g$ Pt/ml) which resulted in a viability index of 50% with alveolar macrophages (Fig. 1C).

As in the case of the macrophages, total ATP per million cells (Fig. 4) was depressed at a lower concentration after 20 hr than that required to alter cell viability. The effective concentration which

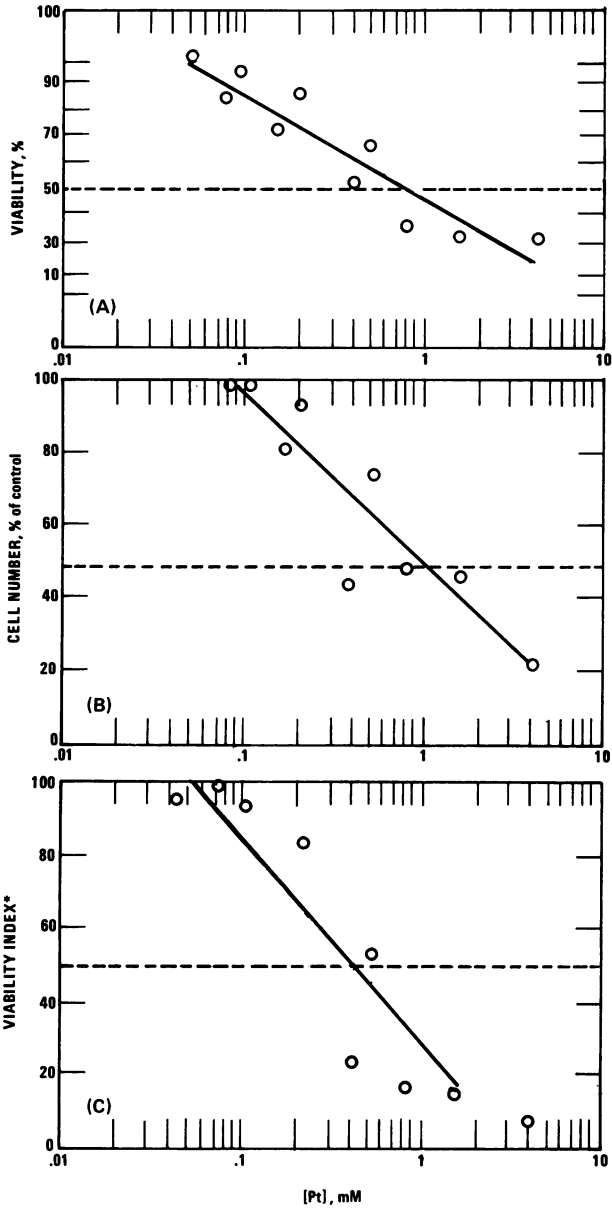


FIGURE 3. Influence of platinum tetrachloride on (A) viability, (B) cell numbers, and (C) viability index in strain WI-38 human lung fibroblasts after 20 hr. Each point represents one culture.

caused a reduction in total ATP to 50% of control was $0.11mM$ or $22.5 \mu g$ Pt/ml.

Subsequent studies were performed to determine the effect of platinum tetrachloride on the uptake and incorporation of ^{14}C -labeled precursors of DNA, RNA, and protein synthesis. Rapidly growing cultures (Fig. 5) were exposed for up to 22 hr to platinum tetrachloride at concentrations ranging from $0.0007mM$ ($0.14 \mu g$ Pt/ml) to $0.14mM$ ($27 \mu g$

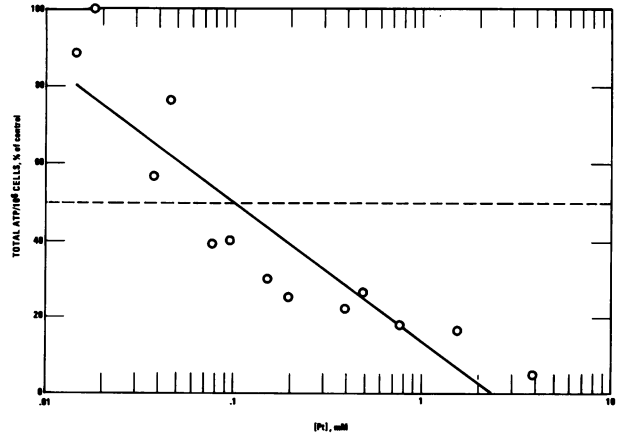


FIGURE 4. Total ATP per 10^6 cell in strain WI-38 human lung fibroblasts exposed to platinum tetrachloride for 20 hr. Each point represents one culture.

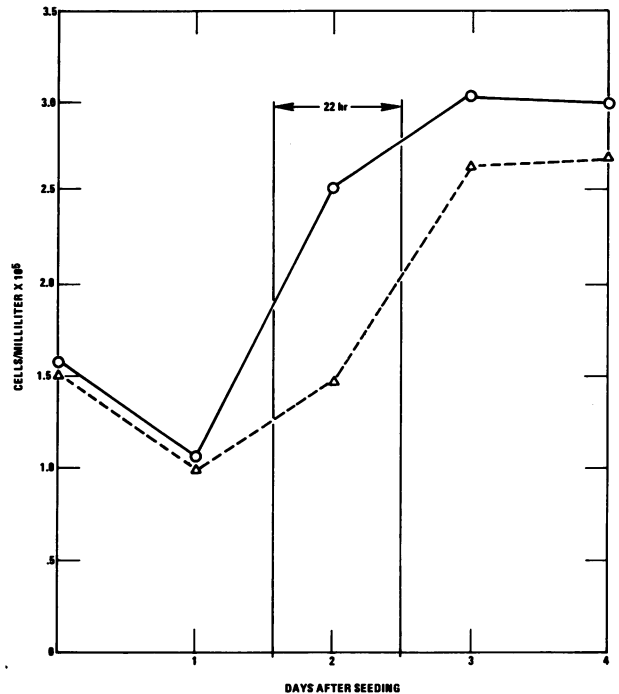


FIGURE 5. Growth curves for strain WI-38 human lung fibroblasts (o) in passage 22 and (Δ) in passage 33. The seeding concentration was 1.75×10^5 cells/ml. Note 22-hr period within rapid growth phase in which experiments described were performed. Each point represents the mean of two cultures.

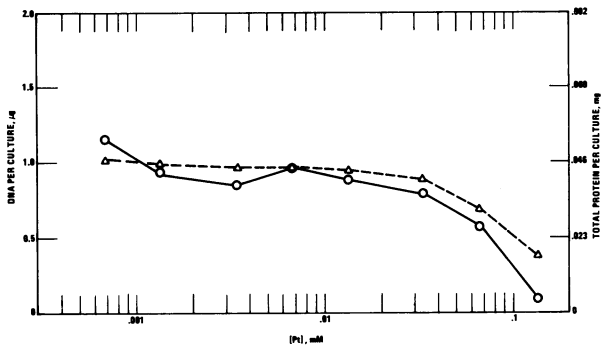


FIGURE 6. Effect of platinum tetrachloride on (o) DNA and (Δ) protein in cultures of strain WI-38 human lung fibroblasts after 22 hr. Each point represents the mean of two to six cultures.

Pt/ml). Thymidine-2- ^{14}C , uridine-2- ^{14}C , or leucine-1- ^{14}C was then added, to remain for only 2 hr as described under Materials and Methods.

The total uptake of the radioactive precursors into cells, their incorporation into tissue macromolecules, and their incorporation into the perchloric acid (PCA) soluble fraction, was standardized on the basis of total culture protein since, statistically, after adjusting for scaling factors, there was no difference in DNA or total protein content over the concentration range studied (Fig. 6). Platinum tetrachloride did not alter the ratio of DNA to total protein in human lung fibroblasts except at concentrations sufficiently high to reduce cell viability ($> 0.07mM$).

As shown in Figure 7, the uptake and the incorporation of radiolabeled thymidine, uridine, and

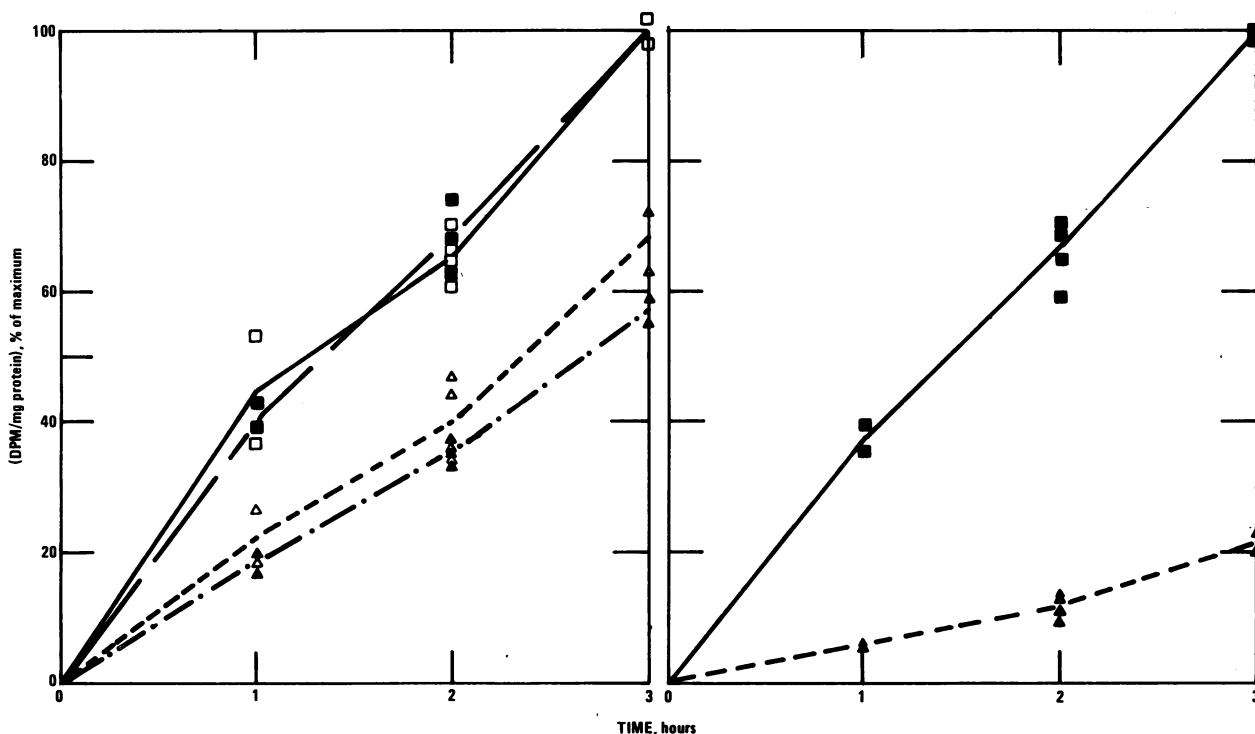


FIGURE 7. Plots of (left) total cellular uptake of (\square) thymidine-2- ^{14}C , and (\blacksquare) leucine-1- ^{14}C and (Δ) incorporation of thymidine-2- ^{14}C and (\blacktriangle) leucine-1- ^{14}C into PCA-precipitate materials by strain WI 38 human lung fibroblasts as a function of time; (right) total cellular uptake of (\blacksquare) uridine-2- ^{14}C and (\blacktriangle) incorporation of uridine-2- ^{14}C into PCA-precipitable materials as a function of time. Each point represents one culture.

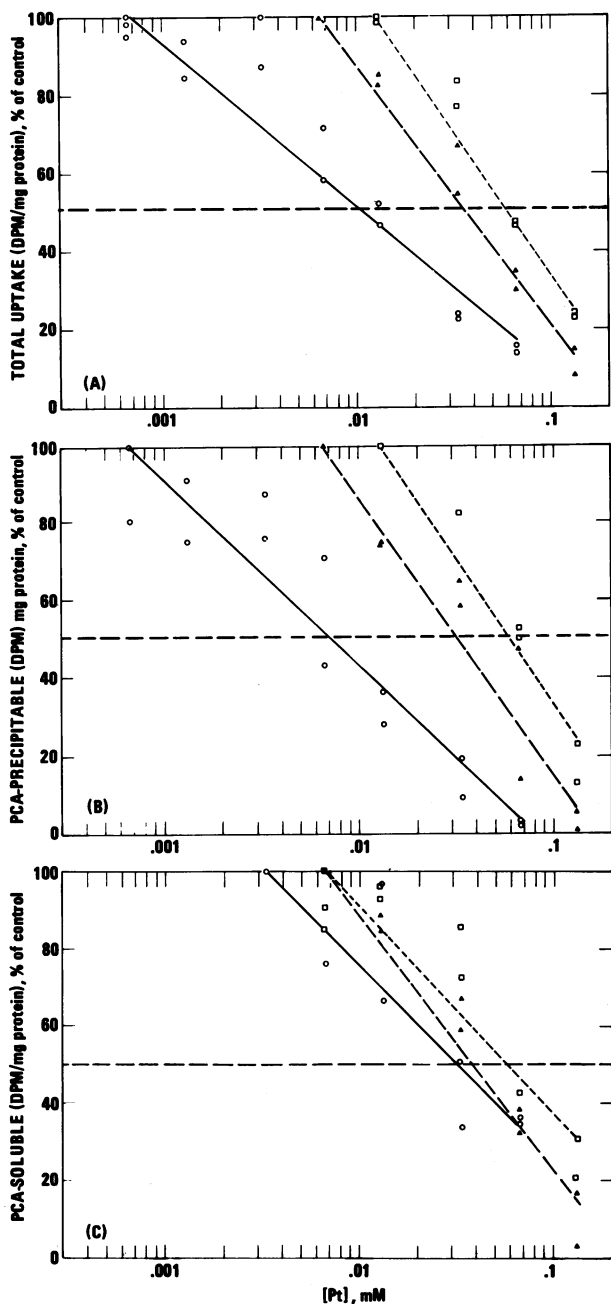


FIGURE 8. Inhibitory effect of platinum tetrachloride on (A) total uptake, (B) incorporation into PCA-precipitable materials, and (C) incorporation into PCA-soluble fractions of (o) thymidine- $2\text{-}^{14}\text{C}$, (Δ) uridine- $2\text{-}^{14}\text{C}$, and (\square) leucine- $1\text{-}^{14}\text{C}$ in strain WI-38 human lung fibroblasts after 22 hr. Radiolabeled precursors were present only during the last 2 hr of the 22-hr total exposure period. Each point represents one culture.

leucine in control cultures was linear with respect to time for at least 3 hr. In control cultures, $1.0N$ PCA-precipitable radioactivity accounted for approximately 65% of thymidine uptake, 55% of leucine uptake, and 20% of uridine uptake.

The inhibition of thymidine uptake was observed to occur at significantly lower concentrations of platinum tetrachloride than required to inhibit uridine or leucine uptake (Fig. 8A). A 50% inhibition of thymidine uptake resulted from a 20-hr exposure to platinum tetrachloride at a concentration of $0.012mM$ or $2.3 \mu\text{g Pt/ml}$. Higher concentrations by 3.5 and 5-fold were required to inhibit uridine or leucine uptake, respectively.

The incorporation of radiolabeled thymidine, uridine, and leucine into PCA-precipitable macromolecules followed essentially the same pattern as observed for total cellular uptake (Fig. 8B). A 50% inhibition of incorporation of each radiolabeled precursor occurred at slightly, but not significantly lower, platinum concentrations than required to inhibit total cellular uptake (Fig. 8B). For example, the incorporation of thymidine into PCA-precipitable material was 50% inhibited at $0.007mM$ or $1.4 \mu\text{g Pt/ml}$.

The incorporation of thymidine, uridine, and leucine into PCA-soluble material was determined by subtraction of the PCA-precipitable radioactivity from the total cellular uptake radioactivity, both corrected to disintegrations per minute per mg protein. The curves thus obtained (Fig. 8C) were quite similar, in that the curve for thymidine incorporation into PCA-soluble material had shifted to the right. The result was that the effective platinum concentration ($0.033mM$) for 50% inhibition of thymidine incorporation into PCA-soluble material was 4.7-fold higher than that required to inhibit incorporation into PCA-precipitable material.

Time-course studies (Fig. 9A) with exposures of 3 and 7 hr indicated that 0.03 and $0.05mM$ concentrations of platinum tetrachloride (5.9 and $11.8 \mu\text{g Pt/ml}$, respectively) inhibited incorporation into PCA-precipitable material almost completely (85–90%) within 7 hr. At these concentrations, uridine incorporation was 40–50% inhibited after 7 hr; leucine incorporation was only 15–25% inhibited after 7 hr. The concentration dependence of the inhibitory effect of platinum tetrachloride on the incorporation process is illustrated in Figure 9B. It can be seen that higher concentrations of platinum tetrachloride are required to inhibit leucine incorporation than are required to inhibit uridine incorporation. Furthermore, 10-fold higher

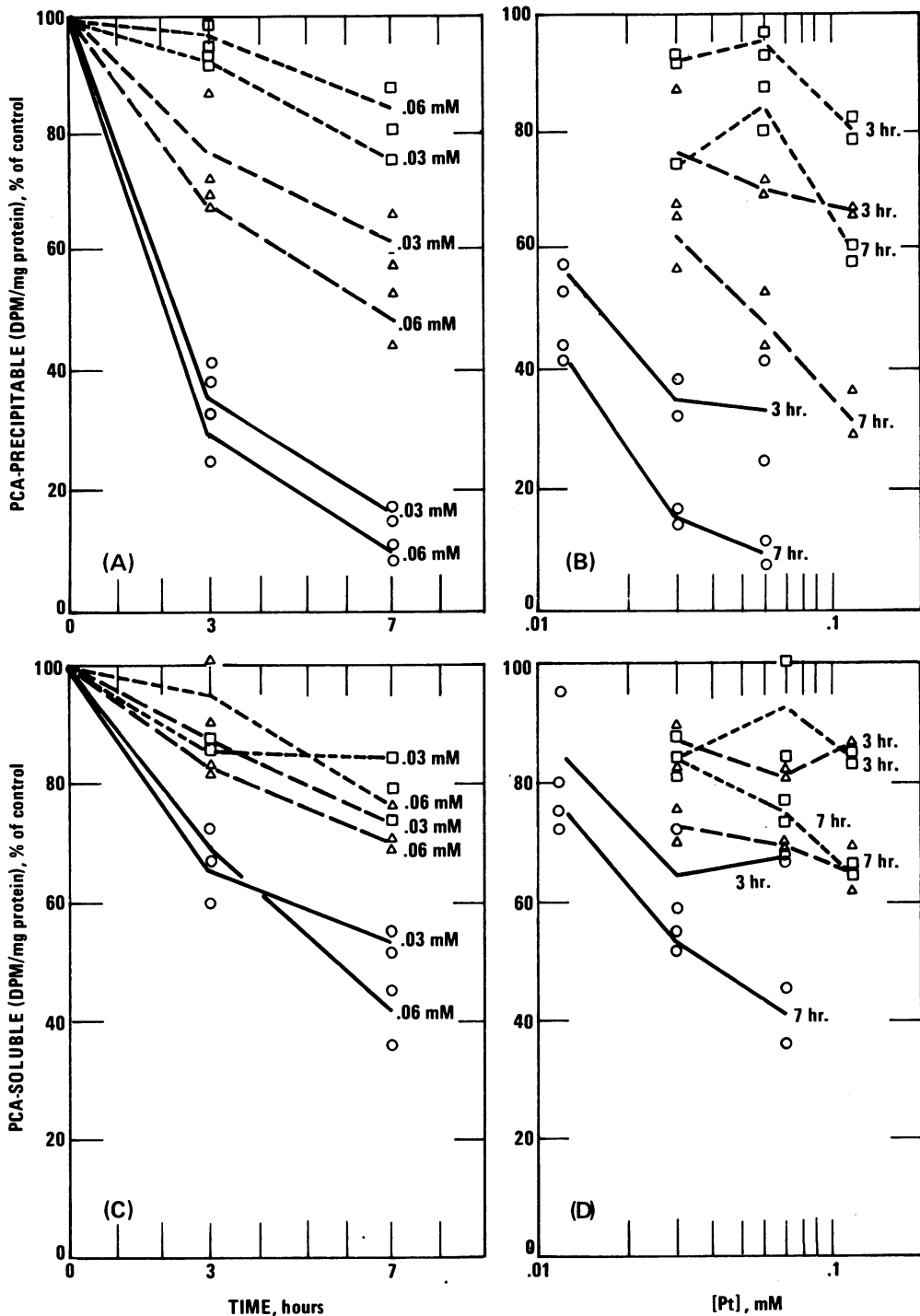


FIGURE 9. Plots of (A) time course and (B) concentration dependence of platinum tetrachloride inhibition of (○) thymidine-2-¹⁴C, (Δ) uridine-2-¹⁴C, and (□) leucine-1-¹⁴C incorporation into PCA-precipitable materials; (C) time course and (D) concentration dependence of platinum tetrachloride inhibition of the same precursors into PCA-soluble fractions in strain WI-38 human lung fibroblasts. Radiolabeled precursors were present only during the last 2 hr prior to indicated time of harvest. Each point represents one culture.

concentrations of platinum tetrachloride were required to inhibit uridine or leucine incorporation than were required to inhibit thymidine incorporation after 3 or 7 hr.

The incorporation of ^{14}C -uridine and especially ^{14}C -thymidine into the PCA-soluble fractions (Fig. 9C) was not as rapidly inhibited for given concentrations of platinum tetrachloride (0.03 and 0.06mM) as was the inhibition of incorporation of the same precursors into PCA-precipitable materials (Fig. 9A). A comparison of Figures 9B and 9D conveys essentially the same information with respect to concentration; i.e., that incorporation of uridine and, to a greater extent, thymidine into PCA-soluble materials was less inhibited by exposure to given concentrations of platinum tetrachloride than was incorporation of these same precursors into PCA-precipitable materials.

The final experiment in this series involved the removal of platinum tetrachloride after a 4-hr exposure by washing the cultures three times with supplemented medium (Fig. 10). The recovery of the processes involving incorporation of thymidine, uridine, and leucine was then determined by use of 2-hr pulse labels followed by the harvesting of replicate cultures 3, 7, and 22 hr after removal of platinum. The results (for one experiment only) indicated at least partial reversibility of all the inhibitory effects of platinum tetrachloride previously studied except at concentrations sufficiently high to alter cell viability.

Discussion

This investigation demonstrates that rabbit alveolar macrophages and human lung fibroblasts are susceptible to the toxicity of soluble platinum tetrachloride in moderately high concentrations. Gross manifestations of the cytotoxic response include changes in cellular morphology with loss in viability by dye exclusion tests (4). In the macrophage system cadmium or mercurous chlorides cause similar effects on cell viability at one-fourth to one-seventh the concentration required for platinum tetrachloride (9). However, platinum tetrachloride is more toxic than nickelous or zinc chlorides in this system by factors of 6 and 10, respectively (9).

Reductions in total ATP levels are observed at lower concentrations of platinum tetrachloride than required to alter cell viability. With the rabbit alveolar macrophage, phagocytic activity, a critical cell function, is inhibited at platinum concentrations which reduce total ATP levels. Such a relationship might be expected since phagocytosis is an

energy-dependent process. Preliminary results indicate that a number of metallic chlorides including those of Pt^{4+} , Hg^{2+} , Cd^{2+} , Zn^{2+} , Ni^{2+} , and Cu^{2+} depress total ATP at concentrations comparable to those which cause a reduction in phagocytic activity (9).

Previous studies with platinum antitumor agents *in vitro* suggest that rapidly dividing cells engaged in DNA synthesis are more likely to exhibit cytotoxic responses to the presence of platinum salts. Harder and Rosenberg (3) clearly demonstrated that *cis*-Pt(II)(NH₃)₂Cl₂ and *cis*-Pt(IV)(NH₃)₂Cl₄ are preferential inhibitors of DNA synthesis in human amnion (strain AV-3) cell cultures. Under the same conditions, these compounds caused lesser and secondary inhibition of RNA and protein synthesis. The present studies demonstrate that platinum tetrachloride causes effects in strain WI-38 human lung fibroblasts similar to those observed by Harder and Rosenberg (3) for the above platinum compounds. Clearly, the inhibition of DNA synthesis by platinum tetrachloride as measured by the incorporation of thymidine into acid-precipitable materials is preferential to the inhibition of RNA and protein synthesis in terms of both concentration and time. To account for such an effect, Harder and Rosenberg (3) have suggested that the active platinum species binds directly to DNA so that at low platinum concentrations only DNA synthesis is affected. At high concentrations they suggested that a higher frequency of platinum-induced lesions might lead to inhibition of RNA production and, in turn, protein synthesis. Rosenberg et al. (10) later found that *cis*-Pt(II)(NH₃)₂Cl₂ does, in fact, bind to nucleoside derivatives. More complex explanations have been proposed to account for the relatively slow but preferential inhibition of DNA synthesis *in vivo* (11) and *in vitro* (12). Pascoe and Roberts have recently demonstrated that DNA interstrand crosslinking by *cis* Pt(II)(NH₃)₂Cl₂ appears to parallel the observed inhibitory effects on DNA synthesis (13); however, this is not the case with the *cis*-Pt(IV)(NH₃)₂Cl₄ compound (14). The authors are not aware of data on DNA crosslinking by platinum tetrachloride in whole cells.

The irreversibility of the inhibitory effects of *cis*-Pt(II)(NH₃)₂Cl₂ and *cis*-Pt(IV)(NH₃)₂Cl₄ was described by Harder and Rosenberg (2) in their studies with human amnion AV-3 cells and by Howle et al. (15) using the former compound with phytohemagglutinin-stimulated rat lymphocytes. These findings were consistent with those of Howle and Gale (11), who found DNA synthesis persistently inhibited in Ehrlich ascites tumor cells

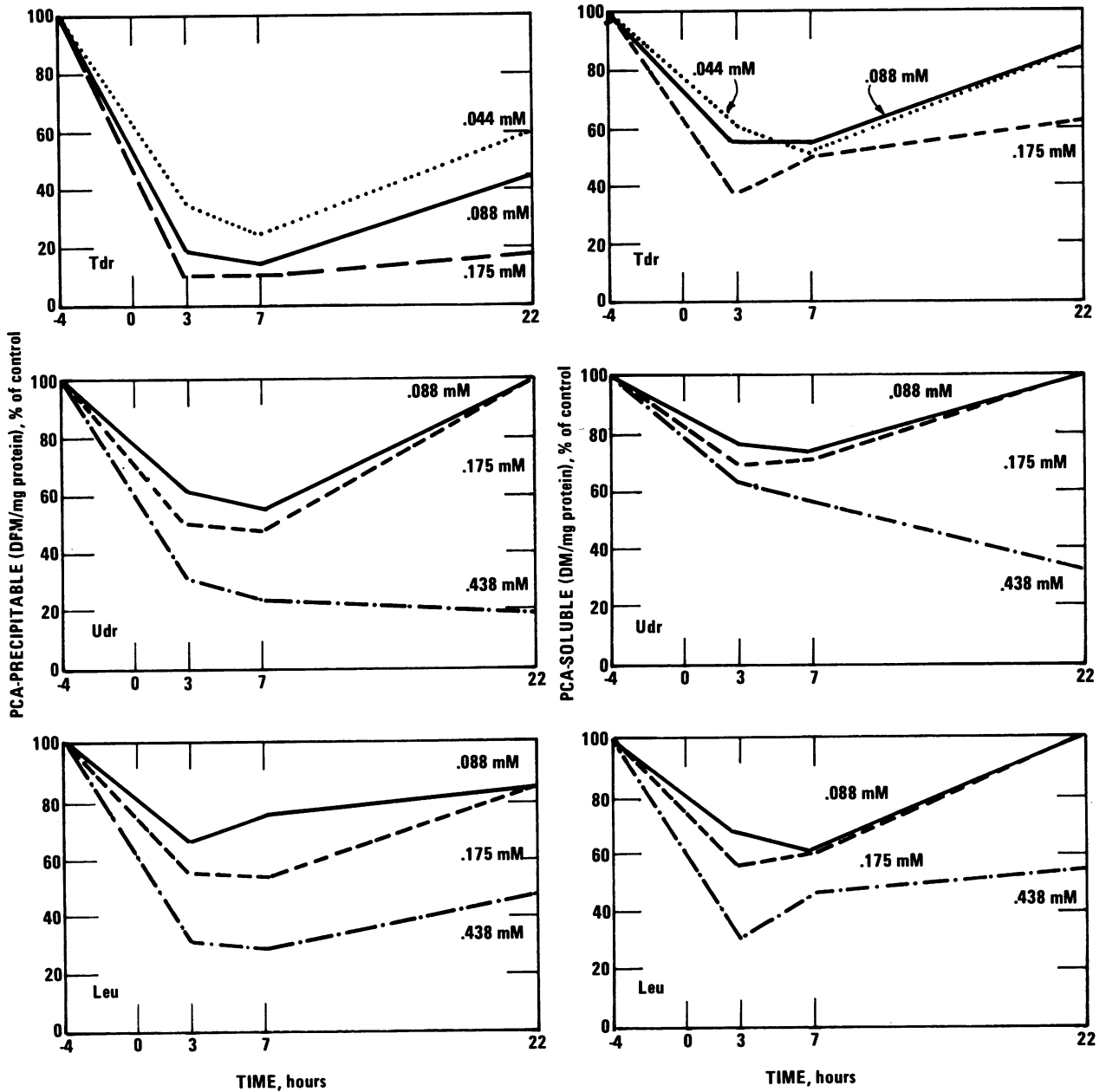


FIGURE 10. Recovery of thymidine-2-¹⁴C (Tdr), uridine-2-¹⁴C (Udr), and leucine-1-¹⁴C (Leu) incorporation into (left) PCA-precipitable materials and (right) into PCA-soluble fractions following a 4-hr exposure to platinum tetrachloride at concentrations indicated. Cultures of strain WI-38 human lung fibroblasts were thrice washed with supplemented media at time zero. Radiolabeled precursors were present only during the last 2 hr prior to indicated time of harvest.

removed periodically from rats up to 4 days after a single injection of *cis*-Pt(II)(NH₃)₂Cl₂ at 10 mg/kg. RNA and protein synthesis were inhibited initially but recovered after 4 days.

Harder and Rosenberg (2) suggest that irreversible inhibition of DNA synthesis by the platinum antitumor agents may result from the gradual limitation of the passage of thymidine into the cell and a slow passage of platinum compounds out of the cell such that their two rinses may not efficiently remove the compounds. The preliminary findings of the present report (Fig. 10) indicate that the inhibitory effects of low concentrations of platinum tetrachloride on DNA, RNA, and protein synthesis after a 4-hr exposure can be reversed with careful washing (thrice) to remove the metal. The apparent reversibility of the observed inhibitory effects of platinum tetrachloride effects may be a function of the strength of binding of the specific platinum compound to DNA or other intracellular components or the effectiveness of the washing procedure.

In conclusion, this investigation has demonstrated that platinum tetrachloride possesses biological activity at the cellular level comparable to that of the more potent platinum antitumor agents. Additional study will be required to define more precisely the nature of the inhibitory effects of this compound on cellular biosynthetic processes and to determine the possible consequences of these effects for the intact animal or man.

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REFERENCES

1. Parrot, J.-L., et al. Platinum and platinosis. *Arch. Environ. Health* 19: 685 (1969).
2. LeRoy, A.F. Interactions of platinum metals and their complexes in biological systems. *Environ. Health Perspect.* 10: 73 (1975).
3. Harder, H.C. and Rosenberg, B. Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein synthesis in mammalian cells *in vitro*. *Int. J. Cancer* 6: 207 (1970).
4. Waters, M.D. et al. Metal toxicity for rabbit alveolar macrophages *in vitro*. *Environ. Res.* 9: 32 (1975).
5. Gardner, D.E., et al. Technique for differentiating particles that are cell-associated or ingested by macrophages. *Appl. Microbiol.* 25: 471 (1974).
6. Lowry, O.H., et al. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 193: 265 (1951).
7. Kissane, J.M., and Robins, E. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* 233: 184 (1958).
8. Finney, D.J. *Probit Analysis* 3rd ed., Cambridge Univ. Press, London, 1972, p. 76.
9. Waters, M.D., et al. Screening studies on metallic salts using the rabbit alveolar macrophage. *In Vitro* 10: 342 (1974).
10. Rosenberg, B. Some biological effects of platinum compounds. New agents for the control of tumors. *Platinum Metals Rev.* 15: 42 (1971).
11. Howle, J. A., and Gale, G.R. *Cis*-Dichlorodiammineplatinum (II): Persistent and selective inhibition of deoxyribonucleic acid synthesis *in vivo*. *Biochem. Pharmacol.* 19: 2757 (1970).
12. Howle, J.A., Gale, G.R., and Smith, A.B. A proposed mode of action of antitumor platinum compounds based upon studies with *cis*-dichloro-(³H dipyrindine) platinum (II). *Biochem. Pharmacol.* 22: 1465 (1972).
13. Pascoe, J. M. and Roberts, J. J. Interactions between mammalian cell DNA and inorganic platinum compounds—I. *Biochem Pharmacol.* 23: 1345 (1973).
14. Pascoe, J. M., and Roberts, J. J. Interactions between mammalian cell DNA and inorganic platinum compounds—II. *Biochem. Pharmacol.* 23: 1359 (1974).
15. Howle, J.A., et al. *Cis*-Dichlorodiammineplatinum (II): inhibition of nucleic acid synthesis in lymphocytes stimulated with phytohemagglutinin. *Proc. Soc. Exp. Biol. Med.* 137: 820 (1971).