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Cisplatin Inhibits *In Vitro* Translation by Preventing the Formation of Complete Initiation Complex

JEFFREY M. ROSENBERG and PAUL H. SATO

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan 48824

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SUMMARY

We previously reported that mRNA loses the ability to direct *in vitro* peptide synthesis after incubation with cisplatin. The present study was designed to determine the step in translation that is affected. The rates of translation reactions inhibited by cisplatin were biphasic, having an initial rate comparable to that of the uninhibited reaction before decreasing. Analysis of cisplatin-inhibited reactions in sucrose density gradients showed a decrease in polyribosome formation. These results are consistent with an inhibition of the initiation step of protein synthesis. Individual steps in initiation were tested by analyzing the formation of ribosomal subunits in sucrose gradients that resolve the incomplete complexes. Cisplatin caused an accumulation of 48 S particles accompanied by a decreased amount of completed 80 S initiation complexes. Similar results were obtained in experiments utilizing radiolabeled methionine or mRNA. We conclude that cisplatin blocks the initiation of translation by preventing the joining of the 60 S ribosomal subunit to the 48 S preinitiation subunit.

Although cisplatin [*cis*-diamminedichloroplatinum(II)] has become one of the most widely used and effective anticancer agents, mechanisms of its action and toxicity are, as yet, not well understood (for reviews, see References 1 and 2). Studies have primarily focused on its interactions with DNA. It has been found that after entering cells cisplatin dechlorinates. The resulting hydrated metabolites react extensively with electron-rich sites on DNA and other intracellular molecules. A principal reaction is the cross-linking of N-7 atoms of proximate guanine nucleotides. DNA reacted with cisplatin has been shown to be structurally altered and to have diminished activity as replication (3) and transcription templates (4).

In addition to inhibiting DNA and RNA synthesis, exposure of cells to cisplatin decreases protein synthesis (5–7). Because of the ability of cisplatin to disrupt the functions of DNA, it had been speculated that the decrease in protein synthesis results from the decreased formation of message. This explanation, however, appears unlikely because the transcription inhibitor actinomycin D does not interfere with the effect (7). Even though this result suggests that cisplatin has a direct effect on translation, the interrelatedness of these processes in cells makes evaluation of the individual mechanisms difficult. For this reason we have studied cisplatin inhibition of translation in reticulocyte lysates, allowing this reaction to be examined without interference from these other processes. It was found that cisplatin inhibits *in vitro* translation activity (8). Studies in which mRNA or mRNA-depleted lysates were exposed to drug separately before translation suggested that the inhibition results from an interaction between drug and message. We therefore argued that, in addition to impairing DNA template functions, cisplatin also reduces the ability of mRNA to direct translation. Binding of drug to RNA had been shown. In fact, analysis of cisplatin-treated cells found substantially higher binding of drug per gram of RNA, compared with DNA or protein (9). More recently, Danenberg *et al.* (10) reported cisplatin binding and inactivation of the catalytic RNA, ribozyme.

Studies that further characterize and more precisely identify the step in translation affected by cisplatin are described in this paper. Inhibition was examined by assaying individual steps in the translation process. Comparisons are made with the actions of other inhibitors of protein synthesis. The results are consistent with an inhibition of the initiation process resulting from impaired formation of complete 80 S initiation complexes.

Materials and Methods

[^{35}S]Methionine (>800 Ci/mmol) and ^{35}S-ATP (>600 Ci/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). Spermidine (free base), GTP (sodium salt), creatine phosphokinase (type 1 from rabbit muscle), cAMP, anisomycin, cycloheximide, and mercuric chloride were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium fluoride was purchased from Fluka A. G. (Hauppauge, NY).

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ABBREVIATIONS: ^{35}S-ATP, 1-[^{35}S]thioadenosine triphosphate; S-ATP, 1-thioadenosine triphosphate; DEPC, diethylpyrocarbonate; TCA, trichloroacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Hemin hydrochloride was purchased from Kodak (Rochester, NY) and prepared as described (11). Micrococcal nuclease (*Staphylococcus aureus*, >6000 units/mg of protein) was purchased from Pharmacia Biotechnology (Piscataway, NJ). Rabbit globin mRNA, poly(A) polymerase, and biosynthesis reaction mixture (without methionine), consisting of 250 mM HEPES, 400 mM potassium chloride, 100 mM creatine phosphate, and 19 amino acids (500 μM each), were purchased from Betheseda Research Laboratories (Gaithersburg, MD). Rabbit reticulocyte lysate was purchased from either Green Hecters (Oregon, WI) or Promega Biotec (Madison, WI). RNasin was purchased from Promega Biotec. S-ATP, tetralithium salt, was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). All solutions used in translation were either treated with 0.1% DEPC or prepared with treated water, and glassware was heated to 250° for 4 hr (11).

Translation assay. Before translation, rabbit reticulocyte lysate was brought to 0.1 mM hemin, 0.5 mM calcium chloride, and 5 units/ml creatine phosphokinase. Translation was measured as the incorporation of [^{35}S]methionine. A reaction cocktail containing 2.7 mM spermidine, 0.7 mM GTP, [^{35}S]methionine (final concentration, 0.5 μCi/ml), and biosynthesis reaction mixture, which was two thirds of the total reaction cocktail volume, was prepared. A typical assay consisted of 16 μl of reticulocyte lysate, 5 μl of reaction cocktail, and a 4-μl addition. The 4-μl addition contained the variables of interest in the appropriate assays, as indicated. Cisplatin and the other reagents added to the assays were dissolved in DEPC-treated deionized water immediately before use. The 4-μl addition was made to lysate. The reaction cocktail was then combined with this solution to initiate the translation reaction. Background counts resulting from nonspecifically bound label were determined by control assays in which endogenous mRNA had been digested with micrococcal nuclease at 5 μg/ml for 7.5 min at 20°.

For translation of exogenous mRNA, the message in the lysates was first digested with nuclease at 4.1 μg/ml (from a 1 mg/ml nuclease stock solution in 50 mM glycine, 5 mM calcium chloride) for 7.5 min at 20°. After incubation, the nuclease activity was stopped by the addition of EGTA to a concentration of 9 mM.

The translation reactions were incubated at 37° for 1 hr unless otherwise noted. After this incubation, 10-μl aliquots were taken from each tube. These samples were treated with 0.5 ml of 1 N sodium hydroxide at 37° for 10 min. The reaction was then precipitated by addition of 3 ml of ice-cold 25% TCA containing 2% (w/v) casein hydrolysate. The precipitate was collected by filtration on a Whatman GF/C glass fiber filter. The precipitate was dissolved by placing the filter in 0.5 ml of tissue solubilizer (TS-1; Research Products International Corp., Mount Prospect, IL) for 30 min at 60°. After the filters had cooled to room temperature, 10 ml of scintillation cocktail (3a20; Research Products International Corp.) were added, the vials were vortexed, and the samples were counted by liquid scintillation counting. Translation activity is expressed as counts incorporated into TCA-precipitable material minus counts precipitated in the nuclease-digested control.

Gradient centrifugation. Translation reactions analyzed by sucrose gradients had a final volume of 150 μl. The reactions were conducted as described above except that they were incubated at 30°. In most instances a 2-μl aliquot was removed from each assay, hydrolyzed with NaOH, and precipitated with TCA as described above, to assess translation activity. Translation was stopped in the remaining lysate by the addition of 600 μl of ice-cold TKM buffer (10 mM Tris·HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$).

Linear sucrose gradients (11.2 ml) at densities described in the figure legends were prepared by dilution of a stock solution of 60% (w/v) sucrose in TKM buffer. The gradients were formed using a Hoefer gradient maker (Hoefer Scientific Instruments, San Francisco, CA). The translation assays were separated by centrifugation at 40,000 rpm in a SW-41 rotor at 4°, as described in the figure legends.

After centrifugation, the bottoms of the tubes were pierced and 0.45-ml fractions were recovered by displacing the gradients from the bottom with 60% sucrose at 0.8 ml/min, using a Beckman Fraction Recovery System (Beckman Instruments, Palo Alto, CA). For analysis of polysomes, fractions were precipitated with 1 ml of 8% (w/v) TCA containing 0.5% (w/v) casein acid hydrolysate and were filtered on glass fiber filters, and the filters were washed with 15 ml of 8% TCA. To analyze the formation of ribosomal subunits, fractions were collected into 1 ml of 0.25 M sodium acetate, pH 5.1, containing 2% cetyltrimethylammonium bromide, followed by the addition of 1 ml of 0.25 M sodium acetate, pH 5.1, containing 500 μg of unfractionated yeast RNA. Samples were vortexed and filtered on glass fiber filters. The filters were washed with 10 ml of 5 mM sodium acetate buffer containing yeast RNA and 10 ml of water. The precipitates were counted as described above.

End labeling of mRNA. Harlan Sprague-Dawley rats were sacrificed and RNA was prepared from testes as described (12). The total RNA pellets recovered were placed in a vacuum to remove residual ethanol and dissolved in DEPC-treated water. Poly(A)-containing RNA was isolated by affinity chromatography of the total RNA on an oligo(dT)-cellulose column (equilibrated with 20 mM Tris·HCl, pH 7.6, 0.5 M NaCl, 0.2% sodium dodecyl sulfate) (11). The integrity of the mRNA was checked by electrophoresis in a 1.15% agarose gel. Poly(A)-containing RNA was end labeled using poly(A) polymerase. Radiolabeling was carried out in a 100-μl reaction containing 40 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 250 mM NaCl, 5 μg of bovine serum albumin, 100 units of RNasin, 247.5 μM S-ATP, 2.5 μM ^{35}S-ATP (200 μCi), 10 units of poly(A) polymerase, and 25 μg of the rat testis poly(A)-containing RNA (13). The reaction was incubated at 37° for 7 min and stopped by the addition of EDTA to 20 mM. The labeled RNA was extracted with Tris-saturated phenol/chloroform/isoamyl alcohol (25:24:1) and spun through a 1-ml G-25 column.

Results

Inhibition of initiation. Formation of the initiation complex and elongation of the nascent polypeptide have been found to be the processes mainly affected by inhibitors of protein synthesis. Agents that affect either of these stages of translation can be distinguished by examining the rate of product formation. Inhibitors of ribosome assembly typically lead to rates of translation that can be divided into two phases. During an initial period labeled amino acids are incorporated by already formed translation complexes and the reaction proceeds at an apparently uninhibited rate. As these functioning ribosomes complete translation they are not replaced by newly formed initiation complexes, because of the inhibitor, and the reaction ceases. In contrast, elongation inhibitors decrease amino acid incorporation from the start of the reaction because even actively translating complexes are not able to continue. The rate, therefore, is linear but slower than that of the uninhibited reaction (14).

In the presence of cisplatin translation proceeded at a rate comparable to that of an uninhibited reaction for the initial 12 min but then declined (Figs. 1 and 2). The initial rate of the reaction was not decreased at even 700 μM cisplatin, which was the highest concentration that could be practically added to the assay in this protocol. This was nearly 9 times the concentration necessary to inhibit the reaction by 60% in this experiment (80 μM) (Fig. 2). To demonstrate that initiation and elongation inhibitors could be discriminated, sodium fluoride and cycloheximide, initiation and elongation inhibitors, respectively, were also tested (Fig. 2). Concentrations of each were adjusted to yield approximately equal inhibition (60%) after 60 min, and the reaction rates with the three inhibitors were compared. Although at the end of the reaction period each sample had incorporated nearly the same amount of the labeled amino acid, the rate of synthesis with the elongation inhibitor was linear,

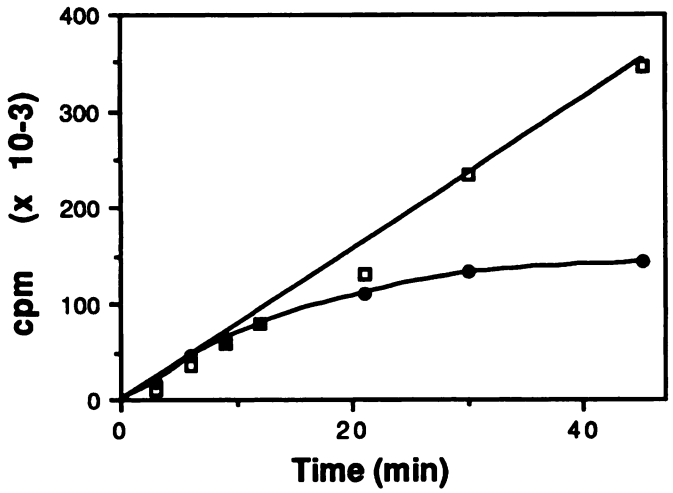

Fig. 1. Decreasing rate of translation in cisplatin-inhibited reactions. At 0 min, solvent (□) or 700 μM cisplatin (●) was added to rabbit reticulocyte lysates. Lysates were allowed to translate endogenous mRNA at 30° for 45 min; aliquots were removed at the indicated times and precipitated to determine the incorporation of [^{35}S]methionine, as described. Lysates were prepared as described in Materials and Methods. Each *point* represents the average of four individual assays. *Error bars*, ±1 SE.

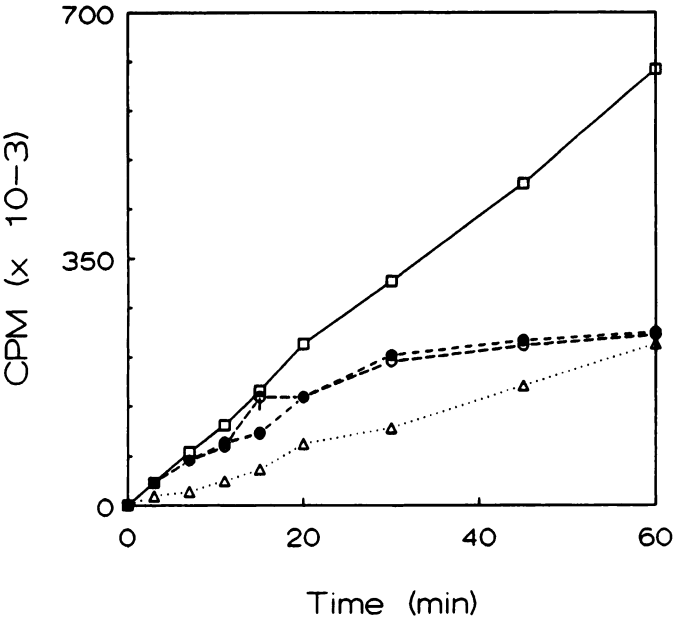

Fig. 2. Similarity of the rate of translation obtained with cisplatin to that obtained with an inhibitor of initiation. At time 0, 80 μM cisplatin (●), 2.0 mM sodium fluoride (○), or 4 μM cycloheximide (△) was added to lysates. An uninhibited reaction is shown for comparison (□). Lysates were allowed to translate endogenous mRNA at 30° for 60 min; aliquots were removed at the indicated times and precipitated to determine the incorporation of [^{35}S]methionine, as described. Lysates were supplemented with 10 mM cAMP and prepared for translation as described in Materials and Methods. Each *point* represents the average of eight control, three sodium fluoride-containing, three cycloheximide-containing, or eight cisplatin-containing assays. *Error bars*, ±1 SE.

whereas those with sodium fluoride and cisplatin decreased. These findings suggest that in cisplatin-inhibited reactions the formation of ribosomal complexes was slowed, relative to the rate of peptide elongation.

The occurance of such a delay preceding inhibition might also be explained if the interaction between cisplatin and the translation system required a period of time before affecting activity. Although monofunctional interactions of this drug with DNA occur quite quickly, it is known that the formation of cisplatin cross-links in both DNA (15) and ribozyme (10) takes place over a period of hours. If, for example, cross-links were required to inhibit translation, a similar biphasic rate of product formation might be expected. Therefore, to verify that initiation is indeed inhibited, polysome formation in cisplatin-containing reactions was examined. The status of the polyribosomes present in the lysates should reflect the stage through which the reaction had been able to proceed before activity was halted. Translation reactions containing [^{35}S]methionine were stopped after 10 min and the polyribosomes were separated by centrifugation on sucrose density gradients.

In comparison with the uninhibited reaction, cisplatin led to a decrease in polyribosomes (Fig. 3a). Apparently, elongation continued, and polypeptides were completed, after ribosome assembly on mRNA had slowed. A similar decrease was observed using the initiation inhibitor sodium fluoride (Fig. 3b). In contrast, inhibition of elongation slows the movement of existing ribosomes, while allowing their continued formation. Fig. 3c shows the resulting accumulation of large polyribosomes in cycloheximide-inhibited reactions. This study, like the kinetics experiment, indicated that in the cisplatin-inhibited reaction the assembly of initiation complexes was slowed, relative to the rate of elongation.

Inhibition of ribosome formation. The effect of cisplatin on specific steps in the assembly of the initiation complex was examined by analysis of the ribosomal subunits formed in inhibited reactions. Lysates containing [^{35}S]methionine were allowed to translate for 10 min. Preinitiation complexes were then separated on linear sucrose density gradients that resolved these ribosomal complexes. Because inhibition of a step in the formation of the ribosomal complex should result in the build-up of subunits completed before that step and the absence of the product, by determining which subunits are present it should be possible to deduce the step or steps that are affected.

The gradient profile of ribosomal subunits formed in cisplatin-inhibited reactions is shown in Fig. 4a. Comparison with the uninhibited reaction shows an accumulation of 48 S subunits and a decrease in 43 and 80 S subunits. This is similar to the gradient observed with the initiation inhibitor sodium fluoride (Fig. 4b) and different from that observed with the elongation inhibitor cycloheximide (Fig. 4c).

An accumulation of label was also noted in fractions between the 48 and 80 S particles. Addition of a cross-linking agent, such as cisplatin, to the reaction mixture might alter the density of translational components or cause them to form artificial associations. To better characterize the ribosomal complexes formed in inhibited reactions these gradients were repeated with labeled mRNA. Message, labeled on the 3′ end by incorporating [^{35}S]thioadenosine monophosphate with poly(A) polymerase, was added to ribonuclease-treated lysates and methionine-supplemented reaction mixtures. After a 10-min incubation period the reactions were fractionated on sucrose density gradients.

As in the reactions with [^{35}S]methionine, cisplatin caused an accumulation of particles sedimenting at 48 S and a decrease in the amount of label at 80 S (Fig. 5). These results suggest that cisplatin blocks formation of the initiation complex by preventing the 60 S ribosomal subunit from joining the 48 S complex. The peak that sedimented between 48 and 80 S,

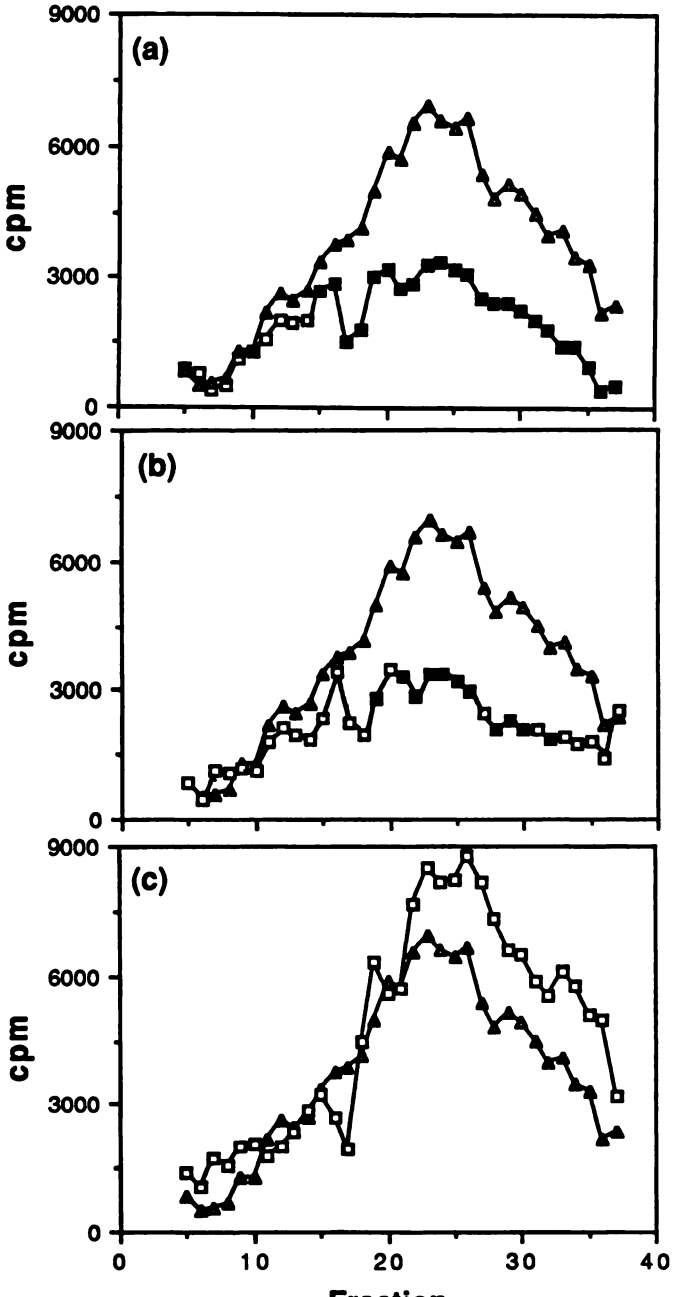

Fig. 3. Decreased polyribosomes in cisplatin-inhibited reactions. Reaction mixtures (150 μl) were allowed to translate endogenous mRNA for 10 min at 30°. Translation was carried out in the absence of inhibitors (△) or in the presence (□) of 200 μM cisplatin (a), 2.5 mM sodium fluoride (b), or 10 μM cycloheximide (c). Lysates were supplemented with 10 mM cAMP and prepared for translation as described in Materials and Methods. Samples were analyzed by sucrose density gradient (15–35%, w/v, linear sucrose gradients) centrifugation for 80 min, as described in Materials and Methods. TCA-precipitable [^{35}S]methionine distribution was determined as described in Materials and Methods. Sedimentation is from *left* to *right*. Each *point* represents the average of 15 control, 12 cisplatin, three sodium fluoride, or three cycloheximide assays. An uninhibited control reaction was analyzed at the same time as each inhibited assay. The gradient profiles were standardized to allow comparisons between the individual analyses. This was done by totalling the counts in each control and multiplying that total by a factor to yield the same amount of counts as all other controls. The counts in fractions of the control and the inhibited reaction analyzed together were then multiplied by this factor. For example, one control gradient had a total of 100,000 cpm precipitated among all of its fractions. All controls were set to 125,000 cpm. Therefore, the counts in each of the fractions of that control gradient as well as of the gradient of the inhibited reaction carried out with that control were multiplied by the factor 125,000/100,000. ■, Significant difference of the point from the corresponding control point at $p < 0.05$ by Student's t test.

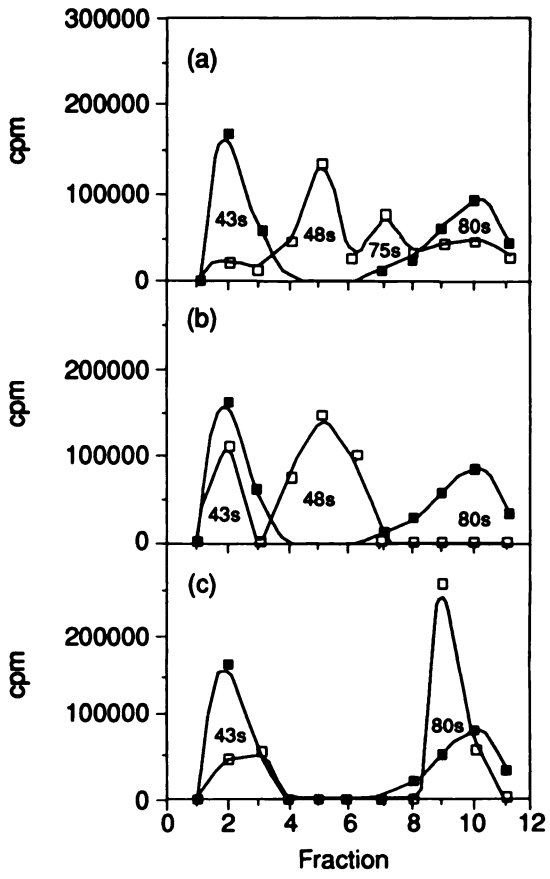

Fig. 4. Ribosomal subunits in inhibited lysates. Rabbit reticulocyte lysate reaction mixtures (150 μl) were allowed to translate endogenous mRNA for 10 min at 30°. Translation was carried out in the absence of inhibitors (■) or in the presence (□) of 200 μM cisplatin (a), 2.5 mM sodium fluoride (b), or 10 μM cycloheximide (c). Samples were analyzed by sucrose density gradient (15–35%, w/v, linear sucrose gradients) centrifugation (180 min), as described in Materials and Methods. Cetyltrimethylammonium bromide-precipitable [^{35}S]methionine distribution was determined as described in Materials and Methods. Sedimentation is from *left* to *right*, with 43 S subunits sedimenting mainly in fraction 2, 48 S subunits in fraction 5, 75 S subunits in fraction 7, and 80 S subunits in fraction 10. Each *point* represents 15 individual control assays, three sodium fluoride assays, three cycloheximide assays, or 16 cisplatin assays. The results from each treatment were standardized as described in Fig. 3. *Error bars*, ±1 SE.

observed in the gradients labeled with [^{35}S]methionine, was not visible in the mRNA-labeled gradient. This may indicate that this particle contained methionine but not message or might be explained by the much lower counts obtained with labeled message.

Although this apparent decrease in the joining of the 60 S ribosome to the 48 S ribosomal precursor could be the cause of the inhibition of translation, 43 S subunits were also decreased by cisplatin (Fig. 4a). The 43 S subunit results from the joining of the Met-tRNA$_i$ ternary complex to the 40 S ribosome. This decrease was accompanied by a nearly compensatory accumulation of 48 S subunits, suggesting that the 43 S particles that had formed became associated with message. Therefore, it

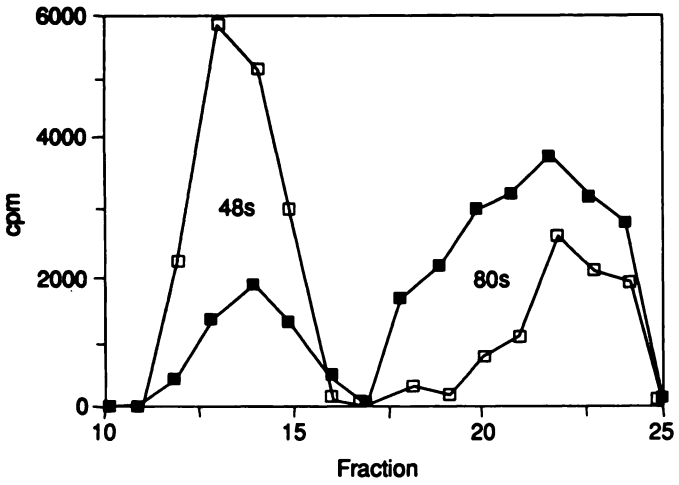

Fig. 5. mRNA is found in the 48 S preinitiation complex formed in cisplatin-inhibited reactions. Labeled mRNA was translated by nuclease-treated lysates for 10 min at 30°. Translation was carried out in the absence (■) or in the presence (□) of 200 μM cisplatin. The isolation and labeling of mRNA was as described in Materials and Methods. Samples were analyzed in sucrose density gradients (16–36%, w/v, linear sucrose gradients) centrifuged for 180 min, as described in Materials and Methods. Cetyltrimethylammonium bromide-precipitable ^{35}S was measured as described in Materials and Methods. Sedimentation is from *left* to *right*, with 48 S subunits sedimenting mainly in fraction 13 and 80 S subunits in fraction 22. Each *point* represents three individual assays.

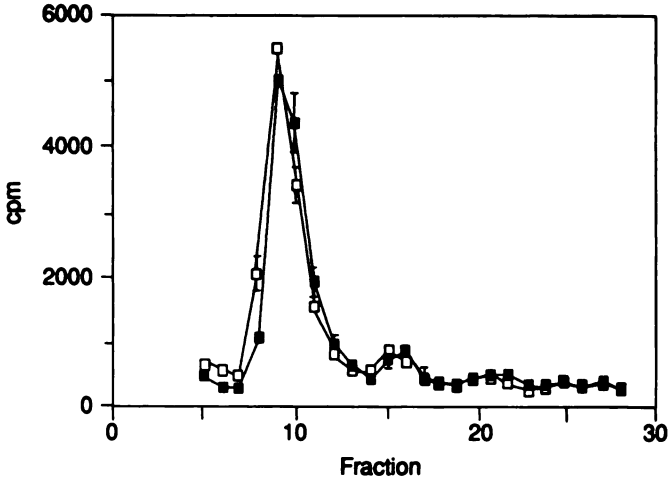

Fig. 6. Ribosomal subunits of 43 S form in cisplatin-inhibited reactions. Rabbit reticulocyte lysate reaction mixtures were allowed to translate endogenous mRNA. Assays were incubated with 100 μM anisomycin in the absence (■) or presence (□) of 200 μM cisplatin for 8 min at 30°. Samples were analyzed by sucrose density gradient centrifugation (15–30%, w/w, linear sucrose gradients) for 180 min, as described in Materials and Methods. Cetyltrimethylammonium bromide-precipitable [^{35}S] methionine was measured as described in Materials and Methods. Sedimentation is from *left* to *right*, with 43 S subunits sedimenting approximately at fraction 10. Each *point* represents three individual assays. *Error bars*, ±1 SE.

appeared unlikely that 43 S subunit formation had been blocked. However, an inability to form this complex could, as well, explain the inhibition of translation.

Further study of 43 S preinitiation complex formation was carried out by characterizing the effect of cisplatin on ribosome formation in reactions inhibited by anisomycin. High concentrations (100 μM) of this agent block the joining of the 43 S complex to mRNA (16). The 43 S subunits that form, therefore, accumulate. Reactions were allowed to proceed for 8 min (a period that was known to precede the decline in the reaction rate) (Fig. 2), after which the amounts of [^{35}S]methionine associated with the ribosomal complex were determined in sucrose density gradients.

Total accumulations of 43 S subunits at the 8-min time point were very similar in the control and cisplatin-containing reactions (Fig. 6). Although this experiment does not rule out the possibility that the formation of 43 S subunits had been slowed, it does demonstrate that substantial amounts of this ribosomal precursor can be formed in the presence of an inhibitory concentration of cisplatin.

The translation reaction appeared to be sensitized to cisplatin by addition of 10 mM cAMP. The apparent IC_{50} of cisplatin in cAMP-stimulated lysates was 25 μM (Fig. 7), compared with 100 μM for the reaction without cAMP in this trial.

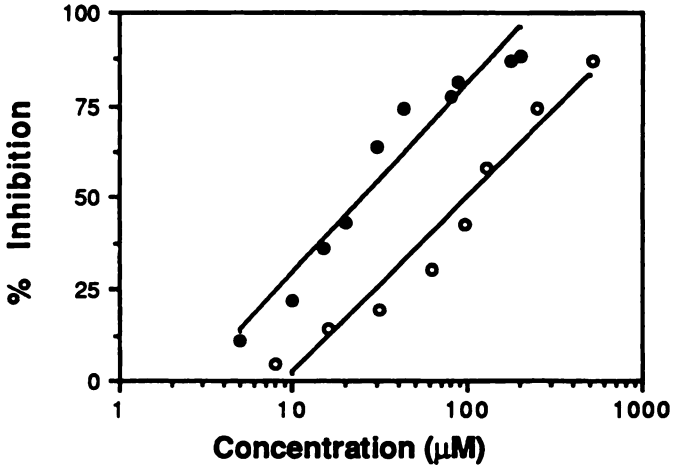

Fig. 7. Concentration versus inhibition of *in vitro* translation curve for cisplatin in the presence of cAMP. Endogenous mRNA was translated after preincubation with increasing concentrations of cisplatin for 30 min at 22°, in the absence (○) or presence (●) of 10 mM cAMP. Inhibition of translation was determined by comparison with assays preincubated in the absence of cisplatin. Each *point* represents six individual assays. *Error bars*, ±1 SE. Best fit *lines* were plotted by first-order linear regression fit to the linear portions of each curve. The slopes of the two lines were not different, by analysis of regression ($p < 0.01$).

Discussion

Cisplatin is known to inhibit protein as well as DNA and RNA synthesis in cells (5–7). Numerous studies, as well as the involvement of DNA synthesis in the actions of many anticancer drugs, have strongly implicated this effect in the mechanism of the therapeutic action of cisplatin. Consistent with that hypothesis, the cisplatin concentrations found to inhibit translation are high, in comparison with those that decrease survival of exponentially growing cell cultures. The IC_{50} value in the absence of cAMP (Fig. 7) is quite similar to that obtained previously (98 μM). At that time a somewhat lower IC_{50} (39 μM) was found using guinea pig reticulocyte lysates (8). These concentrations are similar to the K_i reported for cisplatin inhibition of ribozyme (68 μM) (10). For comparison, cisplatin produces substantial decreases in cell survival and DNA synthesis at concentrations of <10 μM (5, 6, 17).

On the other hand, concentrations that inhibit translation are similar to those necessary to produce toxicity in certain quiescent cultures. For example, Montine and Borch (7) have

used quiescent LLC-PK$_1$ cells as a model to examine cisplatin nephrotoxicity. They reported that cell viability is not affected 72 hr after exposure to ≤100 μM cisplatin for 1 hr and decreases by 32% in cultures exposed to 200 μM cisplatin. Interestingly, in these cells decreases in protein synthesis and cell viability occur over a similar range of concentrations. Although DNA synthesis was inhibited at lower concentrations of the drug, this sensitivity did not correlate with cell viability. Because toxicity did not develop until cisplatin concentrations reached levels that inhibited protein synthesis, the authors concluded that in this system the impaired DNA synthesis is not the major cause of cytotoxicity and that inhibition of protein synthesis is a critical target. Their findings suggest that concentrations of cisplatin that inhibit translation may be relevant to some of the systemic toxicities of this drug.

Whether cisplatin inhibits *in vitro* translation at concentrations comparable to those affecting proliferating cells or DNA synthesis is difficult to evaluate. Although it appears that DNA synthesis is more sensitive, extrapolating the inhibition of translation in these *in vitro* analyses to an effect on protein synthesis in cells is not clear. In Fig. 7 there was a statistically significant decrease in amino acid incorporation over the 60-min reaction with the lowest concentrations of drug tested (5 and 8 μM). Because the rates of inhibited reactions were not different from those of an uninhibited reaction for the first 12 min, before decreasing, measurement of the activity for the initial 60 min of the reaction underestimates the inhibition that might be expected over longer periods. Not until after 12 min did the rate of translation show the effect of the inhibitor. Further, the inhibited rate did not begin in a linear manner at that time but continued to decrease. This gradual slowing of the rate perhaps reflected a phase during which the components of elongating ribosomes dissociate after termination and reassemble into either active or inhibited translation complexes.

The inhibited rate is perhaps best shown by the rate of the reaction near the end of the 60-min incubation period. For example, as shown in Fig. 2, the 60% cisplatin-inhibited reaction produced about 85% of the product formed during the initial 30 min of incubation and only 15% thereafter. Comparing the reactions over the 30–60-min periods, activity with cisplatin was about 10% of the uninhibited control. Unfortunately, maintenance of a constant rate of *in vitro* translation over incubation times longer than 60 min is likely to be complicated by other factors. However, this may be the rate that eventually develops *in vivo*.

In the gradient fractionations a very high (200 μM) concentration of cisplatin was used. This concentration yielded high inhibition and was used to produce more easily observable differences in the polyribosomes and ribosomal subunits. At this concentration of the drug there was almost no additional product formation after the initial 30 min of the reaction. The 700 μM concentration tested in Fig. 1 was used to illustrate that even this very high concentration of cisplatin failed to slow elongation during the initial 12-min period.

Because protein synthesis is a complex process involving a sequence of interacting steps, it is frequently difficult to determine the specific process affected by an inhibitor. A decline in the rate of translation accompanied by a disaggregation of polysomes indicates that, at least under the conditions used in these assays, there was a decrease in the rate of initiation, relative to the rates of elongation and termination. The possibility that elongation was also slowed but to a lesser extent, however, cannot be ruled out. The effect of an inhibitor of both initiation and elongation on the composition of polyribosomes should be determined by the relative amounts of the inhibitions. If initiation is slowed in comparison with elongation, polysomes should decrease. Analysis of the rate of amino acid incorporation resulting from such an inhibitor would be expected to show an initial rate less than that of an uninhibited reaction, followed by a further decrease. A slight slowing of the initial rate of the reaction, although not observed in even near-maximally inhibited assays (Fig. 1), might be difficult to detect.

The reduction in 80 S subunits accompanied by an accumulation of 48 S subunits indicates that the rate at which 48 S subunits formed exceeded the rate at which this complex was further processed. In formation of the initiation complex the 48 S subunit is joined by the 60 S ribosome to form the complete 80 S initiation complex. Therefore, it is concluded that a major mechanism by which translation is inhibited is a decrease in 60 S ribosome attachment.

An earlier study (8) found that translation was fully inhibited when cisplatin was combined separately with mRNA but not with reticulocyte lysates depleted of message. This finding led to the hypothesis that inhibition was dependent on an interaction of drug with mRNA. If this is true, it seems likely that the affected step should not occur before the joining of message to the preinitiation intermediate. The present findings are consistent with this hypothesis, in that 48 S subunits, the product of mRNA joining to the 43 S preinitiation complex, in fact, were increased in inhibited reactions.

One might speculate that cisplatin-mRNA adducts would prevent ribosome progression along the message. The presence of cross-links or adducts might directly block the movement or prevent the melting of the secondary structures in front of the migrating ribosome. Under the conditions and cisplatin concentrations tested here these adducts might form with a high frequency within or close to the 5′ untranslated region of mRNA, so that the 43 S ribosome can attach but is unable to move along the message to the site of 60 S ribosome attachment.

Cisplatin interactions with DNA take place predominantly with nitrogens of guanine and adenine and not the deoxyribose component, forming cross-links between neighboring bases and with the complementary base-paired strand. Similar cross-links in mRNA might be expected. This could be especially true in segments having base-paired structures. Danenberg *et al.* (10) suggest that the binding of cisplatin to the RNA, ribozyme, is promoted by specific secondary and tertiary structures. A number of mRNAs contain elements of secondary structure that influence the rate of their translation. Intramolecular duplex structures and their unwinding have been shown to have the capability of acting in the regulation of ribosome assembly and translation of certain messages (18, 19). Translation of such messages might be especially prone to inhibition by cisplatin.

cAMP caused a substantial potentiation of cisplatin inhibition. cAMP stimulates both the initiation and elongation steps of translation, likely because of effects on the phosphorylation of both initiation and elongation factors (20, 21). Current investigations are considering the possible influence of cisplatin on phosphorylation of translation factors.

References

1. Eastman, A. The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol. Ther.* **34**:155–166 (1987).

2. Pinto, A. L., and S. J. Lippard. Binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (cisplatin) to DNA. *Biochim. Biophys. Acta* **780**:167–180 (1985).
3. Heiger-Bernays, W. J., J. M. Essigmann, and S. J. Lippard. Effect of the antitumor drug *cis*-diamminedichloroplatinum(II) and related platinum complexes on eukaryotic DNA replication. *Biochemistry* **29**:8461–8466 (1990).
4. Corda, Y., J. Job, M.-F. Anin, and D. Job. Transcription by eucaryotic and procaryotic RNA polymerases of DNA modified at a d(GG) or a d(AG) site by the antitumor drug *cis*-diamminedichloroplatinum(II). *Biochemistry* **30**:222–230 (1991).
5. Harder, H. C., and B. Rosenberg. Inhibitory effects of anti-tumor platinum compounds on DNA, RNA and protein syntheses in mammalian cells *in vitro*. *Int. J. Cancer* **6**:207–216 (1970).
6. Howle, J. A., and G. R. Gale. *Cis*-dichlorodiammineplatinum(II) persistent and selective inhibition of deoxyribonucleic acid synthesis *in vivo*. *Biochem. Pharmacol.* **19**:2757–2762 (1970).
7. Montine, T. J., and R. F. Borch. Quiescent LLC-PK$_1$ cells as a model for *cis*-diamminedichloroplatinum(II) nephrotoxicity and modulation by thiol rescue agents. *Cancer Res.* **48**:6017–6024 (1988).
8. Rosenberg, J., and P. Sato. Messenger RNA loses the ability to direct *in vitro* peptide synthesis following incubation with cisplatin. *Mol. Pharmacol.* **33**:611–616 (1988).
9. Pascoe, J. M., and J. J. Roberts. Interactions between mammalian cell DNA and inorganic platinum compounds. I. DNA interstrand cross-linking and cytotoxic properties of platinum(II) compounds. *Biochem. Pharmacol.* **23**:1345–1357 (1974).
10. Danenberg, P. V., L. C. C. Shea, K. D. Danenberg, and T. Horikoshi. Inactivation of tetrahymena rRNA self-splicing by cisplatin proceeds through dissociable complexes. *Nucleic Acids Res.* **19**:3123–3128 (1991).
11. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 187–209 (1982).
12. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**:5294–5299 (1979).
13. Devos, R., E. Gillis, and W. Fiers. The enzymic addition of poly(A) to the 3′-end of RNA using bacteriophage MS 2 RNA as a model system. *Eur. J. Biochem.* **62**:401–410 (1976).
14. Adamson, S. D., E. Herbert, and W. Godchaux III. Factors affecting the rate of protein synthesis in lysate systems from reticulocytes. *Arch. Biochem. Biophys.* **125**:671–683 (1968).
15. Hoffmann, J. S., N. P. Johnson, and G. Villani. Conversion of monofunctional DNA adducts of *cis*-diamminedichloroplatinum(II) to bifunctional lesions. *J. Biol. Chem.* **264**:15130–15135 (1989).
16. Lenz, J. E., and C. Baglioni. Assays for investigating the regulation of met-tRNA$_f$ binding activity. *Methods Enzymol.* **60**:281–290 (1979).
17. Sorenson, C. M., M. A. Barry, and A. Eastman. Analysis of events associated with cell cycle arrest at G_2 phase cell death induced by cisplatin. *J. Natl. Cancer Inst.* **82**:749–755 (1990).
18. Fu, L., R. Ye, L. W. Browder, and R. N. Johnston. Translational potentiation of messenger RNA with secondary structure in *Xenopus*. *Science (Washington D. C.)* **251**:807–810 (1991).
19. Kozak, M. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl. Acad. Sci. USA* **83**:2850–2854 (1986).
20. Suzuki, H., and E. B. Mukouyama. Mechanism of stimulation of globin synthesis by adenosine-3′,5′-monophosphate and guanosine 5′-triphosphate in a rabbit reticulocyte lysate system. *J. Biochem. (Tokyo)* **97**:1289–1300 (1985).
21. Sitikov, A. S., P. N. Simonenko, E. A. Shestakova, A. G. Ryazanov, and L. P. Ovchinnikov. cAMP-dependent activation of protein synthesis correlates with dephosphorylation of elongation factor 2. *FEBS Lett.* **228**:327–331 (1988).

Send reprint requests to: Dr. Paul Sato, Department of Pharmacology and Toxicology, Michigan State University, East Lansing, MI 48824.