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MESSENGER RNA AS A TARGET FOR THE CYTOTOXIC ACTION OF THE ANTITUMOR AGENT CISPLATIN: DRUG INDUCED INHIBITION OF IN VITRO TRANSLATION

By

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ABSTRACT

MESSENGER RNA AS A TARGET FOR THE CYTOTOXIC ACTION OF THE ANTITUMOR AGENT CISPLATIN: DRUG INDUCED INHIBITION OF IN VITRO TRANSLATION

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Translation activity is inhibited *in vitro* by the chemotherapeutic agent cisplatin. Peptide synthesis was measured in assays employing guinea pig and rabbit reticulocyte lysates. Lysates were preincubated with cisplatin (30-240 μM) for various times up to 60 min. Inhibition at each concentration is 60% of its maximum at 1 min and maximal following a 30 min exposure. Concentrations of cisplatin between 3 and 800 μM were incubated for 30 min with guinea pig and rabbit lysates. Translation is inhibited 50% at 39 μM cisplatin in guinea pig and 96 μM in rabbit lysates. Animals treated with therapeutic doses of cisplatin have microsomal concentrations as high as 1.7 mM. The suppression of peptide synthesis is due to an interaction between cisplatin and mRNA. Preincubation of mRNA dependent lysate with 96 μM cisplatin results in slight (20%) inhibition whereas translation was inhibited greater than 80% after treatment of the mRNA prior to its addition to lysate. Furthermore, the migration of mRNA was altered in non-denaturing agarose gels following incubation with cisplatin. A qualitative difference in the

peptide products translated from mRNA exposed to cisplatin was demonstrated. Synthesis of larger peptide products was inhibited to a greater extent than smaller products. Decreased translation activity cannot be explained by digestion of the message by the cisplatin solution. Furthermore, the addition of dithiobiuret (0.6 mM), a chelator which reacts with cisplatin, completely reversed inhibition resulting from 200 μM cisplatin. An increase in translation inhibition occurred when 10 mM cAMP was added to lysates along with cisplatin. This response differs from that found with inhibitors of peptide synthesis that act to stimulate eucaryotic initiation factor 2 kinases. Lysates in the presence of cisplatin demonstrated a disaggregation of polysomes and kinetics of inhibition which suggests the drug acts to prevent the initiation of translation. Cisplatin did not inhibit the amino acylation of methionyl tRNAs or the formation of 43s preinitiation complexes. The distribution of ribosomal subunits, following the separation of cisplatin inhibited lysates on sucrose gradients, showed an accumulation of particles sedimenting at 48s. These data suggest that cisplatin alters mRNA structure to inhibit translation initiation by preventing the joining of the 60s ribosome to the 48s ribosomal preinitiation complex.

To My Family

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INTRODUCTION

Cis-diamminedichloroplatinum (II) (cisplatin) is an anticancer drug that has demonstrated antitumor activity in a number of human neoplasms (Loehrer and Einhorn, 1984). Cisplatin is the first heavy metal coordination compound shown to have antineoplastic activity. Since cisplatin was licensed for clinical use in 1978 it has become one of the most extensively used of all antitumor drugs. Cisplatin is a component of drug combinations used in the treatment of a number of tumors. When used alone, cisplatin is successful in the control of testicular tumors. A number of serious side effects are noted with cisplatin treatment, however. Nephrotoxicity is the dose limiting side effect. Nausea and vomiting also cause many patients to withdraw from cisplatin chemotherapy.

History of Cisplatin Discovery

Cisplatin is a unique drug in the treatment of cancer in that it contains the heavy metal platinum. The discovery of the biological actions of the simple platinum coordination complexes was a case of serendipity. This new class of chemotherapeutic agents was identified during the course of a study of the electric field effects on bacterial growth (Rosenberg, VanCamp, and Krigas, 1965). A set of platinum electrodes, thought to be inert, were used in the cell growth chamber to create the electric field. A bacterial population

of Escherichia coli were allowed to grow until steady state was reached. Electrical power was applied to the electrodes and the density of bacteria declined, when the electric field was terminated, the density returned to normal after a few hours. When the bacterial cells were examined after exposure to the electric field, they appeared as long filaments, up to 300 times the normal length (Rosenberg, et al., 1967a). It was determined that this effect was due to electrolysis products from the platinum electrodes (Rosenberg, VanCamp, and Krigas, 1965). The product was isolated and identified as a platinum containing compound (Rosenberg, et al., 1967b). The neutral compound was found to exist in two isomeric forms: the trans-$[Pt^{IV}(NH_3)_2Cl_4]$ and the cis-$[Pt^{IV}(NH_3)_2Cl_4]$. Platinum has two dominant valence states, (II) and (IV). The lower state forms square planar complexes, and the latter forms octahedral complexes (Fig. 1). Thus, platinum coordination complexes can exist as cis or trans isomers in one of two valence states.

Since the newly discovered platinum compound with the (IV) valence demonstrated the ability to alter cell growth, the (II) valence compound was synthesized in the laboratory for comparison. The (II) compound was also found to be active in causing bacteria to form filaments. The trans structures of the coordination compounds from each of the valences (II and IV) are inactive in low concentrations, but begin to suppress growth at higher

Figure 1. Molecular structures of active anticancer (cis-isomers) and nonactive (trans-isomers) platinum coordination complexes.
(a) cis–diamminedichloroplatinum (II) (cisplatin);
(b) trans–diamminedichloroplatinum (II) (transplatin);
(c) cis–diamminedichloroplatinum (IV);
(d) trans-diamminedichloroplatinum (IV).

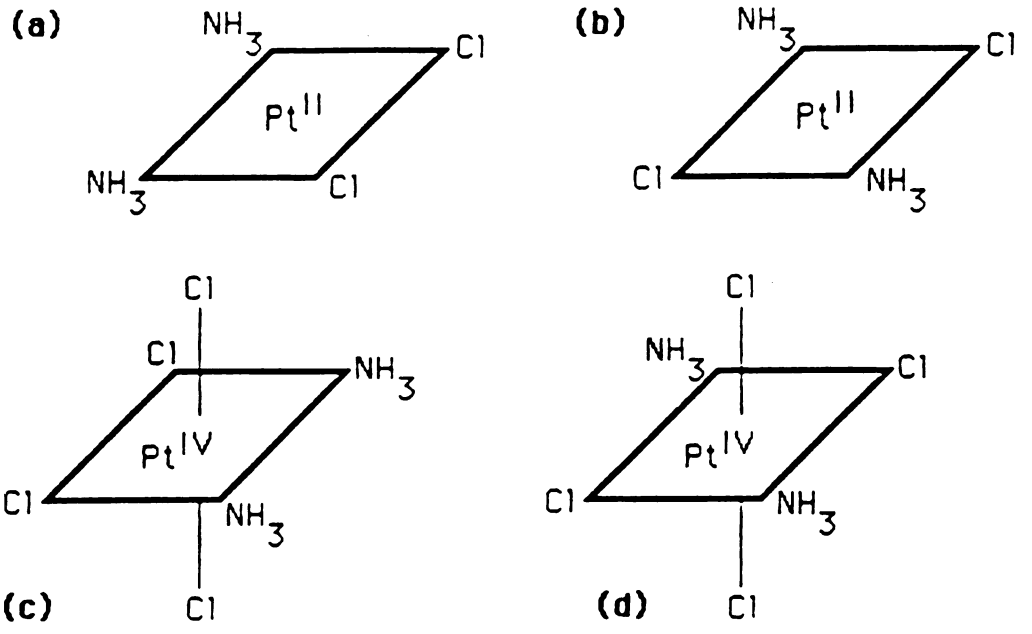
(a)
NH3
Cl
Pt II
NH3
Cl
(b)
NH3
Cl
Pt II
Cl
NH3
Cl
Cl
NH3
Pt IV
Cl
NH3
(c)
Cl
Cl
NH3
Pt IV
Cl
NH3
(d)
Cl

Figure 1

concentrations (Rosenberg, et al., 1967b). Once the structures had been determined, the molecular compound was shown to be one known as Peyrones' Chloride which was first synthesized in 1848 (Rosenberg, 1978).

Trials of many compounds established that those complexes which were neutral in solution inhibited bacterial cell division (Rosenberg, et al., 1967b). The cis configuration was active, and the trans was not. It had been previously determined that bacteria grow into filaments when exposed to hydroxyurea and nitrogen mustard, other antitmor drugs, and low doses of ultraviolet light, and that this was due to a blockade of DNA synthesis (Harder and Rosenberg, 1970). Thus, it appeared that cisplatin and hydroxyurea might have similar mechanisms of action. Bacteria exposed to nitrogen mustard could not recover and form colonies when the drug was removed whereas the bacteria exposed to cisplatin were able to form normal colonies in fresh medium. The DNA of cisplatin exposed bacteria was found to be copied, but in clumps, whereas that of nitrogen mustard was not synthesized. This finding suggests cisplatin does not prevent bacterial DNA synthesis but induces cross-links. This was a clue to a mechanism of action that differed from chemotherapeutic agents of the past.

Studies of cisplatin as a bacteriocidal agent became studies of cisplatin as an anticancer agent when it was found that small amounts of the compound caused de-repression of the viral genome in lysogenic bacteria (Vonka *et al.*, 1972). Other anticancer drugs

which interacted with cellular DNA were also noted as potent inducers of lysis. Thus, cisplatin was tested for anticancer activity (Rosenberg, et al., 1969). It was determined that cisplatin exhibited marked antitumor activity. Further, cisplatin was active against tumors exhibiting slow as well as rapid growth independent of the cell cycle. Cisplatin induced the regression of transplantable as well as chemically and virally induced tumors. There was no demonstrated species specificity and it was useful for disseminated as well as solid tumors. Thus, it was concluded that cisplatin was an active anticancer agent.

Cisplatin Pharmacokinetics

Plasma levels of platinum decline in a biphasic manner after the intravenous administration of cisplatin. The decline is marked by a distribution phase half-life of 43 minutes and an elimination phase half-life of 2 days in the rat (Litterst, *et al.*, 1979). Platinum is initially distributed to nearly all tissues. The highest levels appear in kidney, liver, ovary, uterus, skin, and bone. The rapid phase of the biphasic kinetics most likely reflects cardiac output and regional blood flow with highly perfused organs initially receiving the most drug. Delivery of drug to muscle and skin is slower but moderate concentrations of platinum are found in these tissues. The large relative mass of these latter tissues and their prolonged retention of platinum suggest they act as reservoirs which account for the long, second, half life of this drug. Plasma levels and whole body retention of platinum following administration of cisplatin are

similar in all species studied. Urinary excretion of platinum is extensive on the first day after drug administration with a final urinary recovery of approximately 90% of the administered dose. Fecal excretion of platinum via the bile has been reported at 2-7% of the administered dose (LeRoy, *et al.*, 1979). Platinum is excreted more rapidly from hydrated animals than from controls although total urinary recovery of platinum is nearly equal in both groups (Litterst, *et al.*, 1979). Cisplatin lacks the ability to inhibit hepatic microsomal drug-metabolizing enzymes (Litterst, *et al.*, 1979). Metabolism of cisplatin involves simple chemical reactions, whereby the coordinating ligands (NH_2 and Cl) are replaced during ligand exchange reactions (Rosenberg, 1978). The extent and nature of these reactions is limited by the relative availability and affinity of the substituting ligands. Of the commonly occurring ligands in biological tissues the order of affinity for Pt (II) is: sulfur > nitrogen > chloride > water; whereas the order in terms of concentration is the reverse (Basolo and Pearson, 1967).

Tissue Uptake of Cisplatin

There is no preferential uptake of platinum into tumor tissues that are sensitive to cisplatin. Platinum was not significantly concentrated in Walker 256 tumors in rats over a 72 hr time course, and there was no specific uptake of platinum into the Sarcoma 180 tumor in mice (Hoeschele and Van Camp, 1972). A constant tumor to plasma ratio of platinum of nearly 2 was shown for all times from 4 hr to 5 days after treatment of mice bearing the

Sarcoma 180 tumor. This same ratio was found in non-tumor tissues. When the drug dose was increased by a factor of ten, no significant change in the tumor to plasma ratio was evident (Lange, *et al.*, 1973). However, different organ distributions and whole body clearances of platinum between tumored and non-tumored control animals is reported (Hoeschele and Van Camp, 1972; Wolf and Manaka, 1976).

The prolonged retention of platinum in some organs suggests an irreversible or very tight binding to tissue components. The tissues which accumulate platinum might be expected to suggest potential sites of cytotoxic reactions. The high and sustained kidney levels of platinum following cisplatin administration suggest a correlation between platinum renal toxicity and the organ localization of the drug. A correlation between organ distribution of cisplatin and organ site of antitumor effect might also be expected. High levels of platinum were found in the ovary and uterus of the dog following intravenous cisplatin administration (Litterst, *et al.*, 1976). However, only low levels of platinum have been reported in the testes of rats following intravenous administration of cisplatin (Litterst, *et al.*, 1976; Wolf and Manaka, 1976).

No association of platinum with the cellular elements of blood has been demonstrated (Howle, *et al.*, 1972; Litterst, *et al.*, 1976). No binding to albumin has been demonstrated (Gale, *et al.*, 1973; Guarino, *et al.*, 1979). Following cisplatin administration, 89% of the platinum in the blood is localized in the plasma fraction and

11% in the red blood cells 2 min after infusion. Seventy two hours after treatment only 12% of the platinum was in the plasma (Wolf and Manaka, 1976). Cisplatin is relatively unreactive in extracellular fluids. The hydrolysis of cisplatin is inhibited by chloride ions at the concentrations found in physiological saline and plasma (150 and 100 mM, respectively). Cisplatin enters cells by passive diffusion. Once inside the cell, the lower chloride content (3-4 mM) of the cytosol favors the formation of a series of hydrolysis products, where the Cl ligands are replaced by H_2O and possibly OH. The hydrolysis products are approximately 1000 times more reactive than the parent compound in participating in ligand exchange reactions (Daley-Yates and McBrien, 1982). Thus, it appears that while the drug may have a short half-life in the plasma the metabolite remains in the cell longer as the cisplatin is hydrolyzed to a more reactive species. The subcellular localization of cisplatin in rat kidney and liver was determined over a period of 72 hr (Choie, *et al.*, 1980). Based on atomic absorption spectrometry of platinum, it was found that the cellular cytosol contained the largest quantity of cisplatin. Furthermore, within the cell cisplatin showed the most significant affinity for microsomes. Microsomes are pieces of endoplasmic reticulum that form following the disruption of cells.

Cisplatin Toxicity

The highest incidence of cisplatin toxicity is found in the most metabolically active tissues (Rosenberg, 1971). Nausea and

vomiting are extensive and intense and believed due to stimulation of the chemotactic trigger zone not to gastrointestinal damage (Rosenberg, 1978). Neural toxicities are further demonstrated by destruction of the sound detecting hair cells of the organ of Corti in the inner ear. This leads to transient high frequency hearing loss and possibly total deafness. Ophthalmologic toxicities and seizures are also demonstrated by patients. Myelosuppression, usually a limiting toxicity in anticancer drugs, is mild following the administration of cisplatin (Loehrer and Einhorn, 1984). Gastrointestinal damage and myelosuppression are common limiting toxic effects following the administration of an anticancer drug that effects DNA synthesis. The dose limiting side effect found with cisplatin was nephrotoxicity, more specifically damage to the proximal convoluted tubules (Kociba and Sleight, 1971). This toxicity is not common among chemotherapeutic drugs and particularly important in cisplatin administration since the kidneys are responsible for the excretion of the majority of the drug. This nephrotoxicity is reduced, however, by administrating an osmotic diuretic, such as D-mannitol, while simultaneously hydrating the patient (Cvitkovic, *et al.*, 1977). Prior to the use of this hydration-diuretic therapy further clinical testing of cisplatin was nearly discontinued after phase 1 trials. It had been concluded that cisplatin demonstrated significant toxic side effects and only modest therapeutic benefit (Loehrer and Einhorn, 1984). By utilizing this therapeutic approach, cisplatin can be safely administered at doses 300% higher than previously possible (Cvitkovic, *et al.*, 1977).

Role of DNA Modification in Cisplatin Cytotoxicity

Much evidence suggests that the cytotoxicity of platinum compounds is due to their reactions with DNA or chromatin (Pascoe and Roberts, 1974a,b; Roberts and Pera, 1983). This evidence is based largely on studies of the interaction of platinum compounds with DNA *in vitro* or the DNA of cultured mammalian cells. Supportive data was also obtained from studies of the biochemical responses of both prokaryotic and eukaryotic cells to modifications of their DNA by platinum compounds . There is, however, less evidence implicating DNA as the target for the antitumor effects of these agents either in rodents or in man. Furthermore, it is not yet apparent whether some or all of the toxic side effects of cisplatin are also due to modification of cellular DNA.

To test the possible importance of reactions with DNA, RNA, or protein the log of cell survival was related to the amount of drug bound to each type of macromolecule. (Harder and Rosenberg, 1970; Howle and Gale, 1970; Giraldi and Taylor, 1974; Pascoe and Roberts, 1974a; Roberts and Pera, 1983). At a given level of cell survival there was more binding to RNA than to either DNA or protein over the entire dose range studied (Fig. 2) (Pascoe and Roberts, 1974a). The investigators felt that the estimates of the reactions occurring in the cell should take into account the differences in the molecular weights of DNA, RNA and protein molecules, however. By taking the molecular weights of these macromolecules into account the number of platinum molecules bound to each could be calculated. There were

Figure 2. Extents of binding of cis-diamminedichloroplatinum (II) to RNA (O), DNA (●), cytoplasmic protein (▲), and nuclear protein (□) following treatment of HeLa cells in suspension culture for 2 hr at 37°C. Taken from Pascoe and Roberts (1974a).

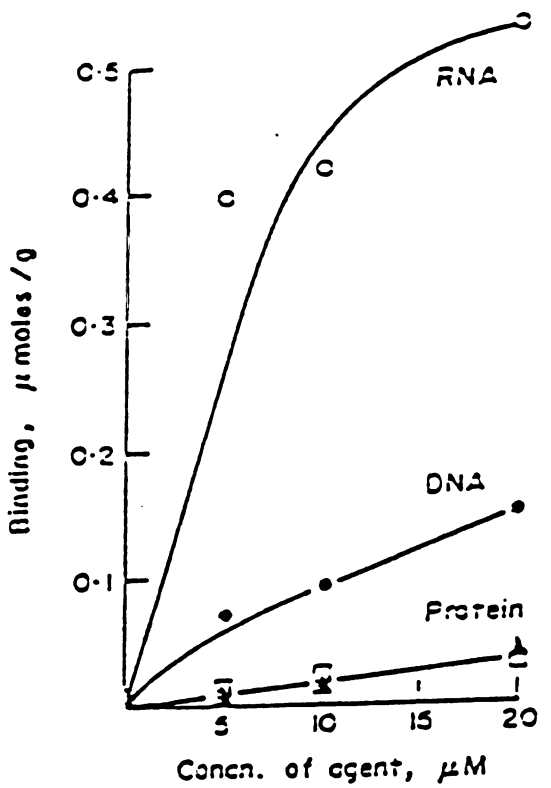
0·5
0·4
0·3
0·2
0·1
Binding, μ moles / 0
RNA
DNA
Protein
5
10
15
20
Concn. of agent, μM

Figure 2.

more platinum molecules bound per mole of DNA than per mole of RNA or protein because the molecular weight of DNA is much greater than that of RNA or protein. It was concluded that the levels of reaction with RNA or protein would be too low to produce their inactivation within the cell presuming no selective reaction with any particular RNA or protein molecule (Pascoe and Roberts, 1974a).

To assess the inherent sensitivities of cells to DNA damage, the binding of platinum to cellular DNA was measured in different cell types in various physiological states. Chinese hamster (Fraval and Roberts, 1979a,b) and human fetal lung (Pera, *et al.*, 1981) cells in stationary culture are more sensitive to treatment with cisplatin than are cells growing exponentially. The greater sensitivity of stationary treated cells is not due to a greater uptake of drug but rather to a decrease in the rate of loss of platinum adducts from their DNA. Cells growing exponentially excise lesions from their DNA four times faster than do cells maintained in stationary phase (Fraval and Roberts, 1979b). This relationship has been interpreted to support the hypothesis that lesions on the DNA are responsible for the cellular toxicity of cisplatin. The further finding that there is a linear relationship between the number of platinum lesions not excised from the DNA during a given period and the logarithm of the survival of stationary phase cells also suggests a role for DNA modification in cisplatin induced cytotoxicity. This relationship implies that the survival of a cisplatin treated cell is related to the amount of platinum bound to DNA during its replication.

A difference between cisplatin sensitive and resistant cells in their capacity to repair DNA would be evidence for DNA lesions as a cause of cisplatin toxicity. A repair deficient mutant Chinese hamster ovary cell line, sensitive to cisplatin, has a decreased ability to remove DNA interstrand cross-links (Meyn, *et al.*, 1982). This finding appears to support arguments indicating the importance of interstrand cross-links in the cytotoxic action of cisplatin. However, both sensitive and resistant Walker tumor cells treated with cisplatin excised total DNA adducts, DNA protein cross-links and DNA interstrand adducts with equal efficiency (Roberts, *et al.*, 1984). Similar results were obtained in two lines of murine leukemia L1210 cells sensitive or resistant to cisplatin (Strandberg, *et al.*, 1982). However, interstrand cross-links in DNA form only a few per cent of the total DNA adducts induced by cisplatin (Eastman, 1983). The major product of the reaction of cisplatin with DNA *in vitro* was found to be cross-links between adjacent guanine residues on the same strand of DNA (Eastman, 1983). However, the half-lives for the loss of overall DNA adducts were essentially the same for both sensitive and resistant Walker tumor cells (Roberts, *et al.*, 1984). Thus, there appears to be no difference in the DNA repair capacities of certain cisplatin sensitive or resistant cells. The methods used to detect DNA repair, however, only measure the first step of repair, that is the removal of one arm of the cisplatin cross-link. These methods give no indication as to whether the remaining steps in repair are carried out properly in cells sensitive or resistant to cisplatin toxicity.

Thus, it could be possible that the sensitive cells are defective in a later step in the repair of DNA cross-links.

The isomers cis-diamminedichloroplatinum and trans-diammine-dichloroplatinum differ in their cytotoxic and anticancer activities. The inactivity of transplatin provides a tool for investigating the biochemical interactions which lead to antitumor activity in these platinum containing compounds. Transplatin has chemical reactivity similar to that of cisplatin and undergoes extensive hydrolysis in low chloride solutions. The trans isomer is inactive at low concentrations in the inhibition of cell growth and division. Furthermore, transplatin was not effective in reducing tumor size in implanted tumors following eight days of treatment whereas cisplatin treatment caused a significant reduction (Rosenberg, 1978). The cis and trans compounds were shown, however, to have an equal ability to react with DNA either *in vitro* or *in vivo* (Pascoe and Roberts, 1974a). However, the trans compound has a decreased ability to form intrastrand cross-links in the DNA of cells in culture (Roberts and Pascoe, 1972; Pascoe and Roberts, 1974a). The close similarity in the interaction of these isomers with DNA and marked difference in their biological actions makes it difficult to relate an interaction with DNA by cisplatin to its proven cytotoxic and antitumor effects.

Alternate Mechanisms of Cisplatin Action

Inconsistencies in the hypothesis that the mechanism of cytotoxic and antitumor action of cisplatin exclusively involves an interaction with cellular DNA are noted. For other cytotoxic agents, cyclophosphamides for example (Fleer and Brendel, 1979), the formation of DNA cross-links is closely related to the cytotoxicity, but with cisplatin, a clear relationship has not been demonstrated (Pera, *et al.*, 1981; Zwelling, *et al.*, 1979). Filamentation of bacteria is thought to be caused by the inhibition of DNA synthesis. This event is lethal when induced by bifunctional alkylating agents which inhibit DNA synthesis even after removing the drug from the growth medium. Following the removal of cisplatin, however, the filaments rapidly revert to normal colonies (Rosenberg, 1975). This suggests a different type of lesion may be induced by cisplatin than by compounds known to cause cytotoxic and antitumor effects by interacting with cellular DNA. These findings, in addition to those discussed above, suggested that other mechanisms may also account for the cytotoxic and/or antitumor actions of cisplatin. Cisplatin has demonstrated an affinity for binding to nucleic acids, especially single stranded DNA (adjacent guanines on the same strand), and has been found to bind to RNA as well (Pascoe and Roberts, 1974a).

Since cisplatin binds RNA, then an effect on the synthesis of polypeptides might be anticipated. Furthermore, cisplatin may bind to one type of RNA selectively. The majority of the RNA in a cell is ribosomal and transfer RNAs with message comprising only about 2%

of the total RNA (Chirgwin, *et al.*, 1979). If cisplatin selectively binds to mRNA, then a large fraction of the template would be affected. Further, the subcellular localization of cisplatin is in the endoplasmic reticulum where translation occurs (Choie, *et al.*, 1980). It was also found that decreases in serum albumin concentration are associated with the accumulation of liver platinum in human patients treated with cisplatin (Nanji, *et al.*, 1986). These studies implied that a relationship between cisplatin and decreased protein synthesis may occur. Therefore, an investigation was undertaken to explore the effect of cisplatin on translation.

Mechanism of Translation

An understanding of translation is necessary to investigate the mechanism by which cisplatin may inhibit peptide synthesis. Protein synthesis can be divided into three phases: initiation, elongation, and termination. Initiation involves the attachment of ribosomal subunits and other necessary components to the 5' end of the coding sequence of mRNA. Initiation involves a series of events that ensure the recognition of the initiator AUG codon of mRNA and the decoding of the template in the correct reading frame. Elongation is the step wise addition of amino acids, in a sequence determined by the base sequence of mRNA, to produce a growing polypeptide chain. Finally, termination of translation involves the release of the ribosome and the newly synthesized polypeptide from the mRNA.

Mammalian ribosome and mRNA concentrations are nearly constant within the cell (Jagus, *et al.*, 1981). Thus, rapid changes in the rate of protein synthesis *in vivo* are achieved by changes in the activity of these components not in their concentrations. Kinetic analyses of mammalian protein synthesis have demonstrated that initiation is the rate limiting step *in vivo* and *in vitro* (Jagus, *et al.*, 1981). In addition, changes in the rate of initiation effect the relative rates of translation of different mRNA species (Bergmann and Lodish, 1979). Thus, the rate of overall protein synthesis is dependent upon the rate of initiation of translation.

The sequence of events comprising the initiation of protein synthesis in mammalian systems can be described in five major steps (Fig. 3) (Moldave, 1985). First, the initiator tRNA species, $tRNA_{f\text{-}met}$, is aminoacylated in a reaction catalyzed by tRNA synthetase. Second, the ternary complex is formed between eucaryotic initiation factor 2 (eIF-2), GTP, and $tRNA_{f\text{-}met}$. Third, the ternary complex is joined by a 40s ribosome to generate a 43s ribosomal subunit. Fourth, this is followed by the binding of the 43s unit to the 5' end of a mRNA to form an intermediate preinitiation complex, which sediments at 48s. The 43s subunit scans the mRNA until it locates the start codon, 5'-AUG. The final step then occurs, the joining of a 60s ribosomal subunit to complete the formation of an 80s initiation complex. The assembly of the 80s initiation complex is catalyzed by specific initiation factors. In addition to

Figure 3. Sequence of events in the initiation of protein synthesis in eucaryotic cells. Steps shown in the formation of the 80s initiation complex are: the aminoacyl tRNA synthetase catalyzed reaction to link methionine (MET*) to its specific transfer RNA ($tRNA_{f\text{-}met}$) to form $tRNA_{f\text{-}met}$; ternary complex ($GTP{\bullet}eIF\text{-}2{\bullet}tRNA_{f\text{-}met}$) is formed by the joining of a GTP and eucaryotic initiation factor 2 (eIF-2) to the methionyl tRNA; the addition of a 40s ribosome to the ternary complex for 43s formation; the binding of 43s to messenger RNA (mRNA) to make a 48s particle; the joining of a 60s ribosome to 48s to complete the 80s complex; and the subsequent regeneration of eIF-2. Initiation of translation proceeds as described in the text.

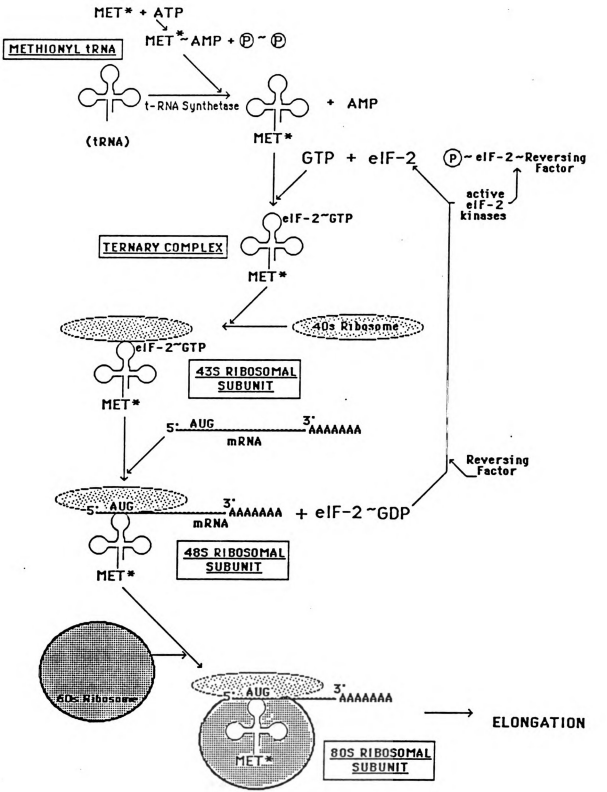
MET* + ATP
METHIONYL tRNA
MET*~AMP + Ⓟ~Ⓟ
t-RNA Synthetase
+ AMP
(tRNA)
MET*
GTP + eIF-2
Ⓟ~eIF-2~Reversing Factor
active eIF-2 kinases
eIF-2~GTP
TERNARY COMPLEX
MET*
40s Ribosome
eIF-2~GTP
43S RIBOSOMAL SUBUNIT
MET*
5' AUG 3' AAAAAAA
mRNA
Reversing Factor
5' AUG 3' AAAAAAA
mRNA
+ eIF-2~GDP
48S RIBOSOMAL SUBUNIT
MET*
40s Ribosome
5' AUG 3' AAAAAAA
MET*
80S RIBOSOMAL SUBUNIT
ELONGATION

Figure 3.

ribosomal subunits, $tRNA_{f\text{-}met}$, mRNA, and the initiation factors, ATP is also required for the initiation of protein synthesis.

Three major steps occur in the elongation process: binding of the incoming aminoacyl-tRNA, peptide bond formation, and translocation (Fig. 4) (Moldave, 1985). There are two sites for tRNA attachment on the 80s ribosomal complex. One contains the peptidyl-tRNA, is closest to the 5' end of the mRNA, and is called the "P" site. Following initiation, $tRNA_{f\text{-}met}$ occupies the P site. The other tRNA binding site is the aminoacyl-tRNA or "A" site and is on the 3' side of the P site. The next aminoacyl-tRNA added to the growing peptide chain is attached to this A site in the presence of GTP and an elongation factor.

The formyl-methionyl group is transferred to the amino group of the aminoacyl-tRNA in the A site in a reaction catalyzed by peptidyl transferase which is associated with the 60s ribosomal subunit. In subsequent steps the growing peptide chain is transferred to the next aminoacyl-tRNA. The result is a peptidyl-tRNA, one amino acid longer, joined to the A site while the P site contains the deacylated tRNA that formerly held the peptide chain. This deacylated tRNA is removed in a reaction catalyzed by a second elongation factor and GTP that is concomitant with the translocation to the P site of the new peptidyl-tRNA which remains hydrogen bonded to its codon on the mRNA. The cycle is completed, and a new codon is brought into the A site.

Figure 4. Sequence of events in the elongation of protein synthesis in eucaryotic cells. Steps shown are: binding of aminoacyl-tRNA with the aid of an elongation factor (EF-Tu); peptide bond formation catalyzed by peptidyl transferase; and translocation of the ribosomal complex along the mRNA template driven by a GTP dependent elongation factor (EF-G). Figure taken from Stryer (1981). Elongation of translation proceeds as described in the text.

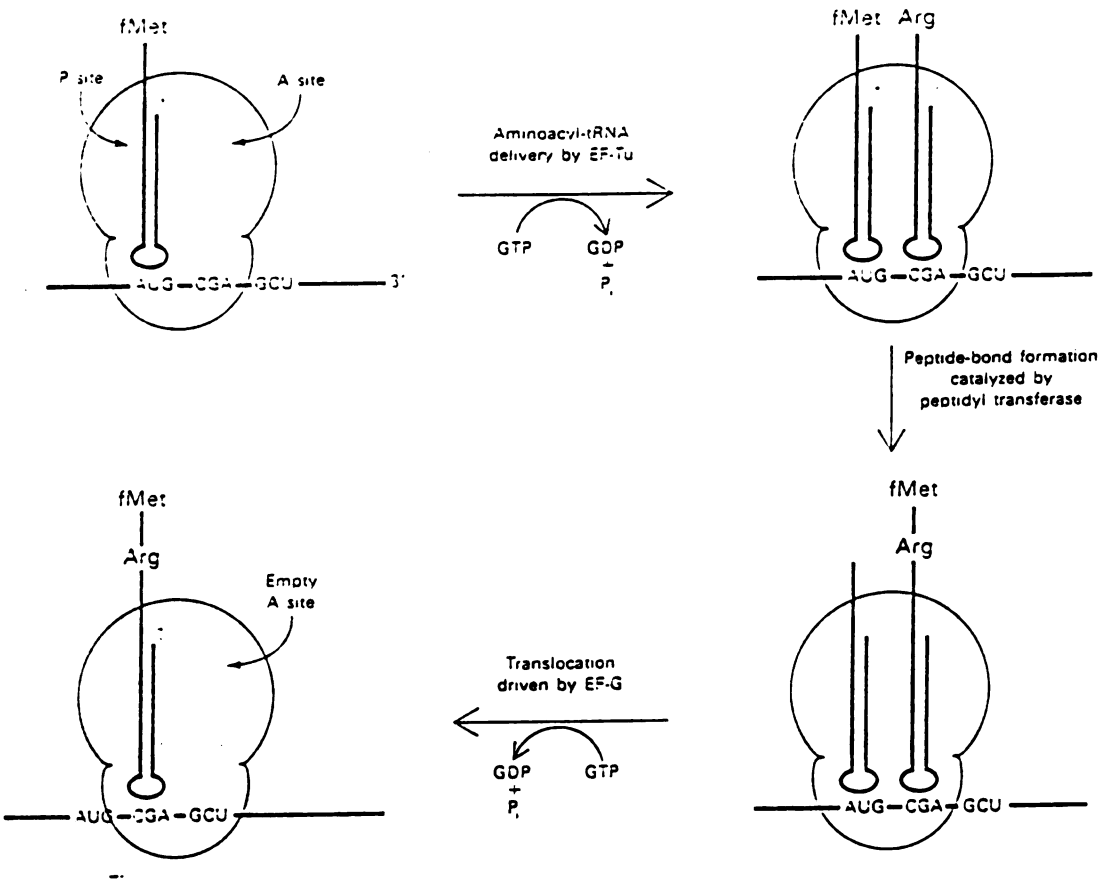
fMet
P site
A site
AUG—CGA—GCU
3'
Aminoacyl-tRNA
delivery by EF-Tu
GTP
GDP
+
Pi
fMet Arg
AUG—CGA—GCU
Peptide-bond formation
catalyzed by
peptidyl transferase
fMet
Arg
AUG—CGA—GCU
Translocation
driven by EF-G
GDP
+
Pi
GTP
fMet
Arg
Empty
A site
AUG—CGA—GCU

Figure 4.

The termination of polypeptide synthesis is signaled by one of three termination codons in the mRNA (Moldave, 1985). After the last amino acid has been added to the polypeptide chain on the ribosome, the polypeptide is still covalently attached to the tRNA which is on the A site of the ribosome. The release of the polypeptidyl-tRNA from the ribosome is promoted by releasing factors. These factors bind to the ribosome to cause a shift of the polypeptidyl-tRNA to the P site. The bond between the polypeptide chain and the last tRNA is then hydrolyzed in an energy dependent reaction. Once the polypeptide is released, the last tRNA and the mRNA leave and the 80s complex dissociates into its 60s and 40s subunits. This dissociation of the 80s complex requires energy and a specific protein factor. Termination affects initiation as one of the major factors controlling the rate of initiation is the availability of free ribosomal subunits.

Objective

The objective of the research presented in this dissertation is to investigate the possibility that cisplatin may inhibit protein synthesis through an interaction with ribonucleic acid. Previous studies examining the relationship between cisplatin and protein synthesis have produced variable results (Zylicz, *et al.*, 1987; Vawda and Davies, 1986; Harder and Rosenberg, 1970; Howle and Gale, 1970; Nanji, *et al.*, 1986). These studies were carried out in whole animal or cell culture preparations. Such *in vivo* analyses of the

translational process are confounded by the interdependence upon related cellular activities such as DNA replication and transcription.

It is possible to study translation and measure *in vitro* peptide synthesis by the use of reticulocyte lysates (Jackson and Hunt, 1983). These preparations contain the enzymes and other components necessary to synthesize peptides in the absence of other cellular processes. The purpose of this study is to examine the effect of cisplatin on the translation of peptides in a cell free system.

MATERIALS AND METHODS

Materials

^{35}S-Cysteine (>600 Ci/mmol), ^{35}S-methionine (>800 Ci/mmol), and ^{35}S-1-thio-adenosine triphosphate (^{35}S-ATP, > 600 Ci/mmol) were obtained from Amersham Corporation (Arlington Heights, IL, USA). L-[3,4,5-^{3}H(N)]leucine (>110 Ci/mmol) was obtained from New England Nuclear Research Products (Boston, MA, USA). Spermidine (free base), GTP (sodium salt), creatine phosphokinase (type 1 from rabbit muscle at 125 units/mg solid), 3':5'-cyclic adenosine monophosphate, puromycin, cycloheximide, transplatin, anisomycin, mercuric chloride, 1-acetyl-2-phenylhydrazine, and phenylhydrazine (95%) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Transplatin was also received from Strem Chemical Company (Newburyport, MA, USA). Sodium Fluoride was obtained from Fluka A. G. (Hauppauge, NY) and 1-thio-adenosine triphosphate (S-ATP, tetra lithium salt) was obtained from Boeringham Mannheim Biochemicals (Indianapolis, IN, USA). Hemin hydrochloride was purchased from Kodak (Rochester, NY, USA) and prepared as described (Maniatis *et al.*, 1982). Micrococcal nuclease (Staphylococcus aureus, >6000 units/mg protein) was purchased from Pharmacia Biotechnology, Molecular Biology Division (Piscataway, NJ, USA). Rabbit globin (5 µg/0.1 ml in sterile water) messenger RNA, poly (A) polymerase and biosynthesis reaction mixture (consisting of 250 mM HEPES, 400 mM potassium chloride,

100 mM creatine phosphate, and a solution of 19 amino acids, 500 μM each) were obtained from Bethesda Research Laboratories (Gaithersburg, MD, USA). Brome mosaic virus (BMV) mRNA (500 μg/ml in water) and RNasin were obtained from Promega Biotec (Madison, WI, USA). Nuclease treated rabbit reticulocyte lysate was purchased from either Bethesda Research Laboratories or Promega Biotec and non-nuclease treated rabbit reticuolcyte lysate was purchased from either Green Hecters (Oregon, WI) or Promega Biotec. Precautions were taken to prevent the contamination of translation assays with ribonuclease. Skin is a major source of contamination so gloves were worn at all times. Furthermore, all the solutions used in lysate preparation and translation were treated with 0.1% diethylpyrocarbonate (DEPC) to inactivate ribonucleases, and glassware was heated to 250°C for at least 4 hr for the same purpose (Maniatis *et al.*, 1982).

Preparation of Reticulocyte Lysates

Reticulocytes were obtained from male, English short-hair guinea pigs *cavia porcellus* (300-500 g; strain, Mdh:(SR[A]) from the Michigan State Health Laboratories, Lansing, MI, USA) made anemic by subcutaneous injections of 1.7% phenylhydrazine at a dose of 17 mg/kg on four consecutive days. The guinea pigs were allowed to recover for four days, then bled through a carotid catheter on the eighth day. Prior to bleeding, the animals were anesthetized (Shucard *et al.*, 1975) and local anesthesia at the site of incision was provided by subcutaneous injection of 2% Lidocaine HCl (Rugby

Laboratories, Incorporated, Rockville Centre, NY, USA). The animal was injected with 0.5 ml of heparinized physiological saline (100 units/ml) through the carotid catheter. After several minutes, the blood was collected in a sterile syringe containing a small amount of heparin solution. The lysate was prepared by isolating the blood cells by centrifugation at 8000 x g for 10 min at 2° C. After removal of the supernatant and buffy white coat by aspiration, the cells were resuspended in physiological saline and centrifuged again. This procedure was repeated for a total of three washes. Following the removal of the final supernatant, the cells were lysed by thoroughly mixing them in an equal volume of ice-cold double distilled water. Following lysis, the cellular debris was removed by centrifugation at 16,000 x g for 20 min at 2° C. The lysate was frozen at –70° C for later use. The frozen lysate retains activity for three months.

Translation of mRNA

Prior to translation, the guinea pig reticulocyte lysate was made 0.1 mM hemin, 0.5 mM calcium chloride, and 10 units/ml creatine phosphokinase (from a creatine phosphokinase stock solution of 325 units/ml in 50% glycerol w/v). Concentrations utilized in rabbit lysates were 0.1 mM, 0.5 mM and 5 units/ml, respectively. For translation of exogenous mRNA, the endogenous mRNA in the lysate was first digested with nuclease at 4.1 µg/ml (from a 1 mg/ml nuclease stock solution dissolved in 50 mM glycine and 5 mM calcium chloride) for 7.5 min at 20° C. Following

incubation, the nuclease activity was stopped by the addition of EGTA to a concentration of 2 mM. Lysate prepared for the purpose of translating endogenous mRNA included water in place of the nuclease and EGTA.

Translation was measured as the incorporation of ^{35}S-methionine, ^{35}S-cysteine, or L-[3,4,5-^{3}H(N)]leucine. To start the translation assay, a reaction cocktail was added containing 2.7 mM spermidine, 0.7 mM GTP, and a labeled amino acid (final concentration in the translation assay of 0.5 μCi/ml). The reaction cocktail also contained biosynthesis reaction mixture (minus the labeled amino acid) which was 2/3 of the total reaction cocktail volume. A typical assay consisted of 16 μl reticulocyte lysate, a 4 μl addition, and 5 μl of reaction cocktail. The 4 μl addition consisted of a variable of interest such as an inhibitor and/or dithiobiuret in the appropriate translation assays as indicated. In translation assays directed by exogenous mRNA, 2 μl of the 4 μl addition consisted of 1.0 μg of the added mRNA. Cisplatin, dithiobiuret, and the other inhibitors were dissolved in DEPC treated deionized water immediately before use. The 4 μl addition was made to lysate and allowed to preincubate according to the experimental protocol. The reaction cocktail was then combined with this solution and the translation assay was initiated. To determine the number of background counts that result from nonspecifically bound label, control assays were performed in which endogenous mRNA was digested with micrococcal nuclease. Translation then proceeded in the absence of added message.

Following the start of the translation reaction, the assay tubes were incubated at 37° C for one hour unless otherwise noted. Following this incubation, 10 µl aliquots were taken from each tube. Assays using ^{3}H-leucine and ^{35}S-methionine were treated with 0.5 ml of 1 N sodium hydroxide at 37° C for 10 min to hydrolyze the amino acyl tRNAs. Samples translated in the presence of ^{35}S-cysteine were alkylated with iodoacetic acid prior to hydrolysis to prevent sulfur exchange (Hirs, 1967). Labeled protein was precipitated by addition of 3 ml of ice-cold 25% trichloroacetic acid containing 2% w/v casein hydrolysate. The protein was collected by filtration on fiberglass filters (Boehringer Mannheim Biochemicals). The filters were dissolved in 0.5 ml of tissue solubilizer (TS-1, Research Products International Corporation, Mount Prospect, IL, USA) for 30 min at 60° C. After the filters cooled to room temperature, 10 ml of scintillation cocktail (3a20, Research Products International Corporation) was added and the samples were counted by liquid scintillation spectrometry. Translation activity is expressed as counts incorporated into trichloroacetic acid precipitable material minus counts precipitated in the control. The protein concentration in the guinea pig and rabbit lysates was determined by the method of Lowry, *et al.* (1951) using bovine albumin as the standard.

Sucrose Gradients

Assays translated for separation on sucrose gradients had a final volume of 150 µl. Peptide synthesis was conducted as described above at 30°C for 10 min. A 2 µl aliquot was removed from each assay, hydrolyzed with NaOH and precipitated with TCA as before to ensure translation activity was as expected. 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, and 1.5 mM $MgCl_2$) which dilutes the assay and provides a temperature unfavorable for translation. The translation assays were then fractionated on 11.2 ml linear sucrose gradients at 40,000 rpm in a SW 41 rotor at 4°C as indicated for the individual experiments. The sucrose gradients were diluted from a stock solution of 60% w/v sucrose prepared in TKM buffer. The linear gradients were formed using a Hoeffer gradient maker (Hoefer Scientific Instruments, San Francisco, CA). Sucrose was delivered to the bottom of the centrifuge tubes by a peristalic pump at 0.5 ml/min. Following the spin, the bottom of the tube was pierced and 0.45 ml fractions were recovered by displacing the gradient from the bottom with 60% sucrose at 0.8 ml/min using a Beckman Fraction Recovery System (Beckman Instruments, Palo Alto, CA). To determine the distribution of ^{35}S-methionine in gradients analyzing 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 of1 ml of 0.25 M sodium acetate, pH 5.1, containing 500 µg of unfractionated yeast carrier RNA. Samples

were vortexed and filtered on Whatman GF/C glass fiber filters. Filtered samples were washed immediately with 10 ml a 1:100 dilution of the 0.5 M sodium acetate buffer containing crude yeast RNA and 10 ml of water. Fractions from gradients used to examine the formation of polysomes were precipitated with 1 ml 8% w/v trichloroacetic acid containing 0.5% w/v casein acid hydrolysate, vortexed, and filtered on glass fiber filters then washed with 15 ml of 8% trichloroacetic acid.

Assay of tRNA Synthetase Activity

Synthetase activity was assayed by charging $tRNA_{f\text{-}met}$ with ^{35}S-methionine. Each 27 µl assay contained 100 mM potassium phosphate buffer, pH 7.0, 10 mM $MgCl_2$, 2 mM ATP, 7 mM ß-mercaptoethanol, 8.5 µCi ^{35}S-methionine, 0.06 A_{260} units $tRNA_{f\text{-}met}$ (Sigma Chemical Company), and 0.55 µg tRNA synthetase. The assay was incubated at 37°C for 15 min, followed by the addition of 8 % trichloroacetic acid containing 0.5% casein acid hydrolysate for 30 min at 0°C. This was then filtered on glass fiber filters which were washed with 8% trichloroacetic acid, solubilized and counted as above for the determination of precipitable ^{35}S-methionine labeled tRNAs.

Isolation and Radiolabeling of mRNA

Harlan Sprague Dawley rats were decapitated and RNA was prepared from testes as described (Chirgwin, *et al.*, 1979). Testes

were removed and immediately homogenized in 5 volumes of guanidinium buffer (4 M guanidinium isothiocyanate, 5 mM sodium citrate, pH 7.0, 0.1 M ß-mercaptoethanol, and 0.5% Sarkosyl). The slurry was spun 2500 rpm to remove cellular debris and made 50 mM sodium acetate, pH 5.2. Nucleic acids were precipitated from the solution by adding 0.75 volumes of ethanol at -20°C for 16 hours. The solution was spun 4000 rpm for 30 min at 20°C. The pellet was dissolved in guanidinium buffer and layered over 6 ml each of 96% w/v, 40%, 20% cesium chloride gradient prepared from a stock solution of 96% w/v cesium chloride in 0.4 mM EDTA, 20 mM tris HCl, pH 7.6. The gradients were spun in a 60 Ti rotor 50,000 rpm for 17 hr. The bottoms of the tubes are cut off, to reduce nuclease contamination, and the pellets were dissolved in 10 mM tris HCl, pH 7.6, 5 mM EDTA, 1% SDS. The solution was extracted with chloroform:n-butanol (4:1). The aqueous layer was removed and re-extracted. The aqueous portions were pooled and precipitated with 0.1 volumes of 2 M sodium acetate, pH 5.2 and 2.2 volumes of ethanol at -20°C overnight. The total RNA was recovered by centrifugation for 20 min at 25,000 x g. The pellets were desiccated to remove residual ethanol and dissolved in DEPC treated water. Poly (A) containing mRNA was isolated by loading the total RNA onto a oligo d(T) cellulose column equilibrated with 20 mM tris HCl, pH 7.6, 0.5 M NaCl, 0.1% SDS (loading buffer) (Maniatis, *et al.*, 1982). The column was washed with loading buffer until no absorbance is detected at 260 nm. The poly (A) RNA was eluted with 10 mM tris HCl, pH 7.6, 1 mM EDTA, 0.05% SDS. Sodium acetate, pH 5.2 (0.1 volumes of 2 M) and 2.2 volumes of ethanol were added to precipitate the mRNA at -

20°C. The RNA is recovered by centrifugation and dissolved in DEPC treated water. The integrity of the mRNA was checked by electrophoresis in a 1.15% agarose mini gel prepared in 10 mM potassium phosphate buffer, pH 7.0, run at 100 V for 30 min. Message was considered intact if it migrated between 18s and 28s.

Poly (A) polymerase is a primer dependent polymerase which catalyzes the addition of AMP to the 3' hydroxyl terminus of RNA. Poly (A) polymerase was used to end label poly (A) containing RNA isolated from rat testes. Radiolabeled RNA was prepared 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 bovine serum albumin, 100 units RNasin, 247.5 µM S-ATP, 2.5 µM ^{35}S-ATP (200 µCi), 10 units poly (A) polymerase, and 25 µg rat testis poly (A) RNA (Devos, *et al.*, 1976). The reaction was incubated at 37°C 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

It was found that the addition of cisplatin results in a large decrease in the ability of reticulocyte lysates to incorporate radiolabeled amino acids into trichloroacetic acid-precipitable material (Rosenberg and Sato, 1988a). This decrease reflects an inhibition of peptide synthesis.

To characterize the effect of cisplatin on peptide synthesis the time course of the inhibition by cisplatin was examined. This effect on translation was observed in *in vitro* preparations from rabbit reticulocyte lysates (Fig. 5). The figure shows the incorporation of radiolabeled amino acid as a function of time. Samples containing the indicated concentrations of cisplatin were incubated with lysate for 60 min at 30°C. Samples were withdrawn following various times of incubation, treated with sodium hydroxide, and precipitated as described. Rabbit lysates translated in the absence of cisplatin demonstrate linear incorporation of label for 60 min. Cisplatin decreases the amount of label incorporated over time in a cell-free translation assay prepared from rabbit reticulocyte lysates. The figure also demonstrates that larger concentrations of the drug result in a proportionate decrease in peptide synthesis.

Figure 5. Time course of cisplatin inhibition of *in vitro* translation. At t=0 min, 0 (□), 40 (♦), 80 (○), or 240 (◊) μM cisplatin was added to rabbit reticulocyte lysates. Lysates were allowed to translate endogenous mRNA at 30°C for 60 min, aliquots were removed at the indicated times and precipitated to determine the incorporation of ^{35}S-methionine as described. Lysates were prepared for the translation of endogenous mRNA as described in Materials and Methods. Each point represents 8 individual assays. Error bars represent ± 1 S.E.

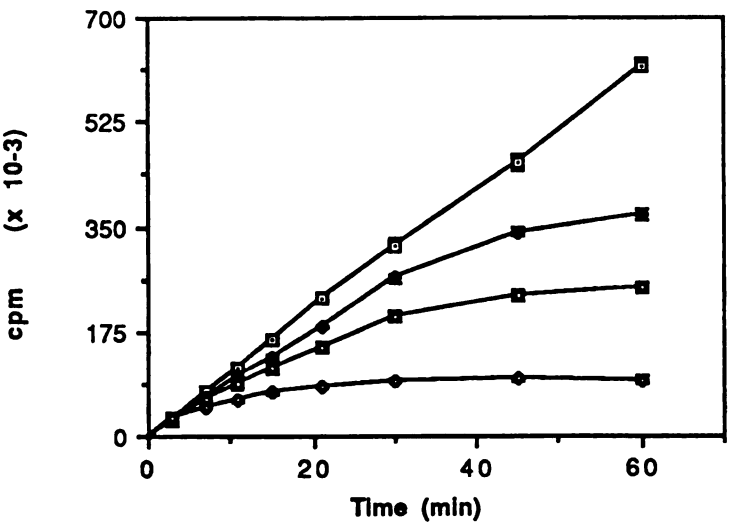

Figure 5.

Inducing Reticulocytes in Guinea Pigs

Cisplatin has demonstrated antitumor activity in a wide range of species tested. To see if the effect of cisplatin to inhibit translation is species dependent, and to facilitate the comparison of translation activity among species, an *in vitro* translation assay was derived from guinea pig reticulocyte lysates (Rosenberg and Sato, 1988b).

The first step in preparing a cell-free translation assay is to induce the formation of reticulocytes in guinea pigs. Subcutaneous administration of phenylhydrazine at a dose of 17 mg/kg daily for 4 days reduces hematocrit by almost 90% and increases the reticulocyte concentration to greater than 80% of the total red blood cell population. This dose and administration schedule are similar to those used in rabbits to induce anemia and stimulate reticulocyte formation (Merrick, 1983). On the other hand, guinea pigs are comparatively resistant to the agent acetylphenylhydrazine. A dose of 200 mg/kg of body weight, on a similar schedule as the non-acetylated form, is necessary to comparably reduce hematocrit and induce reticulocyte formation. In rabbits these agents are about equipotent (Maniatis *et al.*, 1982; Merrick, 1983) . Thus, a large species difference exists in the ability of guinea pigs and rabbits to respond to the acetylated form of this drug. This result indicates that the guinea pig erythrocytes are less sensitive to acetylphenylhydrazine and/or the guinea pig is able to eliminate this

drug more rapidly than the rabbit. Because of its greater potency, phenylhydrazine was used in our subsequent preparations.

In order to prepare lysates with the greatest activity, translation of endogenous mRNA was tested with hemolyzed reticulocytes from guinea pigs on different treatment schedules (Fig. 6). Translation activity is greatest in animals treated with a dose of 17 mg/kg body weight daily for 4 days and bled 8 days after the beginning of phenylhydrazine treatment. The eighth day following initiation of treatment corresponds to a point slightly after the peak reticulocyte concentration. During the course of phenylhydrazine treatment, the hematocrit of animals will typically fall to approximately 12% and return to about 25% at the time of maximum translation activity. Subsequently, guinea pig lysates were prepared on the eighth day from animals treated with 17 mg/kg phenylhydrazine for 4 days.

Translation in Guinea Pig Lysates

Reaction conditions for maximum incorporation of ^{35}S-cysteine, ^{3}H-leucine, or ^{35}S-methionine, into trichloroacetic acid precipitable material were determined. Figure 7 shows an experiment in which magnesium acetate was varied and ^{35}S-cysteine incorporation using endogenous mRNA was measured. The optimal concentrations of the other components of the reaction mixture were examined similarly. The ranges of the concentrations tested and the concentration giving maximal incorporation of

Figure 6. Phenylhydrazine treatment for optimization of translation activity. Guinea pigs (n=24) were bled for lysate preparation on either day 6 (solid bars), day 7 (diagonal bars), or day 8 (horizontal bars) following drug treatment with phenylhydrazine on days 1-4, at 11, 14, 17, or 20 mg/kg each day. The translation activity of each lysate was determined by measurement of the incorporation of ^{35}S-cysteine into trichloroacetic acid precipitable protein minus the unincorporated background counts as described for the translation of endogenous mRNA. The vertical line represents + 1 S.E.

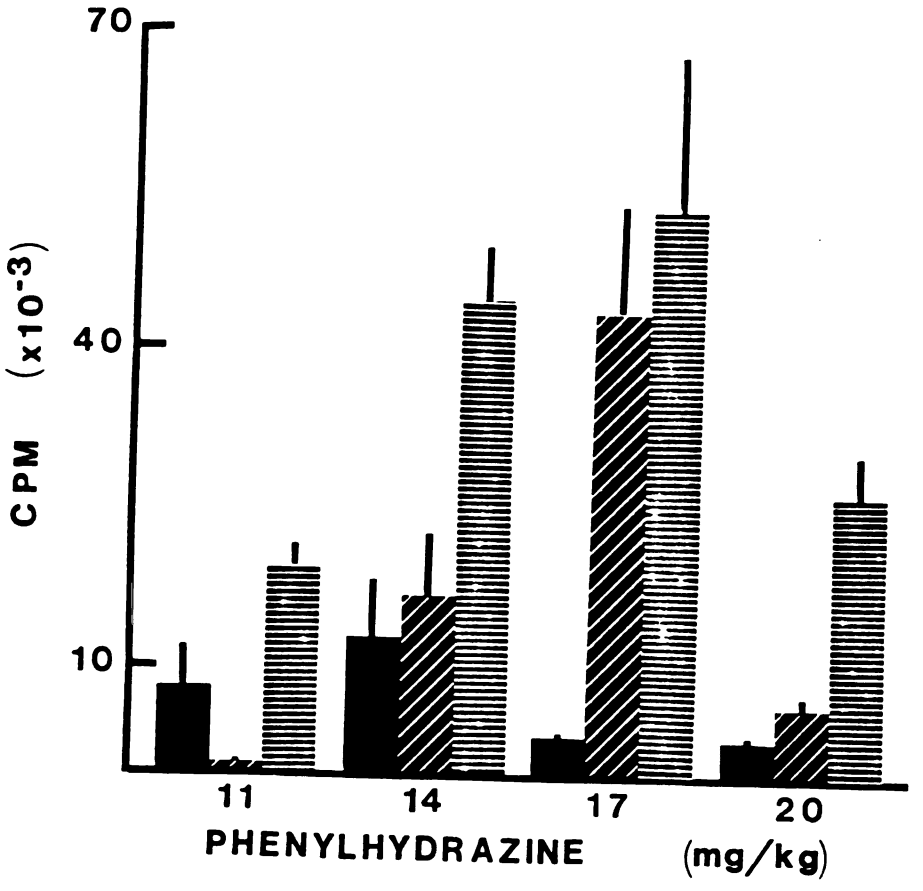

Figure 6.

Figure 7. Effect of varied magnesium ion concentration on translation activity. Endogenous mRNA was translated in the presence of increasing magnesium acetate concentrations. The translation activity of each lysate was determined by measurement of the incorporation of ^{35}S-cysteine into trichloroacetic acid precipitable protein as described for the translation of endogenous mRNA.

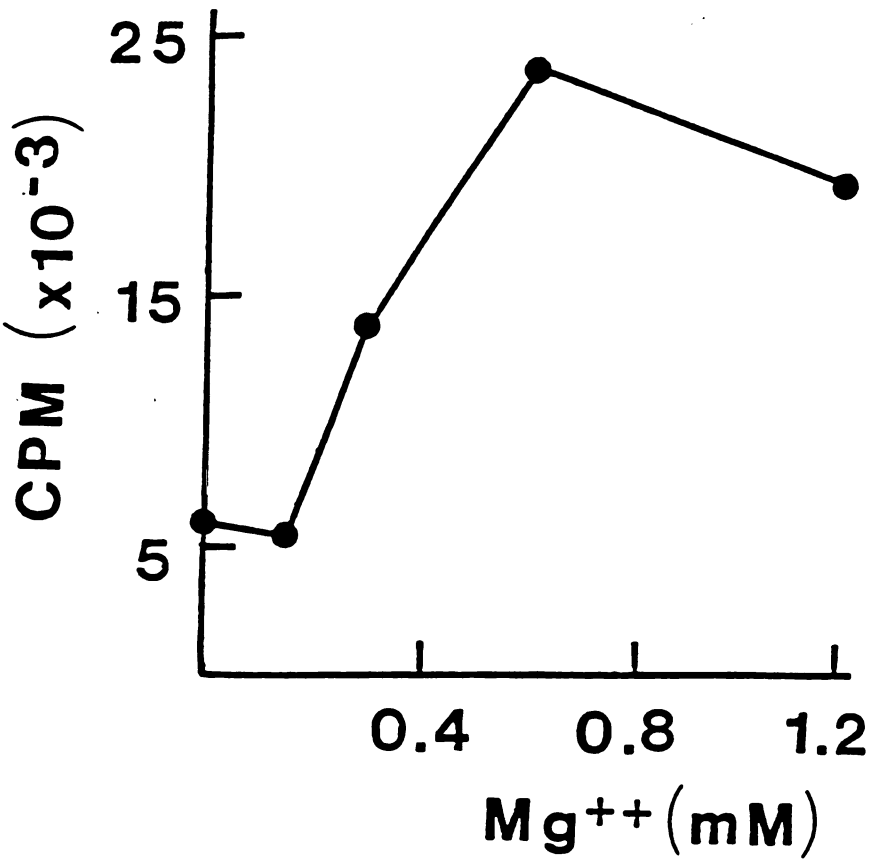

Figure 7.

radioactivity are shown in Table 1. For comparison, the optimal concentrations reported for translation in the rabbit reticulocyte system are also listed. For the most part these concentrations are similar. Some exceptions such as the creatine phosphokinase, ATP, GTP and creatine phosphate concentrations are noted. Differences appear mainly in factors that provide the energy necessary for protein synthesis. Guinea pig translation appears to have a greater dependence upon the regeneration of high energy phosphate bonds via the creatine phosphokinase system. The addition of tRNA to the guinea pig system at 55 µg/ml of lysate stimulates the translation of the exogenous BMV but not rabbit globin mRNA.

The time course of incorporation of radioactivity was determined (Fig. 8). The guinea pig reticulocyte lysate translation activity is the same as that found in rabbit as it is essentially linear for 60 min at 30°C. The two lysates differ in their ability to maintain translation activity following incubation at room temperature prior to the measurement of protein synthesis. Rabbit lysate maintains 75% of its activity after 1 hr whereas the guinea pig lysate retains 43%. This difference in stability may reflect the inactivation of some component necessary for translation or the higher activity of an inhibitory factor in guinea pig lysates. Further evidence supporting this concept is the difference in ATP requirements between rabbit and guinea pig lysates. The addition of ATP to guinea pig lysates inhibits their translation activity. Kinases responsible for inhibiting ternary complex formation and the initiation of translation are activated by ATP (Gross, 1979).

TABLE 1

Concentrations of Different Reagents for Maximal Translation Activity

Reagent	Range Tested	Concentration* Guinea Pig Optimal	Concentration* Rabbit Optimal ‡
Hemin (mM)	0-0.07	0.03	0.06
CPK (units/ml)	0-50	3.5	1.5
Calcium chloride (mM)	0-0.5	0.21	0.32
Nuclease (units/ml)	9.53-85.3	20.6	23.7
EGTA (mM)	0-33.3	0.84	0.63
Potassium acetate+ (M)	0-1	0.15	0.10
Magnesium acetate+ (M)	0-1.24	0.62	1.50
Spermidine (mM)	0-3.2	0.40	0.50
ATP (mM)	0-32	0	0.83
GTP (mM)	0-17	0.11	0.33
HEPES (mM)	0-143	27.2	20.0
Creatine phosphate (mM)	0-80	10.9	6.7
Amino acids (each)(μM)	0-286	54.5	20.0
Dithiothreitol (mM)	0-8	0	1.7

*The concentration units listed are the same across a row and represent the concentration of the reagent in the final translation volume.

+Various mRNAs differ in their requirements for these ions. The values listed are optimal for the translation of endogenous mRNA.

‡Pelham and Jackson, 1976

Figure 8. Time course of *in vitro* protein synthesis. Endogenous mRNA was translated at 30°C and samples were withdrawn at 10 min intervals. The translation activity at each time point was determined by measurement of the incorporation of ^{35}S-cysteine into the trichloroacetic acid precipitate as described for the translation of endogenous mRNA.

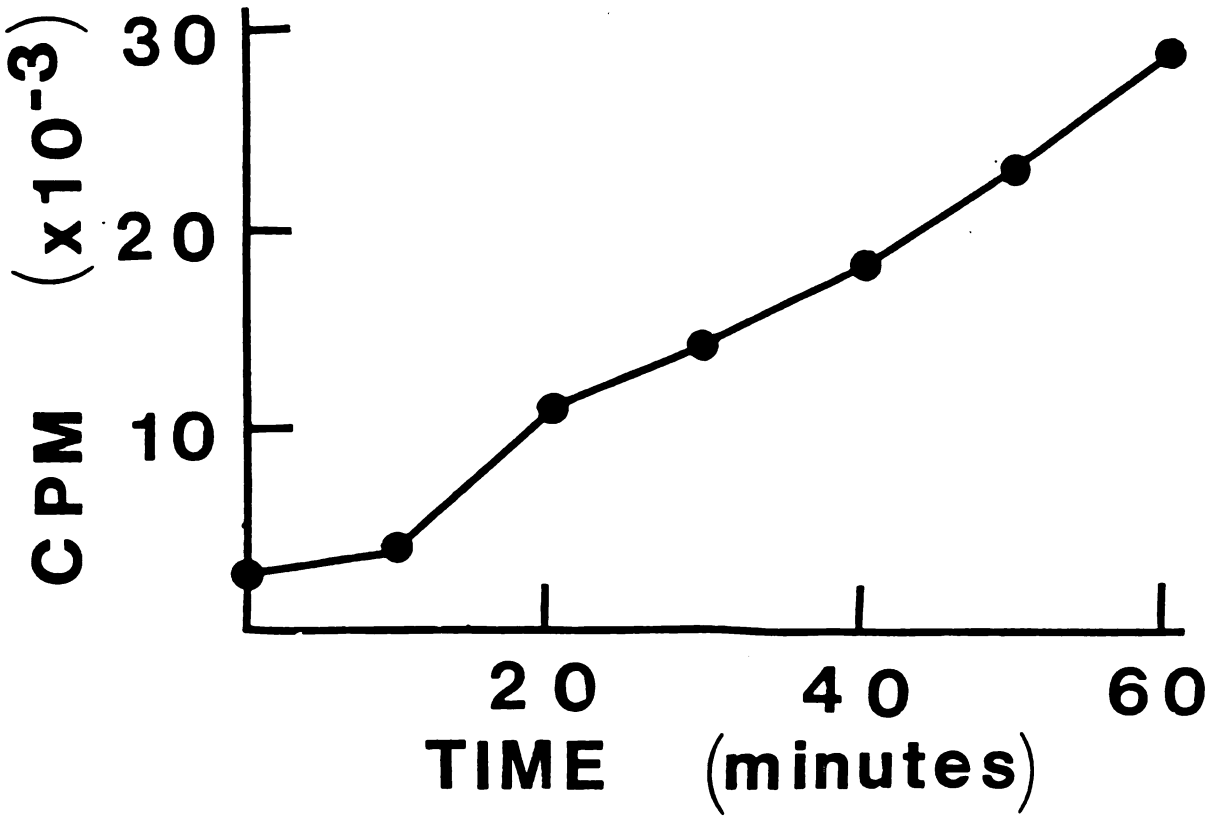

Figure 8.

Higher translation activity was obtained by fractionation of the cells from phenylhydrazine treated guinea pigs by albumin gradient centrifugation (Piomelli, *et al.*, 1967). Table 2 compares the incorporation of label by fractionated lysates. Separation of cells prior to hemolysis produces a lysate that is 10 to 200 fold more active in synthesizing protein when directed by either endogenous or exogenous mRNAs. Centrifugation through a Percoll (Sigma Chemical Company) gradient (Branch *et al.*, 1983) failed to separate cells with greater translation activity.

Translation of Exogenous mRNA in Guinea Pig Lysates

In order to translate exogenous mRNA, the message present in the reticulocyte lysate is digested by a calcium-dependent nuclease from *Staphylococcus aureus*. This nuclease is subsequently inactivated by chelating calcium with EGTA (Pelham and Jackson, 1976). Following this treatment, there is minimal incorporation of amino acids into the trichloroacetic acid precipitate in the absence of added mRNA. Unincorporated background counts for guinea pig lysates are 8 counts per minute per µl of assay volume per µg of lysate protein compared to 7 for the rabbit lysates. Incorporation of a labeled amino acid into trichloroacetic acid precipitable material by the guinea pig reticulocyte lysates is minimized at a nuclease concentration of 20.6 units/ml lysate and a calcium chloride concentration of 0.21 mM. For rabbit lysate these values are 23.7 units/ml and 0.32 mM, respectively, which is similar to the values determined for guinea pig lysates. The lysates were treated for 7.5

TABLE 2

Enrichment of Translation Activity by Density Gradient Centrifugation of Reticulocytes

mRNA	Cellular Preparation	
	Non-Fractionated	Fractionated*
Endogenous	595±102 (n=4)	7888±3450 (n=4)+
Globin	38± 38 (n=4)	7700± 249 (n=4)+
BMV	145±0.7 (n=4)	13,097±3150 (n=4)+

Values are CPM ^{35}S-methionine incorporated per µl of translation assay minus control ± S.E.M.

*Cells were fractionated by albumin density centrifugation at 130,000 x g for 2 hr through 1.076 and 1.083 g/ml layered gradients. The pellet fraction has the greatest translation activity and was used to prepare lysate as described in Materials and Methods.

+Denotes significance at $p < 0.05$.

min before translation was tested. Maximum translation of mRNA added to the nuclease treated lysate requires 2 mM EGTA. This concentration was used to inactivate the calcium-dependent nuclease.

At the optimal concentration tested in nuclease treated lysates with added rabbit globin mRNA, incorporation is usually greater than 75% of the activity with endogenous mRNA as determined using lysates not treated with nuclease. Rabbit reticulocyte lysates also translate exogenous mRNA at about 75% of the rate they translate endogenous message (Pelham and Jackson, 1976). Incorporation of ^{3}H-leucine and ^{35}S-methionine are dependent on the amount of RNA message added. At messenger RNA concentrations less than approximately 0.4 µg per 30 µl assay, rabbit globin stimulates more protein synthesis in guinea pig lysates than does BMV mRNA (Fig. 9). Translation is more effectively stimulated at low concentrations of rabbit globin mRNA in guinea pig reticulocyte lysate than at the same low concentrations of BMV mRNA. At higher message concentrations, however, translation with globin mRNA reaches a plateau whereas BMV mRNA has repeatedly demonstrated a greater ability to direct the incorporation of labeled amino acid into trichloroacetic acid precipitable material. Similar results are found with rabbit reticulocyte lysates (data not shown). The comparable translation activity of this guinea pig derived reticulocyte lysate is further demonstrated in Table 3. Both rabbit globin and BMV mRNAs direct

Figure 9. Dependence of translation on mRNA concentrations. Increasing concentrations of rabbit globin (●) and BMV (O) mRNAs were translated by a micrococcal nuclease treated guinea pig reticulocyte lysate. The translation activity of each lysate was determined by measurement of the incorporation of ^{3}H-leucine into trichloroacetic acid precipitable protein as described for the translation of exogenous mRNA.

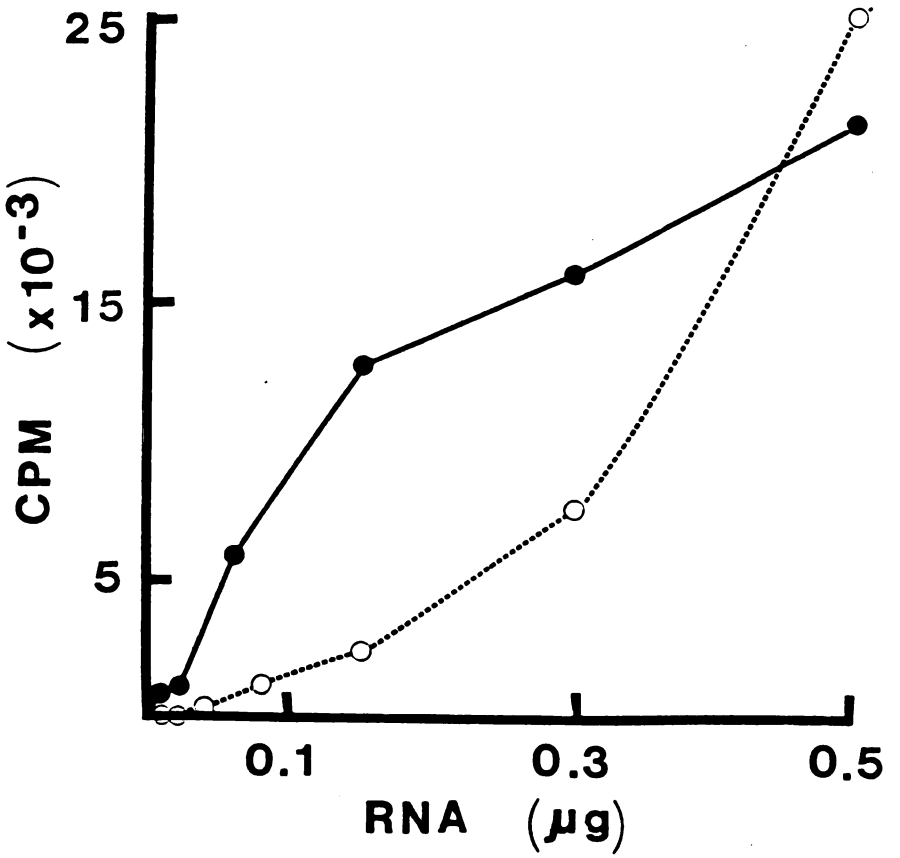

Figure 9.

TABLE 3

Comparison of the mRNA-Dependent Translation Activities in Guinea Pig and Rabbit Retibulocyte Lysates

	Lysate			
	Guinea Pig		Rabbit	
BMV mRNA*				
^{3}H-Leucine	138+ 16	(n=13)	99+ 22	(n=8)
^{35}S-Cysteine	394+ 33	(n=18)	429+ 40	(n=8)
^{35}S-Methionine	2290+123	(n=4)	3840+ 100	(n=5) ‡
Rabbit Globin mRNA+				
^{3}H-Leucine	292+ 66	(n=4)	418+ 23	(n=3)
^{35}S-Cysteine	4230+349	(n=6)	1020+ 78	(n=11) ‡
^{35}S-Methionine	10600+345	(n=4)	8120+3250	(n=4)

Values are CPM incorporated per µl of assay volume per µg lysate protein per µg mRNA ± S.E.M.

*The range of rabbit globin mRNA tested was 0.01 to 0.5 µg per assay.

+The range of BMV mRNA tested was 0.1 to 3.5 µg per assay.

‡Denotes significance at $p < 0.05$.

translation in the guinea pig system with activity similar to that found in the rabbit system.

Protein products of a mRNA dependent translation assay directed by BMV mRNA were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 10). This is a qualitative comparison of the translation products of the guinea pig and rabbit systems. The proteins synthesized by the same mRNA in the two lysates are shown to be alike as the electrophoretic patterns and intensities are similar. In this fluorograph the volume of samples electrophoresed is the same; thus, the similarity of the intensity of the equal molecular weight bands further emphasizes the quantitative similarity of protein synthesis of each protein translated.

Effective Cisplatin Concentrations for Inhibition of Translation

The cisplatin effect on translation was then observed in *in vitro* preparations from two species, rabbit and guinea pig (Fig. 11). Lysates were preincubated with concentrations of cisplatin for various periods of time up to 60 min. As shown, cisplatin inhibition increases during the time of preincubation with the lysates. Furthermore, the figure demonstrates increased inhibition with increasing concentrations of the drug. Lysates from the two species behave similarly, both in terms of the effective concentrations and the time course of the effect.

Figure 10. Qualitative similarity of translation products from guinea pig and rabbit reticulocyte lysates. The translation products of BMV mRNA in rabbit and guinea pig reticulocyte lysates were electrophoretically separated on a 7.5% SDS-polyacrylamide gel with a 2.5% stacking gel (Laemmli, 1970). The gel was fixed in 10% acetic acid and fluorographed as described (Chamberlain, 1979). The mRNA was translated in the presence of ^{35}S-methionine as described for the translation of exogenous mRNA. Lanes 1 and 2 are minus mRNA controls for rabbit and guinea pig lysates, respectively; lanes 3 and 4 contain BMV mRNA with rabbit and guinea pig lysates, respectively.

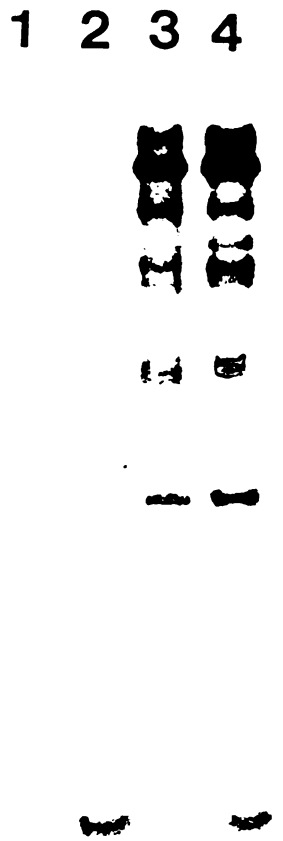

Figure 10.

Figure 11. Time course of cisplatin inhibition of *in vitro* translation. Rabbit reticulocyte lysates were preincubated with 30 (●), 60 (▲), 96 (■), and 240 (▼) μM cisplatin for 0, 7.5, 15, 30, or 60 min at 22°C. Guinea pig reticulocyte lysates were preincubated at 30 μM cisplatin (0) for 0, 2.5, 5, 10, 20, and 40 min and at 100 μM cisplatin (Δ) for 2.5, 5, 10, and 20 min at 22°C. Lysate was prepared for the translation of endogenous mRNA as described in Materials and Methods. In order to examine the progression of cisplatin inhibition at several time points yet minimize the loss of translation activity that occurs at room temperature, lysate was aliquoted into assay tubes that were placed on dry ice. At the appropriate time before the start of translation, assay tubes were thawed and combined with cisplatin. Inhibition of translation was determined by comparison to assays preincubated for identical times in the absence of cisplatin. Each point represents six individual assays. Error bars represent ± 1 S.E. Best fit lines were plotted by second order regression.

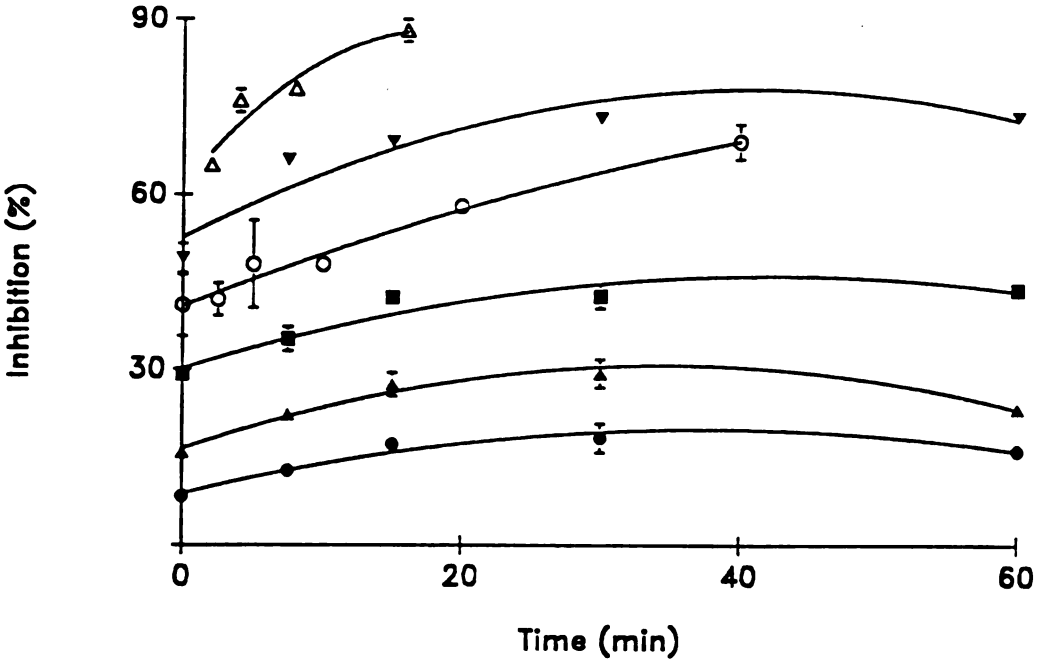

Figure 11.

The relationship between cisplatin concentration and inhibition of peptide synthesis was more thoroughly examined. As shown in figure 11, the cisplatin effects on translation are near maximal after 30 minutes, therefore, this length of time was chosen for the experiments in which cisplatin preincubation was utilized. Concentrations of cisplatin between 3 and 800 μM were incubated with guinea pig and rabbit reticulocyte lysates. These were then assayed for their ability to incorporate amino acids into trichloroacetic acid precipitable material. The concentration dependence of the cisplatin inhibition of translation of endogenous messenger RNA into peptides is shown in figure 12. In the guinea pig system, cisplatin detectably inhibits peptide synthesis at a concentration as low as 3 μM. A concentration of 39 μM gives fifty percent inhibition. Synthesis with rabbit lysate is also detectably inhibited at the lowest concentration of cisplatin tested (4 μM). The IC_{50} for cisplatin inhibition of translation of endogenous mRNA is 98 μM in rabbit reticulocyte lysates. Best fit lines are plotted for both species. The guinea pig and rabbit best fit lines are parallel having no significant difference in the slopes of their linear regions by analysis of regression at $p < 0.01$.

Cisplatin inhibition of the incorporation of labeled amino acid was also demonstrated in translation assays directed by exogenously supplied mRNA. The endogenous mRNA in the reticulocyte lysate was digested with nuclease. Translation was then carried out by adding BMV mRNA. Peptide synthesis is inhibited with an IC_{50} of 59 μM cisplatin in these translation assays.

Figure 12. Relation of cisplatin concentration to the extent of inhibition of translation activity in reticulocyte lysates. Endogenous mRNA was translated by guinea pig (O) and rabbit (●) lysates after preincubation with increasing concentrations of cisplatin for 30 min at 22°C. Inhibition of translation was determined by comparison with assays preincubated in the absence of cisplatin. Each point represents at least six individual assays. Error bars represent ± 1 S.E. Best fit lines were plotted by third order regression.

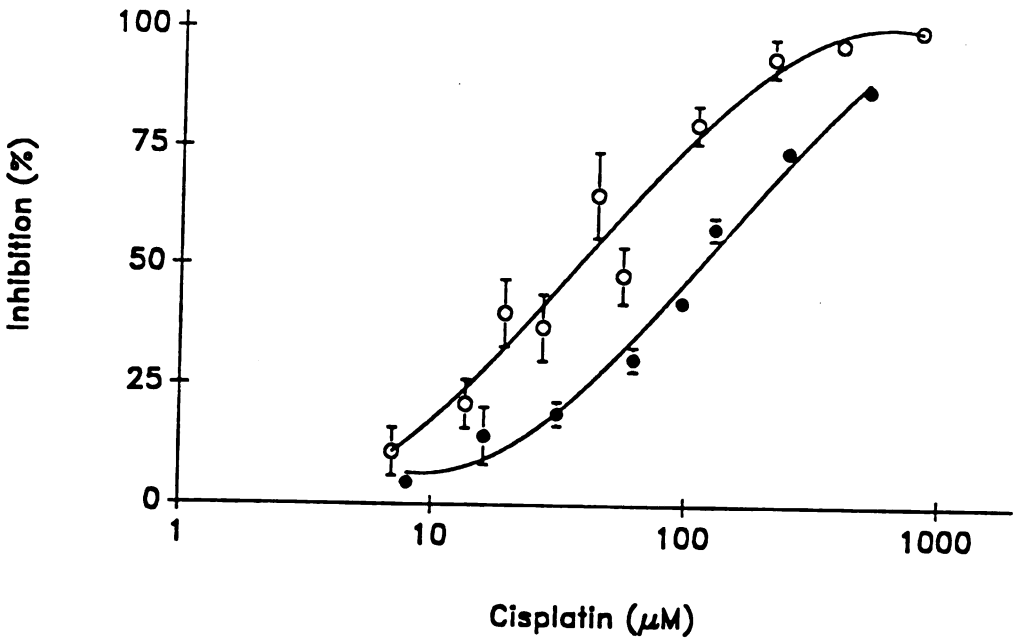

Figure 12.

This IC_{50} is on the same order as those from rabbit and guinea pig assays of endogenous mRNA.

Cisplatin Inhibition is Not Caused by a Contaminant

Trans-diamminedichloroplatinum (II) (Transplatin) was utilized to investigate the importance of stereochemistry in the inhibition of protein synthesis by cisplatin. The relationship between transplatin concentration and peptide synthesis was examined. Concentrations of transplatin between 30 and 350 μM were added to rabbit reticulocyte lysates and allowed to translate endogenous mRNA for 45 min. As shown in figure 13, transplatin inhibits peptide synthesis. A concentration of 80 μM transplatin gives 50% inhibition of translation. The fifty percent inhibitory concentration of transplatin is similar to that found for cisplatin. The slope of the linear region of the transplatin log dose response curve is, however, significantly different from that of the cisplatin curve by analysis of regression at $p < 0.01$. This difference implies that cisplatin and transplatin inhibit translation by different mechanisms.

Inhibition may be dependent upon the particular label used to measure peptide synthesis. Numerous experiments have demonstrated an interaction between sulfur containing compounds and cisplatin (Nakagawa, *et al.*, 1988; Milano, *et al.*, 1987). If cisplatin directly interacts with the labels used to measure translation then a greater effect with sulfur containing amino acids

Figure 13. Cisplatin and transplatin have different dose-response relationships. Endogenous mRNA was translated by rabbit lysates after preincubation with increasing concentrations of cisplatin (□) or transplatin (◆) for 30 min at 22°C. Inhibition of translation was determined by comparison with assays preincubated in the absence of cisplatin and transplatin. Each point represents at least six individual assays. Error bars represent ± 1 S.E. Best fit lines were plotted by first order linear regression fit to the linear portions of each curve.

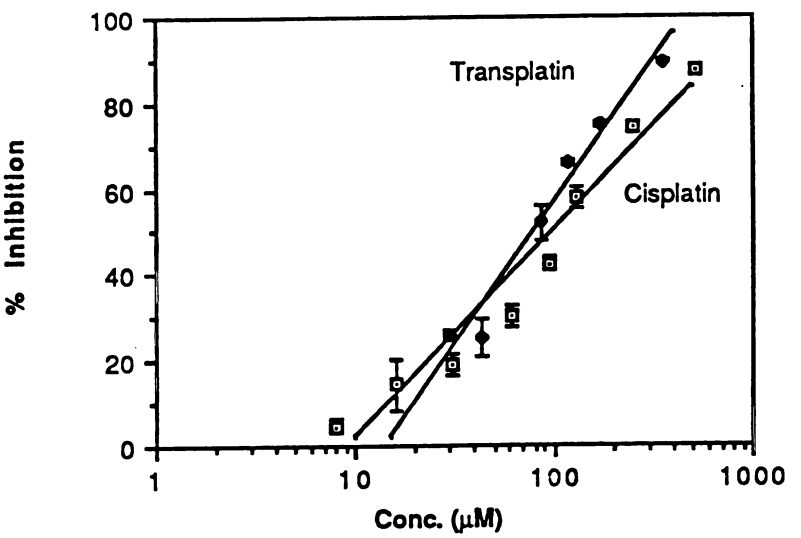
100
80
60
40
20
0
% Inhibition
Transplatin
Cisplatin
1
10
100
1000
Conc. (μM)

Figure 13.

would be anticipated. Three different amino acids, ^{3}H-leucine, ^{35}S-cysteine, and ^{35}S-methionine, were added in separate assays to observe the relationship between cisplatin inhibition and the label incorporated. Translation was measured in lysates preincubated with 114 μM cisplatin as the incorporation of ^{3}H-leucine, ^{35}S-cysteine, or ^{35}S-methionine into trichloroacetic acid precipitates. In these assays, essentially the same inhibition of translation was found at 73, 76, and 72%, respectively. This indicates that the observed inhibition of protein synthesis is not an artifact of the assay protocol caused by a reaction between a labeled amino acid and cisplatin.

It was shown that the cisplatin or transplatin solutions appear to be pure and are not contaminated with their isomer or another substance which is blocking peptide synthesis. First, the cisplatin has been assayed for purity by Bristol and is the reference standard used in manufacturing the drug for commercial use. Second, when injected onto HPLC, both cisplatin and transplatin are detected as single sharp peaks (Fig. 14). Peak heights and areas under the curve are proportionate to the concentrations of cisplatin and transplatin injected onto the HPLC column. No other peaks are found. Furthermore, cisplatin and transplatin demonstrate different retention times so cross contamination with an isomer can be detected.

Figure 14. High performance liquid chromatographic (HPLC) analysis of cisplatin and transplatin solutions. Cisplatin (a), transplatin (b), or a combination of both (c) were dissolved in the mobile phase (0.3 mg/ml hexadecyltrimethylammonium bromide and 9 mg/ml sodium chloride in deionized water). Cisplatin and transplatin, 17.5 μg each, were separated following injection onto a C_8 zorbax 4.6 mm x 25 cm column with a C_8 butterfly precolumn at a flow rate of 1.2 ml/min. Eluted products were detected by absorbance at 313 nm.

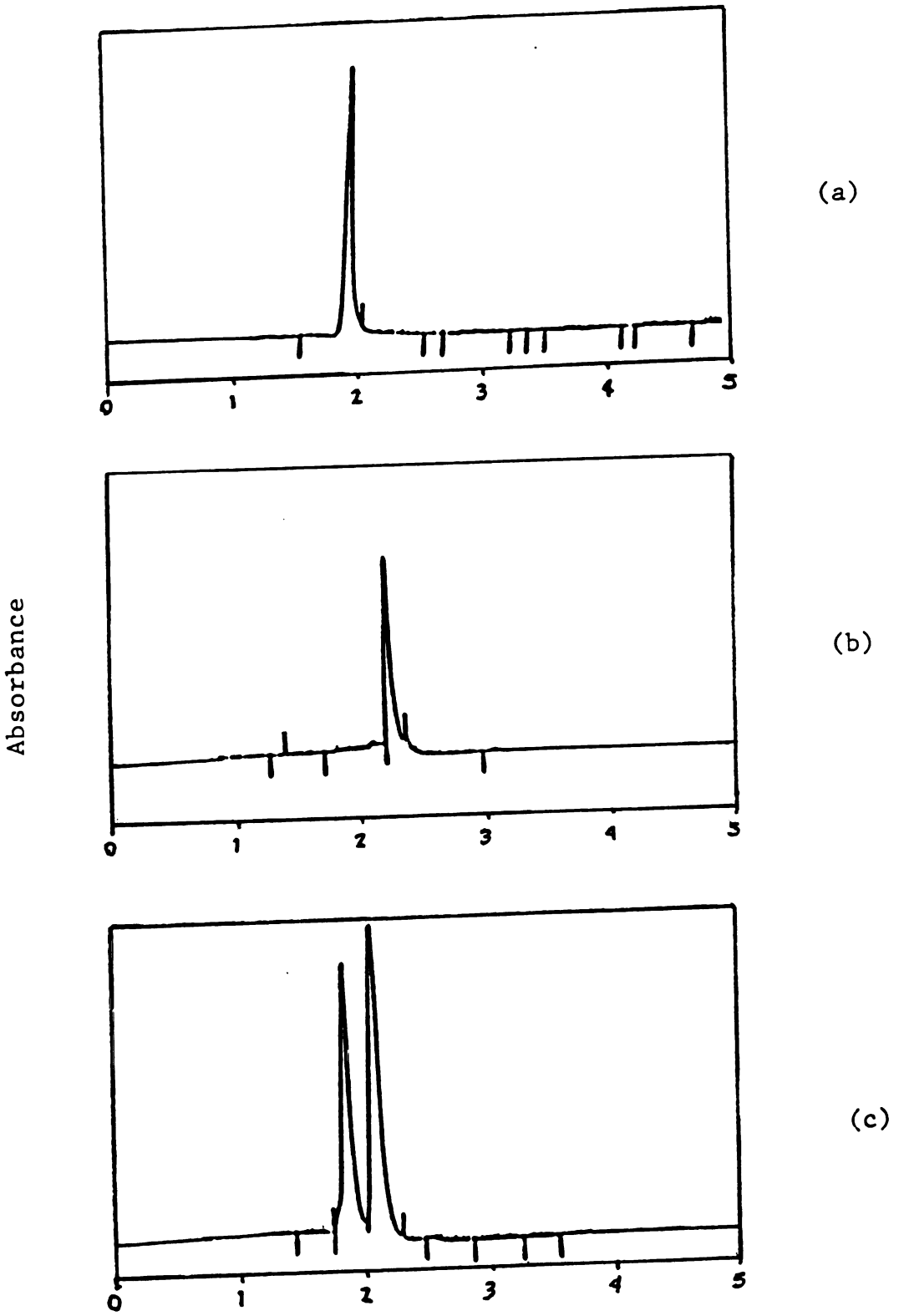

Figure 14.

As HPLC is limited to detecting only substances able to pass the column under the prescribed conditions, thin layer chromatography (TLC) was utilized to further assay the purity of cisplatin and transplatin used to inhibit translation. Cisplatin and transplatin were each dissolved in dimethylformamide at 2 mg/ml. Following spotting (10-40 μg) on a Whatman K5F TLC plate, the compounds were separated with an acetone:nitric acid mobile phase (9:1). The chromatograms were visualized by developing the plate with iodoplatinate spray. Cisplatin showed a single peak without smearing or detectable contaminants. Transplatin purchased from Strem chemical company demonstrated a similar pattern and was without apparent contamination. Transplatin purchased from Sigma Chemical, however, showed considerable smearing and appeared to be contaminated. The latter preparation was not used in experiments to inhibit translation. Impurities were not evident in cisplatin from Bristol or transplatin purchased from Strem chemical. Thus, inhibition caused by the previous two compounds appears to be caused by cisplatin and transplatin and not a contaminant.

Cisplatin Selectively Inhibits Peptide Products

To determine whether cisplatin caused qualitative changes in the peptide products being synthesized they were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 15). Samples in lanes 1-6 were withdrawn from rabbit endogenous mRNA assays exposed to various concentrations of the drug for different lengths of time.

Figure 15. Qualitative differences in translation products from cisplatin-exposed assays. Products from cisplatin inhibited translation assays were electrophoretically separated on 7.5% SDS-polyacrylamide gels (Laemmli, 1970). The gels were fixed in 10% acetic acid and fluorgraphed as described (Chamberlain, 1979). The lanes were loaded as described in the text. Lanes 1-6 were each loaded with an equal number of dpm. These show the products of translation directed by endogenous rabbit mRNA. Lane 1 was preincubated 30 min at 22°C in the absence of cisplatin while lanes 2, 3, and 4 were preincubated with 16, 96, and 512 μM cisplatin, respectively. Product in lanes 5 and 6 were each preincubated with 100 μM cisplatin for 2.5 and 40 min, respectively. Lanes 7 and 8, each loaded with an equal number of dpm, show products from the translation of BMV mRNA by message dependent rabbit lysate. Both assays were preincubated at 22°C for 30 min, lane 7 in the absence and lane 8 in the presence of 96 μM cisplatin.

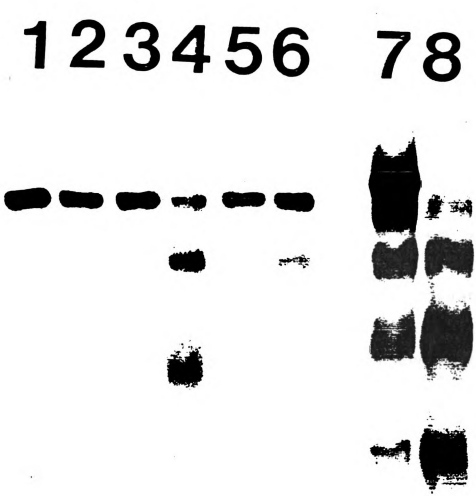

Figure 15.

Larger volumes of the inhibited translation assays were electrophoresed so that comparable amounts of incorporated radioactivity were placed in each lane of the gel. The total trichloroacetic acid precipitable radioactivity loaded into each of the lanes 1-6 was 5200 dpm. The assay in lane 1 was not exposed to cisplatin. The lanes 2-4 are analyses of translation assays incubated with 16, 96, and 512 μM cisplatin, which were inhibited 9, 45, and 87%, respectively. Lanes 5 and 6 show the translation products of lysates preincubated with 100 μM cisplatin for 2.5 and 40 minutes, which were inhibited 45%, and 71%, respectively. The intensity of the bands, or amount of radioactivity associated with each polypeptide of a given size, decreases more for the larger, more slowly migrating bands, than for the rapidly migrating peptides. Because equal counts were loaded into each lane, the differences in the banding are emphasized. Loading the same number of counts of samples containing less large product means that more label is carried in the form of smaller peptides. Products of a mRNA dependent translation assay directed by BMV mRNA also demonstrate a more profound decrease in high molecular weight products. Equal amounts of incorporated amino acid (35,700 dpm each lane) were also compared in lanes 7 and 8. Lane 7 contains product from an assay not exposed to cisplatin. The assay in lane 8 was preincubated with 96 μM cisplatin which resulted in 85% inhibition.

The extent of synthesis inhibition of the various peptide products was quantitated. Another gel was prepared to separate equal volumes from assays translating BMV mRNA. Two lanes were

loaded with product, one from an assay without drug and in the other the mRNA was preincubated with 96 μM cisplatin which resulted in 85% inhibition. These lanes were each cut from the gel and sliced into fractions of equal length so that the counts per minute in fractions with the same migration could be compared. For each fraction from the cisplatin exposed assay, the amount of inhibition was calculated relative to the uninhibited assay (Fig. 16). Large peptide products, those with molecular weights greater than approximately 130,000, an M_r on the order of 0.25, are inhibited to the same degree, nearly 90%. The relationship between molecular weight and percent inhibition is linear for those peptides migrating faster than 130,000 and slower than approximately 50,000. The synthesis of peptides of 53,000 molecular weight is inhibited 50% by cisplatin. Peptides smaller than 50,000 are inhibited on the order of 40% when compared to assays run in the absence of cisplatin.

Cisplatin Inhibition is mRNA Dependent

By using nuclease treated lysates exogenous mRNA-dependent lysates, it was possible to test whether cisplatin inhibits peptide synthesis by an interaction with mRNA or with other components of the translation system. Various concentrations of cisplatin were preincubated with either mRNA, the messenger dependent lysate fraction, or both fractions (Fig. 17). The final concentration of cisplatin was the same for each experiment. Only when cisplatin was preincubated with the mRNA was the full inhibitory effect

Figure 16. Cisplatin preferentially inhibits translation products with slower electrophoretic mobility. An equal volume of products from two translation assays were electrophoretically separated on a 7.5% SDS-polyacrylamide gel (Laemmli, 1970). The gel was fixed in 10% acetic acid and fluorographed as described (Chamberlain, 1979). The assays were preincubated 30 min at 22°C, one with 96 μM cisplatin and the other in the absence of drug. The lanes were cut from the gel and then into 0.7 x 1.0 cm fractions. The fractions were rehydrated in 0.15 ml water for 1 hr at 22°C and dissolved by incubation with 0.5 ml of 30% hydrogen peroxide at 80°C for 4 hr. After the vials cooled, 10 ml of scintillation cocktail were added to each sample and they were counted by liquid scintillation spectrometry. Inhibition of translation of the separated products was determined by comparing the counts in segments representing equal migration from the cisplatin pretreated and untreated translation assays.

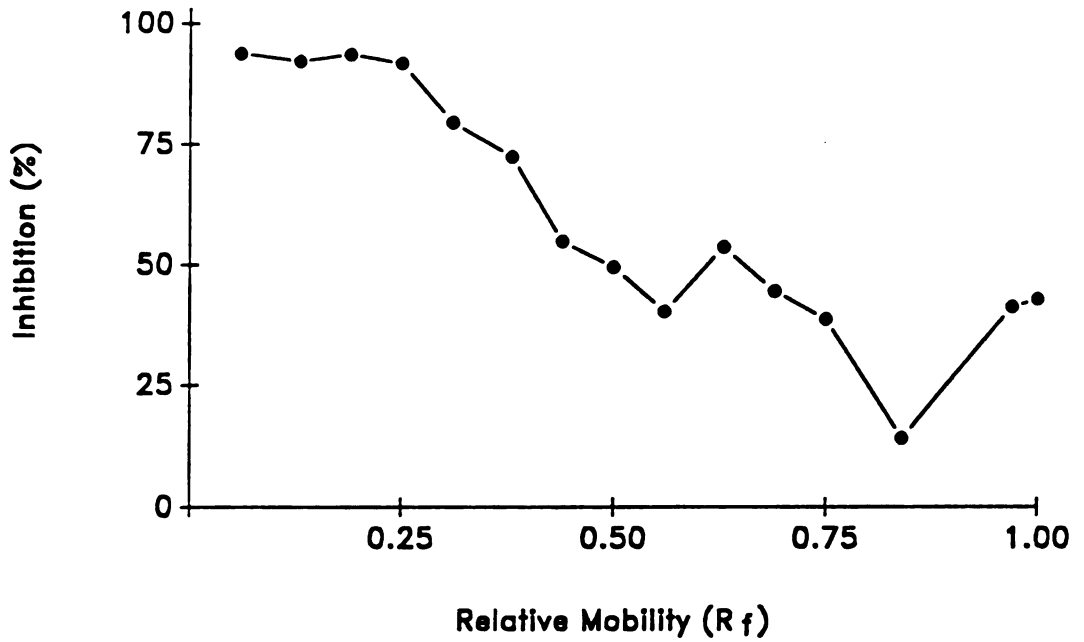
100
75
50
25
0
Inhibition (%)
0.25
0.50
0.75
1.00
Relative Mobility (R f)

Figure 16.

Figure 17. Inhibition of translation by cisplatin follows preincubation with exogenous mRNA in a message dependent translation assay. Cisplatin at concentrations of 0, 30, 60, 96, or 240 μM was preincubated with either rabbit mRNA dependent lysate (0), BMV mRNA (●), or both fractions (Δ) for 30 min at 22°C. After the preincubation, the mRNA and lysate were combined and translation was measured. Inhibition of translation was determined by comparison with the assays preincubated in the absence of cisplatin. Each point represents six individual assays. Error bars represent ± 1 S.E. Best fit lines were plotted by third order regression.

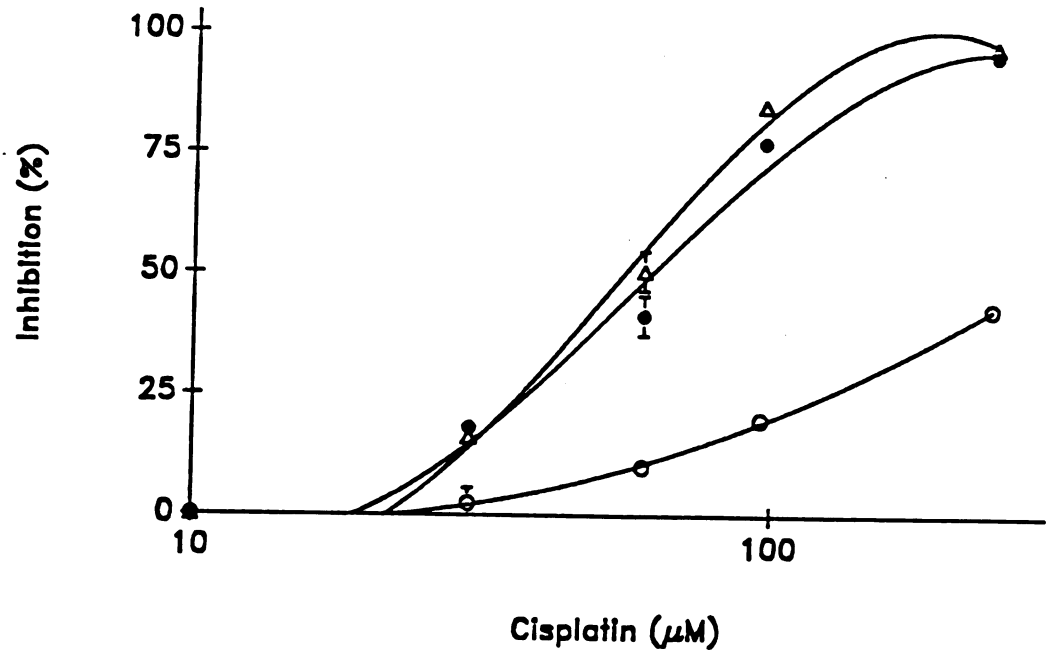

Figure 17.

observed. When the mRNA fraction was incubated with 30 μM cisplatin, then added to a nuclease treated lysate, peptide synthesis was inhibited 85%. In contrast, when a nuclease treated lysate was incubated with the same concentration of cisplatin, then mRNA was added, translation was inhibited only 19%. The fact that inhibition is much greater when the drug is preincubated with the exogenous mRNA than it is when lysate without message is preincubated with the drug suggests that an interaction between cisplatin and the mRNA is critical for the reduction in peptide synthesis activity.

The inadvertent addition of a ribonuclease is a major concern when conducting cell free translation assays. Such contamination would cause the inhibition of peptide synthesis by degrading the mRNA template. Ribonucleases are ubiquitous, they are found on the skin, in water, and on glassware. Therefore, great care is taken in treating solutions and glassware prior to their use to denature ribonucleases. Thus, it was necessary to demonstrate that the cisplatin was not contaminated by ribonuclease. Furthermore, the possibility that cisplatin acts to degrade mRNA was also ruled out by analyzing a sample of mRNA, preincubated with cisplatin, by denaturing agarose gel electrophoresis. This sample was compared to an identical sample of mRNA not preincubated with the drug (Fig. 18). The resulting electrophoretic patterns are not distinguishable. Additionally, the cisplatin inhibition of translation is not reversed by RNasin. RNasin is a protein isolated from human placenta which specifically inhibits ribonucleases. It functions by binding noncovalently to a wide spectrum of ribonucleases. RNasin was

Figure 18. Messenger RNA is not degraded by incubation with a cisplatin solution. BMV mRNA (3.5 μg), after an incubation in the absence (lane 2) or presence (lane 3) of 300 μM cisplatin for 30 min at 22°C, was analyzed by electrophoresis in a 0.8% denaturing agarose gel. RNAs were denatured with glyoxal and dimethyl sulfoxide before elecrophoresis and were stained with ethidium bromide as described (Maniatis, *et al.*, 1982). Glyoxylated total RNA was loaded in lanes 1 and 4; the 18s and 28s ribosomal bands are visible.

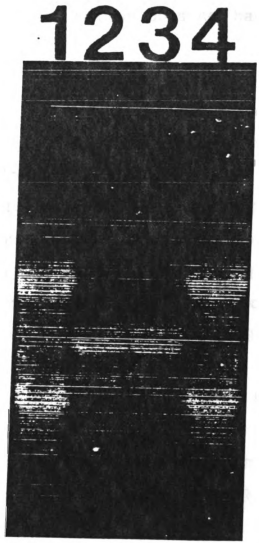

Figure 18.

added to certain lysates at 1 unit/µl of assay volume. Cisplatin inhibited translation to the same extent in the presence and absence of added ribonuclease inhibitor. The apparent lack of degradation of the mRNA incubated with the drug and the inability of RNasin to alter its effect indicates that the cisplatin solution does not have a ribonuclease activity.

The inhibition of translation by cisplatin appears to be the result of a reaction between the drug and mRNA that does not result in the digestion of the message. An interaction between mRNA and cisplatin was suggested in the fractionated mRNA dependent lysates and it is now known that this relationship does not involve digestion of the message. Therefore, message preincubated with the drug was separated on non-denaturing agarose gels to demonstrate a direct interaction. The migration of mRNA is altered following treatment with cisplatin and electrophoresis in non-denaturing agarose gels (Fig. 19). Lane 1 was loaded with 1.5 µg BMV mRNA, lane 2 with BMV mRNA preincubated with 100 µM cisplatin 30 min at room temperature, and in lane 3 the message was preincubated with 200 µM cisplatin before being loaded into the non-denaturing 1.35% agarose gel. The resulting migration pattern suggested that cisplatin acted to slow the movement of BMV mRNA.

To further demonstrate the effect of cisplatin on the movement of RNA in a non-denaturing agarose gel, the RNA, detected as A_{260} absorbance, was scanned in each lane (Fig. 20). In these gels, the migration of identical samples of RNA varies with changes

Figure 19. Migration of mRNA is altered following incubation with cisplatin. BMV mRNA (1.5 μg), was incubated in the absence of cisplatin (lane 1), presence of 100 μM cisplatin (lane 2), or presence of 200 μM cisplatin (lane 3) for 30 min at 22°C. The products of these reactions were analyzed by non-denaturing electrophoresis in a 1.35% agarose gel prepared in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) (Maniatis, *et al.*, 1982). Following electrophoresis at 1.5 V/cm for 16.5 hr, the gel was stained with ethidium bromide (Maniatis, *et al.*, 1982).

Figure 19.

Figure 20. Migration of native RNA is altered following incubation with cisplatin. BMV mRNA (2.5 μg) (panel a) or total RNA (10 μg) (panel b) was incubated in the absence of cisplatin (broken lines) or presence of 200 μM cisplatin (solid lines) for 30 min at 22°C. The products of these reactions were separated by non-denaturing electrophoresis in a 1.35% agarose gel prepared in TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA) run in the presence of 5 μg/ml ethidium bromide (Maniatis, *et al.*, 1982). Following electrophoresis at 1.5 V/cm for 16.5 hr, the individual lanes were sliced from the gel and scanned at 260 nm using a Beckman recording spectrophotometer (model UV 5260) with a gel scanning attatchment. Absorbance was recorded at 260 nm as a function of the gel length. Migration is from right to left in the tracings.

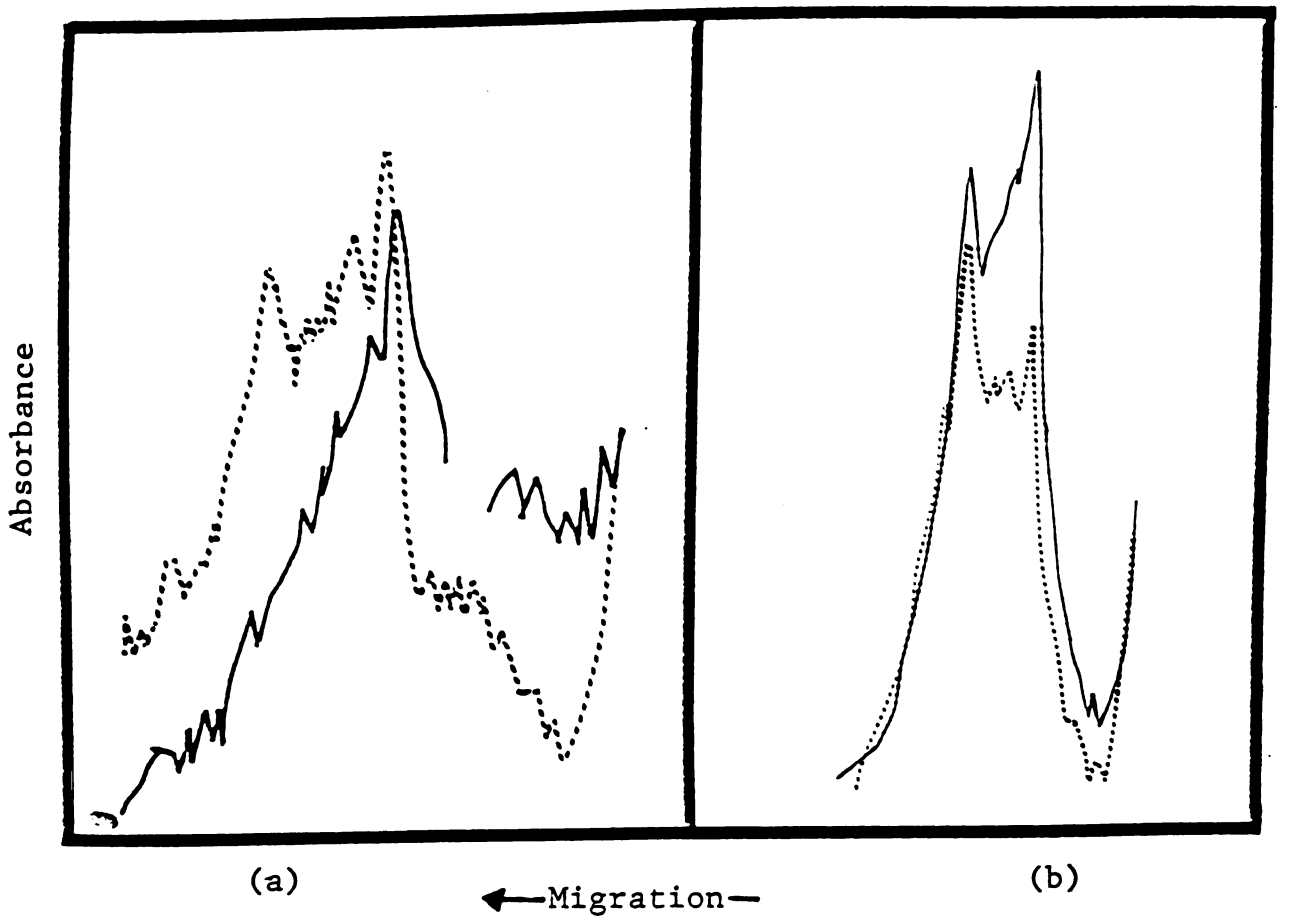

Figure 20.

in its structural configuration. Panel a shows a A_{260} scan of a lane loaded with 2.5 μg of BMV mRNA (broken line), the mRNA in another lane was preincubated with 200 μM cisplatin before being separated in the gel (solid line). The A_{260} absorbing material is shown to be shifted nearer to the top of the gel which indicates migration of RNA is much slower after preincubation with cisplatin. In panel b the scan demonstrates the pattern of A_{260} absorbing material found after separating 10 μg of total RNA in a non-denaturing agarose gel following preincubation in the absence (broken line) or presence (solid line) of 200 μM cisplatin. Ribosomal RNA bands predominate total RNA preparations and migrate at 18s and 28s with messenger RNA migrating in between the two. The RNA preincubated in the presence of cisplatin in panel b shows no apparent shift in the two major absorbance peaks whereas the A_{260} absorbing material between them is noticeably shifted toward the top of the gel. The effect of cisplatin to slow the migration of RNA appears to be limited to the BMV mRNA and the material between the two major A_{260} absorbance peaks in the total RNA. A RNA ladder is a mixture of non-messenger RNAs used as molecular weight markers in an agarose gel. When preincubated with 200 μM cisplatin and separated in a non-denaturing 1.35% agarose gel, RNA ladder shows no alteration in its migratory pattern (data not shown).

Dithiobiuret Prevents Cisplatin Inhibition

Experiments were then conducted to reverse the effects of cisplatin on translation. Substances that specifically interact with

this drug should alter its ability to inhibit peptide synthesis. A reaction of cisplatin with the chelator dithiobiuret can be demonstrated spectrophotometrically (Fig. 21). Neither cisplatin nor dithiobiuret show any increase in absorbance over time at 300 nm. When cisplatin is combined with dithiobiuret, however, absorbance increases over time. This increase in absorbance suggests a reaction between the two compounds. A reaction also occurs between dithiobiuret and the isomer transplatin. When incubated under conditions similar to those used for cisplatin, transplatin and dithiobiuret also result in an increase in absorbance over time at 300 nm. When compared to the dithiobiuret-cisplatin reaction, the addition of dithiobiuret to transplatin shows a greater initial increase in absorbance with time. This difference in absorbance suggests the isomers interact differently with dithiobiuret.

The reaction between dithiobiuret and cisplatin and between dithiobiuret and transplatin can also be demonstrated and quantitated by injecting the reaction mixture onto HPLC (Fig. 22). The injection of 8.75 μg of cisplatin and a trace of dithiobiuret resulted in the first chromatograph (a). Following a preincubation of cisplatin with a 10 fold molar excess of dithiobiuret for at least 15 min, an amount of cisplatin equal to that in the first chromatograph (a) was injected. A significant reduction in the cisplatin peak is noted while dithiobiuret demonstrates a large absorbance peak at 313 nm (c). A small, broad, region of absorbance is also noted at a retention time of approximately 4 min. The third chromatograph (c)

Figure 21. Reaction of cisplatin and transplatin with dithiobiuret. The change in absorbance was recorded at 310 nm as a function of time using a Beckmann UV 5260 recording spectrophotometer. Changes in absorbance with time are shown for solutions of 3.3 mM dithiobiuret (line A) and 1.0 mM cisplatin and 1.0 mM transplatin (line B). Equal volumes of the dithiobiuret and cisplatin solutions (line C) and dithiobiuret and transplatin solutions (line D) were combined and the change in absorbance was measured. Dithiobiuret, cisplatin, and transplatin solutions were prepared in 0.1 M sodium acetate buffer, pH 5.0. The reference cell contained the same buffer.

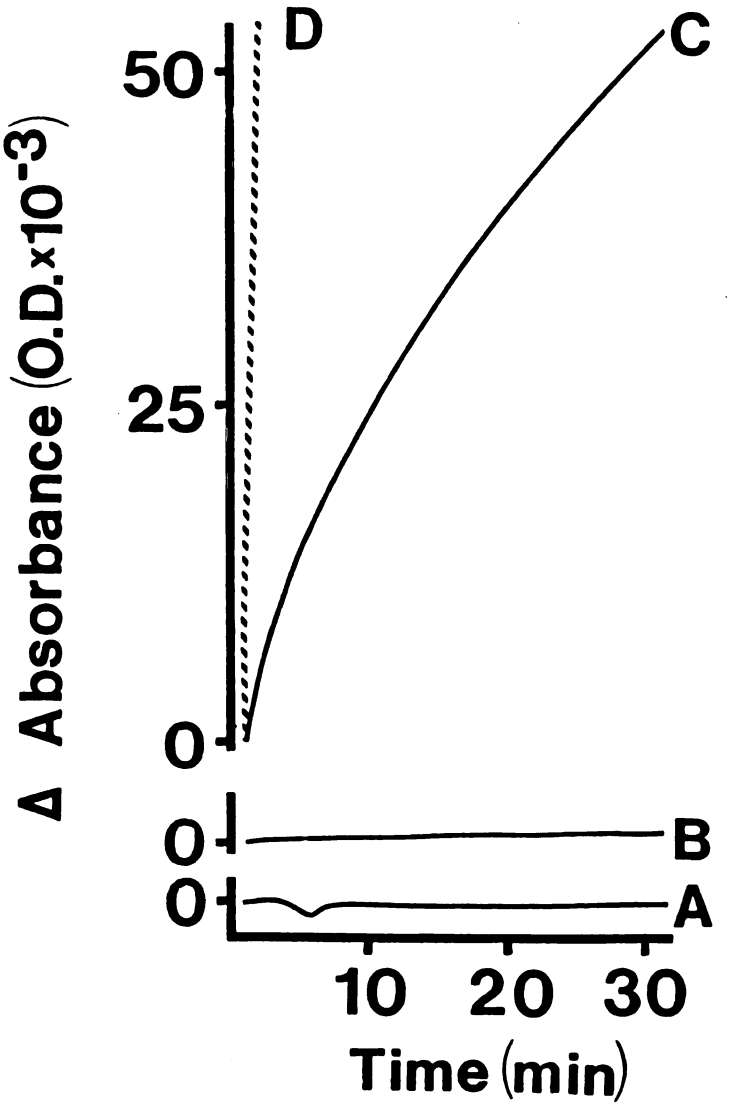

Figure 21.

Figure 22. Dithiobiuret reacts with transplatin more completely than cisplatin. Equal volumes of 0.6 mM cisplatin or transplatin were reacted with 6.0 mM dithiobiuret dissolved in mobile phase. Following the combination of dithiobiuret and cisplatin (chromatograph b) or dithiobiuret and transplatin (chromatograph d) for 15 min at 22°C, the reaction was analyzed following separation by HPLC as described for figure 14. The amount of cisplatin or transplatin injected onto the column following the reaction with dithiobiuret was 17.5 μg. Chromatographs a and c demonstrate the absorbance at 313 nm of 17.5 μg each cisplatin and transplatin, respectively, incubated in the absence of dithiobiuret.

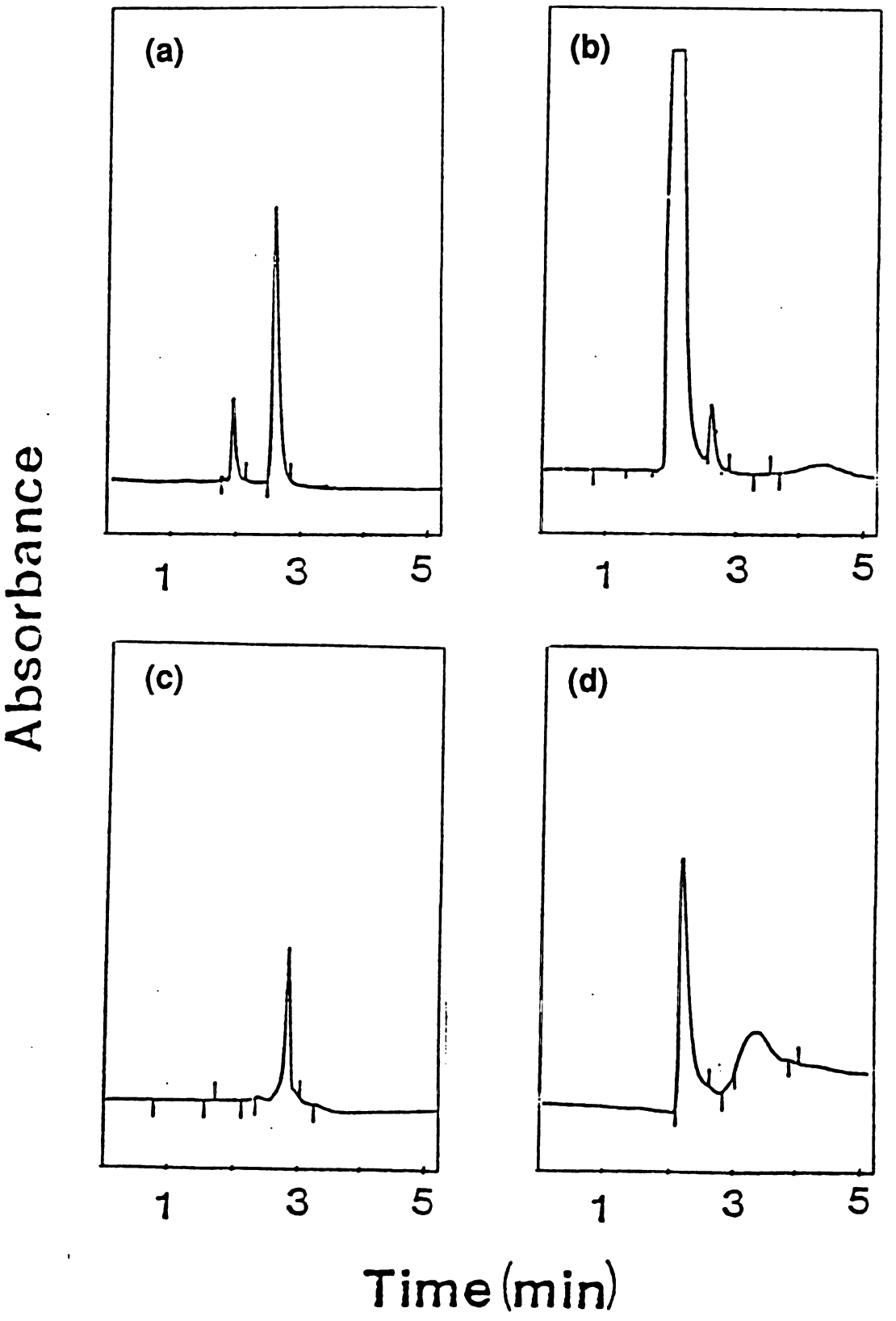

Figure 23

is the result of the injection of 8.75 µg of transplatin while the fourth chromatograph (d) follows the preincubation of transplatin with a 10 fold molar excess of dithiobiuret and the injection of 8.75 µg of transplatin. The transplatin peak completely disappears and a broad absorbance peak is evident having a retention of approximately 3.3 min. These results suggest a more complete reaction occurs between dithiobiuret and transplatin than dithiobiuret and cisplatin. Following preincubation with dithiobiuret, free, unreacted, cisplatin remains whereas no unreacted transplatin is detected after a similar reaction.

The possibility that dithiobiuret could reverse the inhibition of translation by cisplatin or transplatin was then examined. Addition of dithiobiuret to reticulocyte lysate has neither an inhibitory nor a stimulatory effect on peptide synthesis. When dithiobiuret and cisplatin are combined prior to addition to a translation assay, the inhibitory action of cisplatin on peptide synthesis is reduced (Fig. 23). Higher concentrations of this chelator more greatly reverse the cisplatin effect. If lysate and dithiobiuret are combined before cisplatin, then reversal of the cisplatin effect does not occur and translation activity is inhibited. In the mRNA dependent assay, the addition of precombined cisplatin and dithiobiuret to mRNA causes a reversal of the cisplatin effect. However, if cisplatin and mRNA are combined before addition of dithiobiuret, reversal does not occur.

Figure 23. Dithiobiuret reverses the inhibition of translation by cispaltin. Dithiobiuret at 0.1, 0.2, 0.6, or 1.8 mM was preincubated with 200 μM cisplatin (Δ) for 7 min at 22°C. In another set of assays, rabbit lysate containing endogenous mRNA (●) was preincubated with the same concentrations of dithiobiuret for 7 min at 22°C. The dithiobiuret-cisplatin solution was then added to lysate containing endogenous mRNA and further preincubated 30 min at 22°C. The dithiobiuret-lysate mixture was further preincubated with 200 μM cisplatin for 7 min at 22°C. In a third set of assays, lysate containing endogenous mRNA was preincubated with 200 μM cisplatin for 7 min at 22°C followed by another 30 min preincubation with either 0.1, 0.2, 0.6, or 1.8 mM dithiobiuret (0). The inhibition was calculated for each set of experiments by comparison with a similarly combined preincubated assay with water substituted for either dithiobiuret or cisplatin.

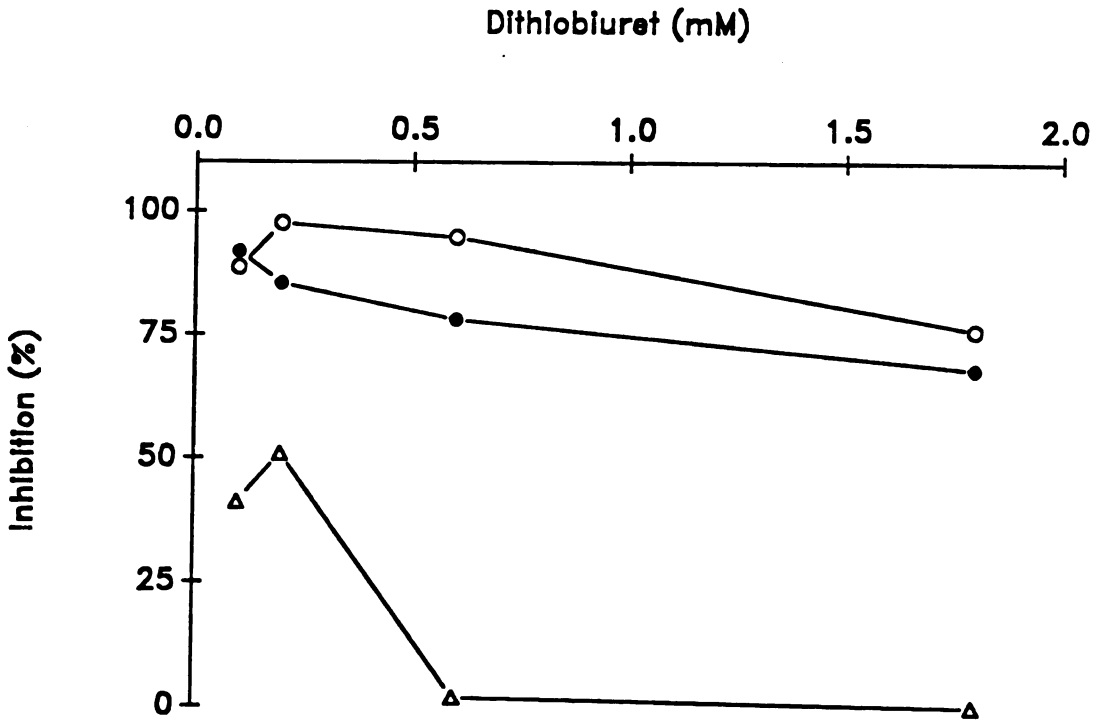

Figure 23.

Other substances were also tested for their ability to reverse the inhibition of peptide synthesis by cisplatin. The binding of cisplatin to DNA is well documented (Lippard, 1982). Therefore, it is not surprising that the preincubation of cisplatin with DNA prior to their addition to the translation assay blocks the ability of the drug to inhibit peptide synthesis (Table 4). Other metal chelators were tested as well. At the concentrations used EDTA and EGTA do not antagonize inhibition by cisplatin. Aluminum is reported to react with and inactivate cisplatin (Gilman, *et al.*, 1985) but it also lacked the ability to reverse the inhibition.

Although transplatin appears to react more completely with dithiobiuret, their precombination does not reverse the inhibition of translation to the same extent as that found with cisplatin (Table 5). Transplatin concentrations of 30 and 120 μM were preincubated with 1.2 mM dithiobiuret. Following the addition of the mixtures to translation assays, it was found that the inhibition was reversed on the order of 25%. After mixing cisplatin and dithiobiuret, the inhibition due to cisplatin is reversed approximately 80%. Thus, completeness of the reaction between dithiobiuret and the isomers of diamminedichloroplatinum (II) does not correlate with the inhibitory activity of the reacted isomers.

Cisplatin Inhibition is Increased by cAMP

It has been previously demonstrated that the addition of cAMP at 1 mM or more stimulates cell free protein synthesis (Sitikov, *et*

TABLE 4

Ability of Different Compounds to Reverse Inhibition of *in vitro* Translation by Cisplatin

Compound	Concentration	%Inhibition -Cisplatin	%Inhibition +Cisplatin (45 μM)
Control		0	73.0 ± 9.4 (n=4)
Aluminum	0.125 μg/ml	1.7 ± 0.5 (n=4)	91.3 ± 2.3 (n=4)
Aluminum Chloride	150 μM	14.0 ± 8.0 (n=6)	94.2 ± 3.4 (n=6)
DNA	0.0825 μg/ml	0.4 ± 0.1 (n=4)	4.1 ± 1.4 (n=4) *
Dithiobiuret	600 μM	0.4 ± 0.3 (n=4)	14.7 ± 2.1 (n=4) *
EDTA	150 μM	0.2 ± 0.1 (n=4)	62.5 ± 4.1 (n=4)
EGTA	150 μM	0.1 ± 0.1 (n=4)	93.0 ± 1.1 (n=4)

* Denotes significance at $p < 0.05$

The compounds listed above were preincubated for 15 min at 22°C in the presence or absence of cisplatin (final concentration in the assay of 45 μM) to yield a final concentration in the translation assay as listed above. The mixtures were added to rabbit reticulocyte lysates, incubated 30 min further at 22°C and the translation of endogenous mRNA was tested as described in Materials and Methods.

TABLE 5

Lesser ability of Dithiobiuret to Reverse Inhibition of *in vitro* Translation by Transplatin

Transplatin Concentration	% Inhibition -Dithiobiuret	% Inhibition +Dithiobiuret
30 μM	19.5 ± 2.9 (n=4)	11.5 ± 1.9 (n=4)
120 μM	69.2 ± 1.0 (n=4)	63.3 ± 0.2 (n=4)*

Transplatin was preincubated for 15 min at 22°C in the presence or absence of dithiobiuret (final concentration in the assay of 1.2 mM) to yield a final concentration in the translation assay as listed above. The mixtures were added to rabbit reticulocyte lysates, incubated 30 min further at 22°C and the translation of endogenous mRNA was tested as described in Materials and Methods.

*Denotes significance at $p < 0.05$.

al., 1988; Ernst, *et al.*, 1976). Addition of the cAMP is known to stimulate protein synthesis in lysates where protein chain initiation is inhibited due to the activation of eIF-2 kinases (Hurst, *et al.*, 1987). Translation assays stimulated by the addition of cAMP show an increase in inhibition by cisplatin (Fig. 24). Concentrations of cAMP were added up to 20 mM. Increasing concentrations of cAMP stimulated translation in rabbit reticulocyte lysates more than 2 fold when cAMP was added to greater than 10 mM. The addition of increasing concentrations of cAMP in lysates translated in the presence of 87.5 μM cisplatin stimulated the inhibition of peptide synthesis more than 35% over control. Thus, cAMP increases both translation activity and inhibition in cell free assays which implies the eIF-2 kinases do not mediate the effect of cisplatin on peptide synthesis.

The relationship between cAMP stimulated assays and cisplatin inhibition was more thoroughly investigated. Lysates were translated in the presence of 10 mM cAMP and increasing concentrations of cisplatin were added (Fig. 25). Cisplatin was titrated between 5 and 250 μM. The slope of the linear region of the log-dose response curve is not significantly different from that found in the absence of added cAMP by analysis of regression at $p < 0.01$. The results indicate that cAMP stimulated lysates are inhibited by cisplatin via the same mechanism as in nonstimulated assays. The line resulting from added cAMP is significantly shifted to the left, however, which indicates an increase in cisplatin

Figure 24. The addition of cAMP to lysates increases both translation activity and inhibition by cisplatin. Endogenous mRNA was translated by rabbit reticulocyte lysates after preincubation with increasing concentrations of cAMP for 30 min at 22°C in the absence (left axis, □) or presence (right axis, ♦) of 87.5 μM cisplatin. Stimulation of translation was determined by comparison with assays preincubated in the absence of cAMP and inhibition of translation was determined by comparison with assays preincubated in the absence of cisplatin. Each point represents six individual assays. Error bars represent ± 1 S.E. Significant differences in the presence of added cAMP in the absence (*) and in the presence (+) of added cisplatin are denoted at $p < 0.05$ by Student t-test.

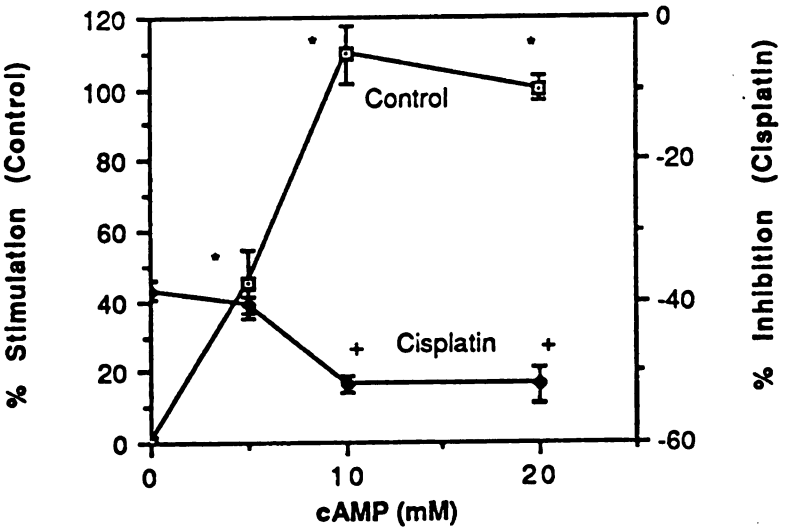
% Stimulation (Control)
% Inhibition (Cisplatin)
120
100
80
60
40
20
0
0
-20
-40
-60
0
10
20
cAMP (mM)
Control
Cisplatin

Figure 24.

Figure 25. Addition of cAMP increases the inhibition of *in vitro* translation by cisplatin. Endogenous mRNA was translated by rabbit reticulocyte lysates after preincubation with increasing concentrations of cisplatin for 30 min at 22°C 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 represent ± 1 S.E. Best fit lines were plotted by first order linear regression fit to the linear portions of each curve.

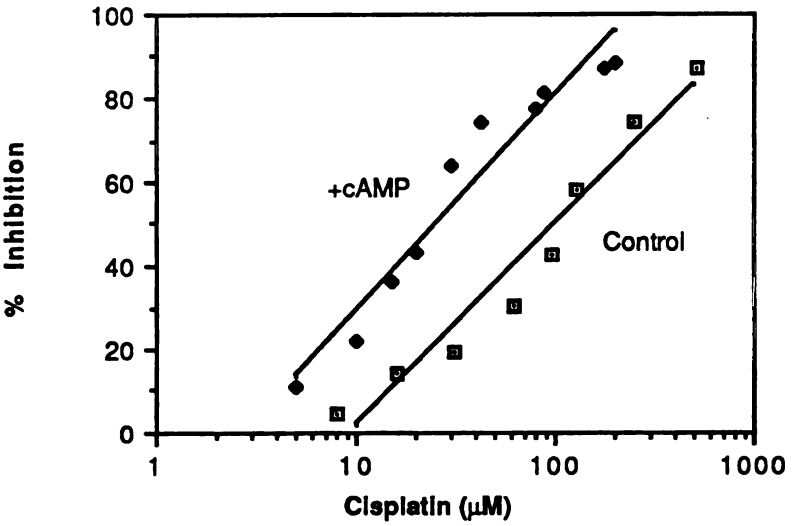
100
80
60
40
20
0
% Inhibition
+cAMP
Control
1
10
100
1000
Cisplatin (μM)

Figure 25.

potency in the presence of cAMP. The IC_{50} of cisplatin in rabbit lysate is more than 4 fold greater than in a cAMP stimulated assay.

The response of other inhibitors of translation to added cAMP was examined for comparison to cisplatin. Similarity in the response of these other inhibitors to that of cisplatin may yield results which aid in identifying the mechanism by which cisplatin inhibits peptide synthesis. Concentrations of transplatin were titrated between 20 and 250 μM in assays translated in the presence of 10 mM cAMP (Fig. 26). The curve representing the inhibition which resulted from the addition of transplatin to assays translated in the presence of cAMP is not significantly different from that found in the absence of added cAMP by analysis of regression at $p < 0.05$. Thus, the addition of cAMP did not alter the inhibition found with added transplatin to the same degree as in assays incubated with cisplatin. This difference in response to cAMP suggests a difference in the mechanisms by which the isomers of diamminedichloroplatinum (II) inhibit translation.

Other well characterized inhibitors of translation were also added to assays in the presence and absence of 10 mM cAMP (Fig. 27). Sodium fluoride is known to inhibit the initiation of translation by preventing the 60s ribosomal subunit from joining the 48s complex (Holland, 1979). The addition of cAMP enhances the inhibition which results from the addition of sodium fluoride. The action of sodium fluoride in the presence of 10 mM cAMP is similar to that found when cisplatin is added to assays containing cAMP. Inhibition of

Figure 26. Addition of cAMP does not significantly increase the inhibition of *in vitro* translation by transplatin. Endogenous mRNA was translated by rabbit reticulocyte lysates after preincubation with increasing concentrations of transplatin for 30 min at 22°C in the absence (□) or presence (♦) of 10 mM cAMP. Inhibition of translation was determined by comparison with assays preincubated in the absence of transplatin. Each point represents six individual assays. Error bars represent ± 1 S.E. Best fit lines were plotted by first order linear regression fit to the linear portions of each curve.

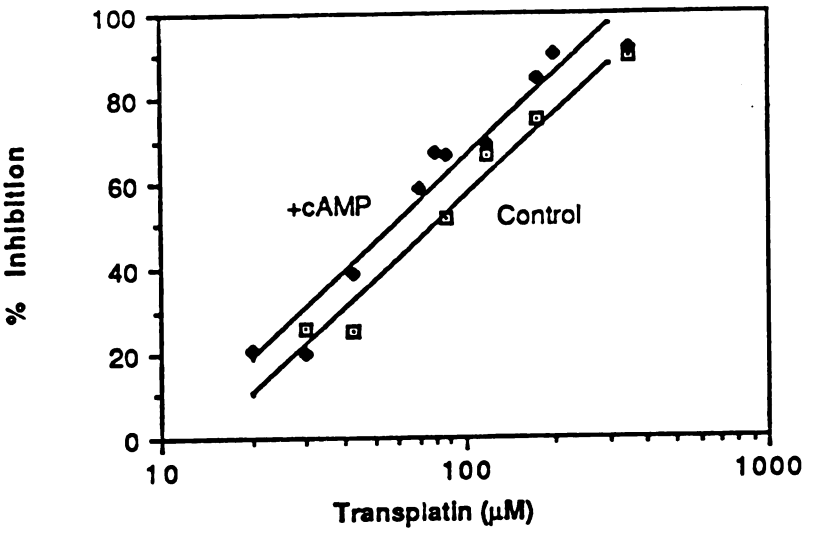
100
80
60
40
20
0
% Inhibition
+cAMP
Control
10
100
1000
Transplatin (μM)

Figure 26.

Figure 27. Comparison of the effect of cAMP on other translation inhibitors. Endogenous mRNA was translated by rabbit reticulocyte lysates in the presence of 87.5 μM cisplatin, 2.5 mM sodium fluoride (NaF), 25 μM mercuric chloride ($HgCl_2$), or 60 μM puromycin added at the start of translation. Translation was carried out in the absence (white columns) or presence (hatched columns) of 10 mM cAMP for 45 min at 30°C. Inhibition of translation was determined by comparison with assays preincubated in the absence of inhibitor and in the absence or presence of cAMP. Each point represents four individual assays. Error bars represent ± 1 S.E. Significant differences in inhibition for an inhibitor in the presence of added cAMP are denoted (*) at $p < 0.01$ by Student t-test.

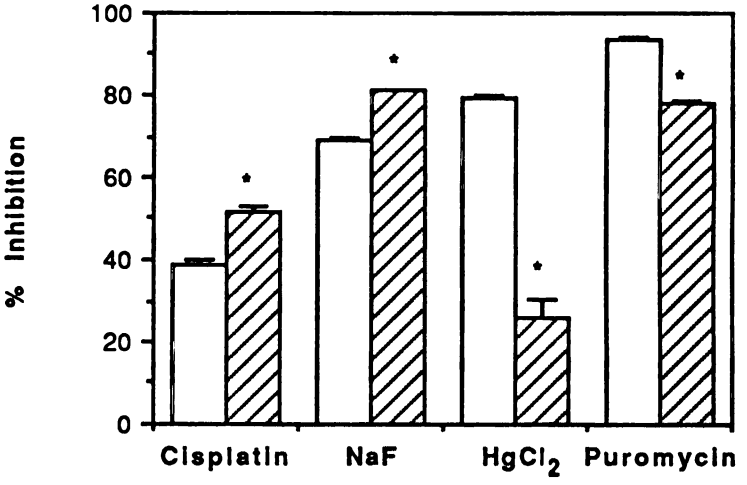

Figure 27.

protein synthesis has also been shown to occur in lysates exposed to heavy metal ions such as Hg^{2+}. Heavy metals inhibit the initiation of peptide synthesis by activating the eIF-2 kinases. Addition of 10 mM cAMP was recently shown to prevent the inhibition of protein synthesis when added to lysates in the presence of Hg^{2+} (Hurst, *et al.*, 1987). These same results were found in translation assays tested in the presence of 25 μM $HgCl_2$ supplemented with 10 mM cAMP (Fig. 27). Cisplatin inhibition, therefore, does not respond to the addition of cAMP in the same manner as a heavy metal ion. Finally, the elongation inhibitor puromycin was translated in the presence and absence of added cAMP. The inhibition caused by puromycin is reversed by the addition of 10 mM cAMP. This inhibitor is also unlike cisplatin in response to added cAMP. Thus, the enhancement of inhibition following the addition of 10 mM cAMP is found in lysates inhibited by cisplatin and sodium fluoride while reversal of inhibition results in assays translated in the presence of the heavy metal Hg^{2+} and the elongation inhibitor puromycin.

Kinetics of Inhibition by Cisplatin are Biphasic

The mechanism by which a compound inhibits protein synthesis can be gained from the analyzing the kinetics of translation. An inhibitor was added to lysate at t=0 min and aliquots were removed and precipitated at the indicated times up to 60 min. Inhibitors of initiation are said to have "biphasic" kinetics. That is, translation initially proceeds at the uninhibited rate for several minutes and then declines abruptly. During the initial linear region elongation

occurs and already synthesized initiation factors and ribosomal subunits are utilized catalytically. When these previously synthesized initiation subunits are exhausted they are not replenished in the presence of an initiation inhibitor and translation halts (Fig. 28a). Elongation inhibitors slow or completely block translation from the start of protein synthesis. The kinetics resulting from an assay inhibited by an elongation inhibitor are linear as they reduce the incorporation of radiolabeled amino acid throughout the entire time course (Fig. 28b). The kinetics of inhibition of translation in the presence of 80 μM cisplatin were determined to be biphasic (Fig. 28c). The results demonstrate an incorporation of ^{35}S-methionine into trichloroacetic acid precipitable material that is initially linear. Following approximately the first 10 min of the translation assay, an abrupt decline of incorporation was observed. Thus, the time course of inhibition by cisplatin is similar to that found with sodium fluoride, an initiation inhibitor, (Fig. 29a) and dissimilar to that found with the elongation inhibitor cycloheximide (Fig. 29b). The characteristic kinetics and similarity to an inhibitor with a known mechanism of action are strong evidence to suggest that cisplatin acts to inhibit translation by blocking the initiation of peptide synthesis. Transplatin demonstrates an apparently linear increase in the incorporation of label with time (Fig. 28d) consistent with the kinetics of an elongation inhibitor.

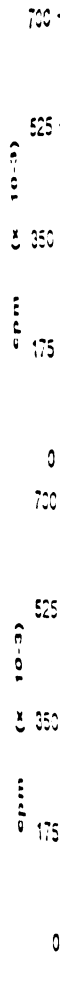

Figure 28. Kinetics of translation in the presence of cisplatin and other protein synthesis inhibitors. At t=0 min, water (□, panels a-d), 2.5 mM sodium fluoride (♦, panel a), 10 μM puromycin (■, panel b), 4 μM cycloheximide (◊, panel b), 80 μM cisplatin (♦, panel c), 70 μM transplatin (♦, panel d), or 280 μM transplatin (○, panel d) was added to lysates. Lysates were allowed to translate endogenous mRNA at 30°C for 60 min, aliquots were removed at the indicated times and precipitated to determine the incorporation of ^{35}S-methionine as described. Lysates were prepared for the translation of endogenous mRNA as described in Materials and Methods. Each point represents 8 individual control assays, 3 sodium fluoride, 3 cycloheximide, 6 puromycin, 8 cisplatin, and 8 transplatin assays. Error bars represent ± 1 S.E.

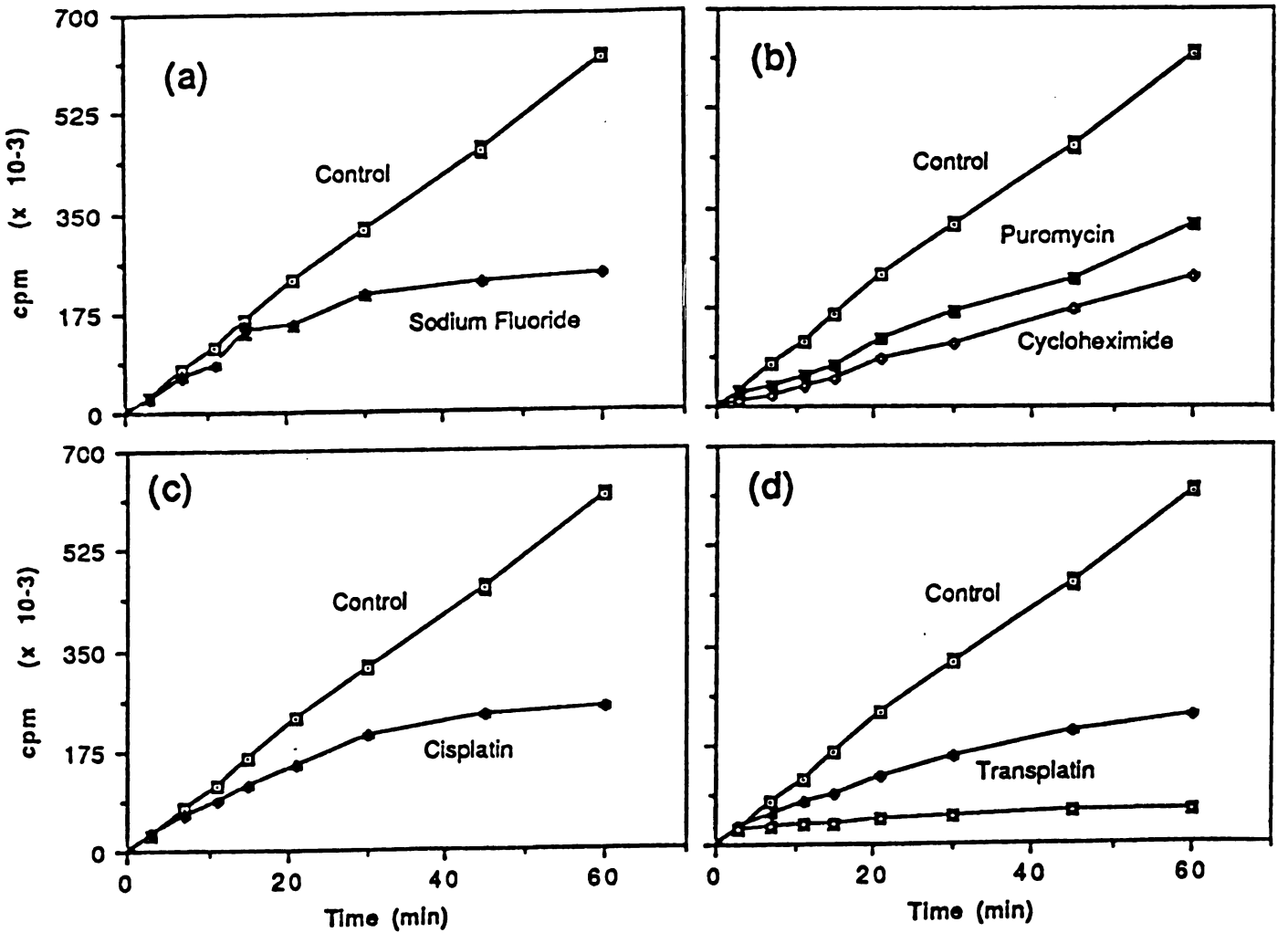
(a)
Control
Sodium Fluoride
(b)
Control
Puromycin
Cycloheximide
(c)
Control
Cisplatin
(d)
Control
Transplatin
cpm (x 10-3)
700
525
350
175
0
0
20
40
60
Time (min)

Figure 28.

Figure 29. Time course of *in vitro* translation in the presence of cisplatin compared to other inhibitors. At t=0 min, either 80 µM cisplatin (▫, panels a,b), the initiation inhibitor sodium fluoride (2.5 mM, ♦, panel a), or the elongation inhibitor cycloheximide (4 µM, ♦, panel b) was added to lysates. Lysates were allowed to translate endogenous mRNA at 30°C for 60 min, aliquots were removed at the indicated times and precipitated to determine the incorporation of ^{35}S-methionine as described. Lysates were prepared for the translation of endogenous mRNA as described in Materials and Methods. Each point represents 3 sodium fluoride assays, 3 cycloheximide, and 8 cisplatin assays. Error bars represent ± 1 S.E.

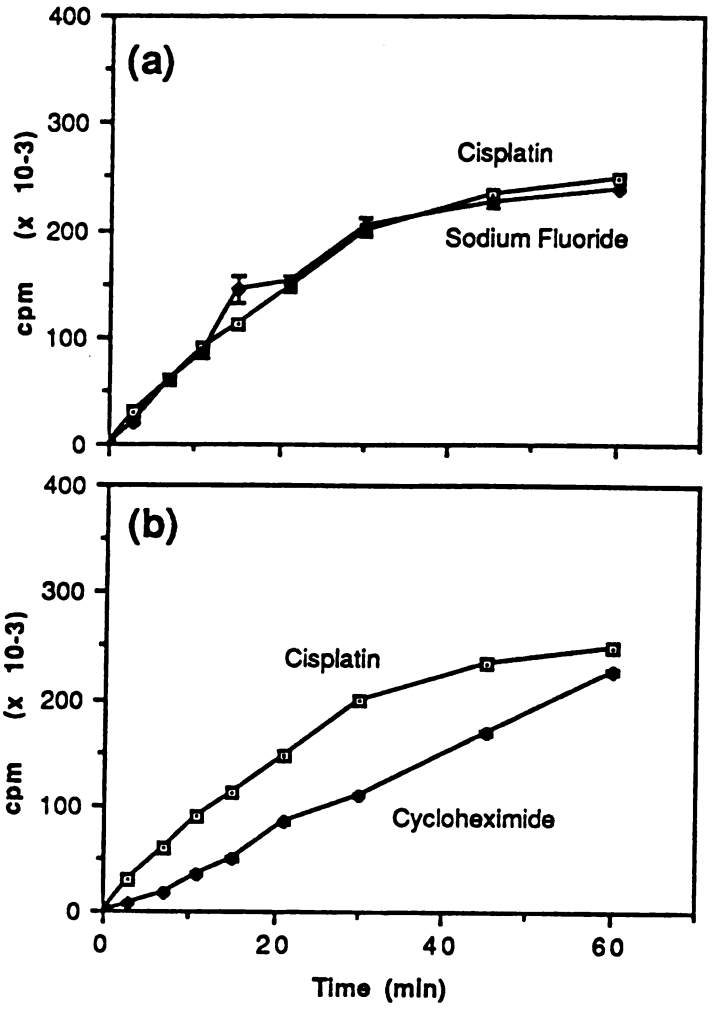
(a)
Cisplatin
Sodium Fluoride
cpm (x 10-3)
400
300
200
100
0
(b)
Cisplatin
Cycloheximide
cpm (x 10-3)
400
300
200
100
0
0
20
40
60
Time (min)

Figure 29.

Cisplatin Reduces Polyribosome Formation

Inhibitors of initiation demonstrate decreased polyribosome formation in translation assays performed in their presence. As initiation complexes are not formed, ribosomes are not assembled onto mRNA. In contrast, inhibitors of elongation slow the movement of ribosomes along the mRNA template and cause the accumulation of large polysomes. Thus, the lack or accumulation of polyribosomes is used to separate an inhibitor which acts to block the initiation of translation from one which affects the elongation of nascent peptides. Following translation in the absence and presence of various inhibitors of protein synthesis, the formation of polysomes was determined by linear sucrose density centrifugation. Polyribosome synthesis is assayed as the incorporation of ^{35}S-methionine. The label is linked to $tRNA_{f\text{-}met}$ by enzymes present within the reticulocyte lysates. This tRNA is always the first utilized in every newly initiated protein. This labeled methionine is precipitable in trichloroacetic acid. Thus, it is possible to assay the formation of polyribosomes for the purpose of better understanding the mechanism by which a compound inhibits translation in reticulocyte lysates.

The initiation inhibitor sodium fluoride significantly reduced the formation of polysomes, when compared to a control assay translated in the absence of inhibitor, as expected (Fig. 30a). The elongation inhibitor cycloheximide demonstrates an accumulation of larger polysomes when compared to the control (Fig. 30b). Assays

Figure 30. Cisplatin inhibits the formation of polysomes. Rabbit reticulocyte lysate reaction mixtures (150 µl) were allowed to translate endogenous mRNA for 10 min at 30°C. Translation was carried out in the absence of inhibitors (■) or the presence (□) of 2.5 mM sodium fluoride (panel a), 10 µM cycloheximide (panel b), 200 µM cisplatin (panel c), or 200 µM transplatin (panel d). Samples were analyzed by sucrose density gradient centrifugation (15-35% w/v linear sucrose gradients) in a Beckman SW 41 rotor for 80 min as described in Materials and Methods. Trichloroacetic acid precipitable ^{35}S-methionine distribution was determined as described in Materials and Methods. Sedimentation is from left to right. Each point represents 15 individual control assays, 3 sodium fluoride, 3 cycloheximide, 12 cisplatin, and 3 transplatin assays. The results from each treatment were standardized. Each inhibitor was run with a control. As there was variation in the total number of counts among individual gradients, the sum of the counts in each control was determined. The total sum of the counts was made the same in all the controls. The counts in each fraction of the control and its corresponding inhibited assay were then multiplied by the ratio of the standard number to which all the controls were compared divided by the number of counts in the control of an individual experiment. For example, the control in the first sample had 100,000 cpm that were specifically precipitated among all its fractions. All the controls were set to 125,000 cpm. Therefore, the counts in each of the fractions of that control gradient and each of the fractions collected from the matched inhibited gradients, were multiplied by 125,000/100,000. Filled squares represent a significant difference of the point from control at $p < 0.05$ by Student t-test.

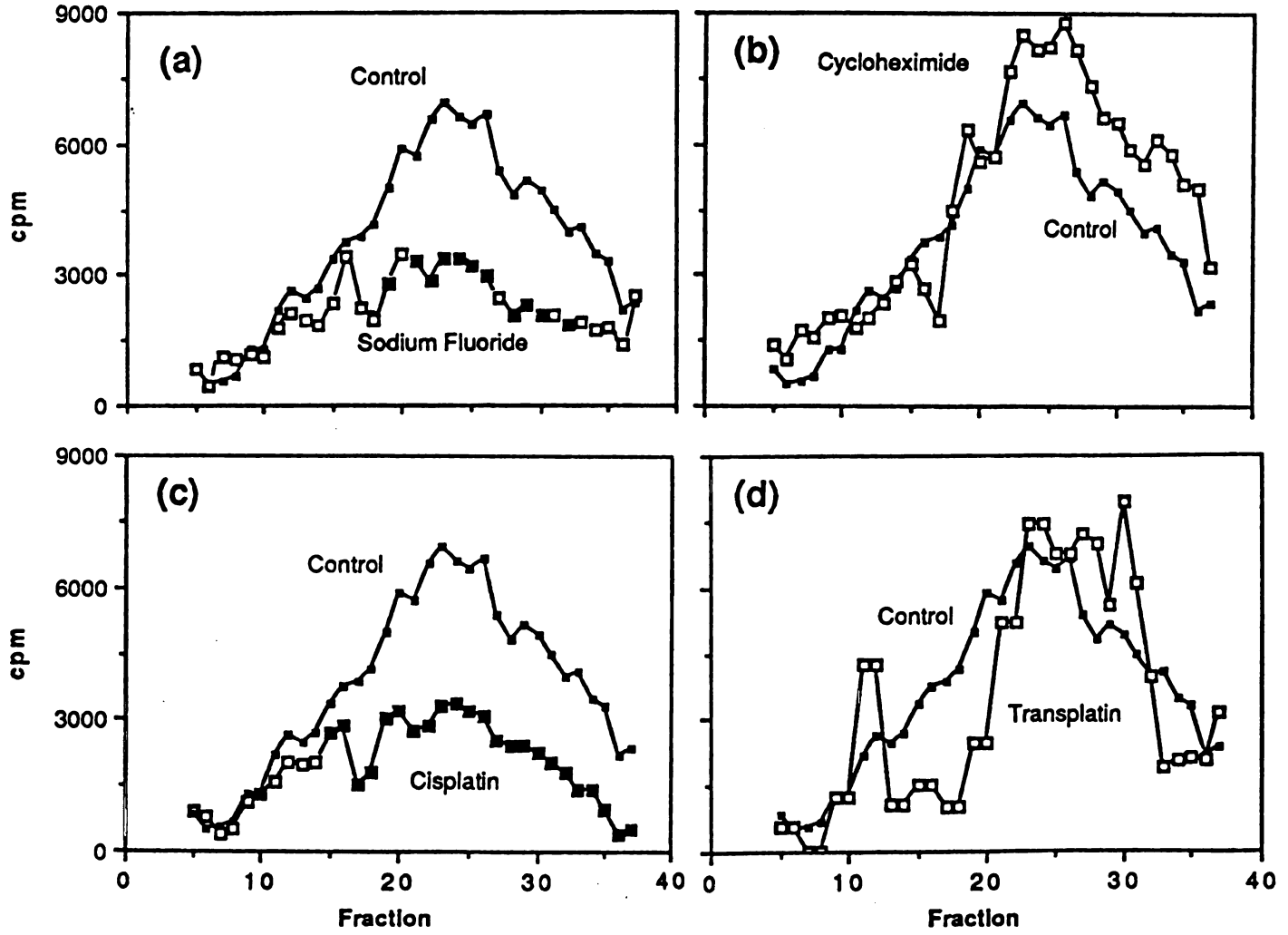
(a)
Control
Sodium Fluoride
(b)
Cycloheximide
Control
(c)
Control
Cisplatin
(d)
Control
Transplatin
cpm
9000
6000
3000
0
0
10
20
30
40
Fraction

Figure 30.

translated in the presence of cisplatin demonstrated a significant decrease in polysome formation (Fig. 30c). When compared to the known inhibitor of initiation, sodium fluoride, the polyribosome formation in cisplatin inhibited assays are not distinguishable from that found in assays translated in the presence of sodium fluoride (Fig. 31a). Polysome formation in cisplatin inhibited lysates is significantly different from that found in assays inhibited by the elongation inhibitor cycloheximide (Fig. 31b). The sedimentation of polysomes from assays performed in the presence of transplatin appear to resemble those found in lysates translated with elongation inhibitors (Fig. 30d, 31d). Further, the sedimentation of polysomes in cisplatin inhibited assays is significantly different from the sedimentation pattern found following inhibition with transplatin (Fig. 31c). These results in addition to the biphasic kinetics demonstrated by cisplatin suggest cisplatin inhibits protein synthesis by interfering with the initiation of translation.

Formation of 43s is Not Inhibited by Cisplatin

To identify which step in the initiation process was affected by cisplatin, specific assays were conducted. First in the process of initiation is the linking of the amino acid methionine to its tRNA by an enzymatic reaction catalyzed by tRNA synthetase. The possibility that cisplatin inhibition of protein synthesis can be explained by an effect on the tRNA synthetase reaction was investigated. Increasing concentrations of cisplatin were added to an assay measuring the incorporation of ^{35}S-methionine into trichloroacetic acid

Figure 31. Formation of polyribosomes in the presence of cisplatin compared to other translation inhibitors. Rabbit reticulocyte lysate reaction mixtures (150 µl) were allowed to translate endogenous mRNA for 10 min at 30°C. One set of translation assays was carried out in the presence of 200 µM cisplatin (▫) while other assays were conducted in the presence of other inhibitors (■): 2.5 mM sodium fluoride (panel a), 10 µM cycloheximide (panel b), 200 µM transplatin (panel c). Panel (d) shows a comparison of polysome formation in the presence of 200 µM transplatin (●) to that found following translation in the presence of 10 µM cycloheximide (◇). Samples were analyzed by sucrose density gradient centrifugation (15-35% w/v linear sucrose gradients) in a Beckman SW 41 rotor for 80 min as described in Materials and Methods. Trichloroacetic acid precipitable ^{35}S-methionine distribution was determined as described in Materials and Methods. Sedimentation is from left to right. Each point represents 15 individual control assays, 3 sodium fluoride, 3 cycloheximide, 12 cisplatin, and 3 transplatin assays. The results from each treatment were standardized to equalize the areas under the curves as described in the legend of Figure 30. Filled squares represent a significant difference of the point from control at $p < 0.05$ by Student t-test.

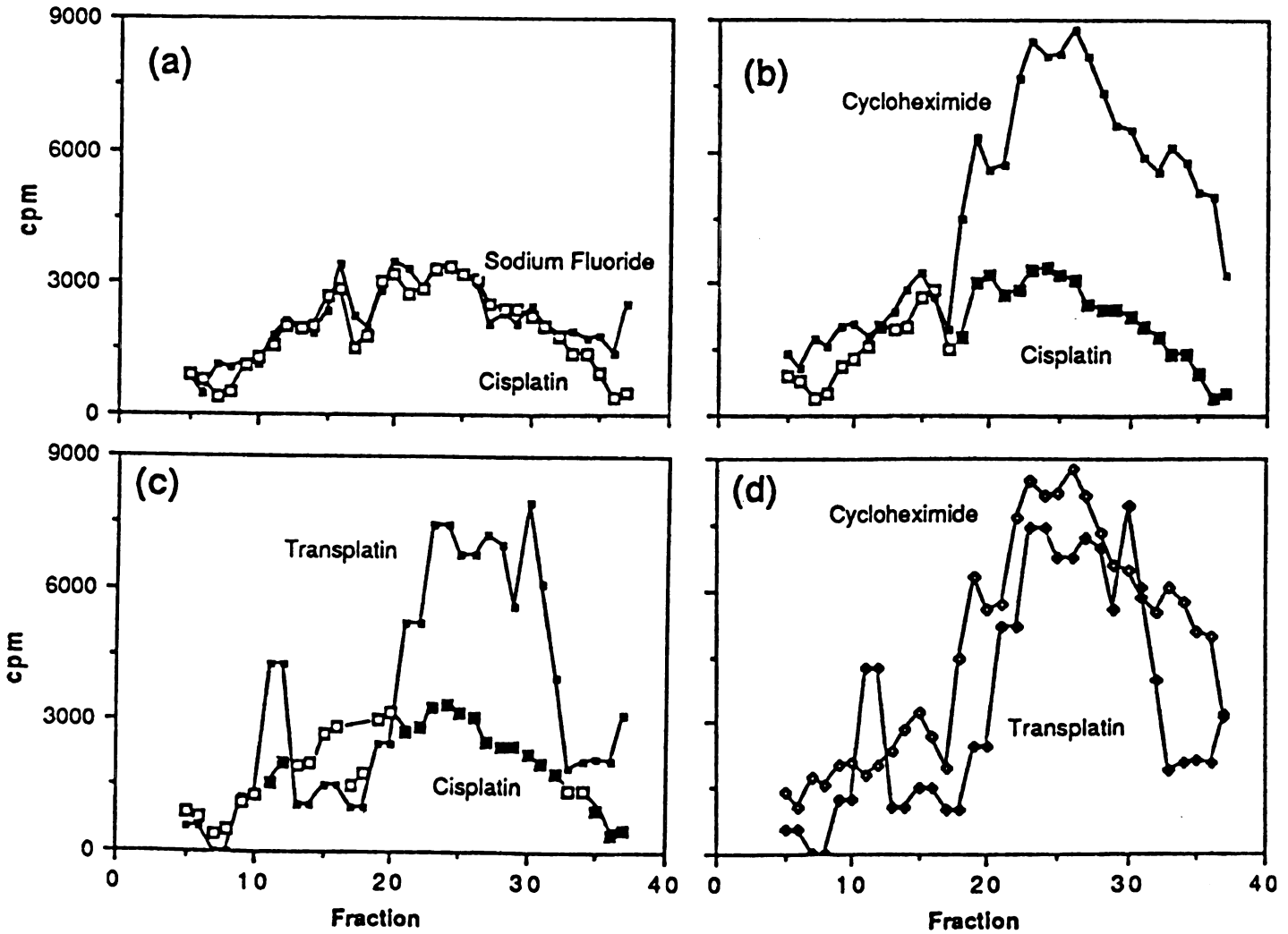
(a)
Sodium Fluoride
Cisplatin
(b)
Cycloheximide
Cisplatin
(c)
Transplatin
Cisplatin
(d)
Cycloheximide
Transplatin
cpm
9000
6000
3000
0
0
10
20
30
40
Fraction

Figure 31.

precipitable tRNAs. As shown, the amount of labeled amino acid linked to tRNA is not significantly reduced when the reaction is allowed to proceed in the presence cisplatin (Fig. 32). At concentrations of cisplatin which produce inhibition of translation greater than 80%, the incorporation of methionine into tRNA is reduced less than 3% of control values. Therefore, cisplatin does not interfere with the enzymatic joining of ^{35}S-methionine to its tRNA.

The next step in the formation of the 80s initiation complex involves the formation of the ternary complex and its joining to the 40s ribosomal subunit to make a 43s initiation unit. To assay the formation of these complexes in the presence of cisplatin, the antibiotic anisomycin is utilized. In low concentrations anisomycin is an elongation inhibitor. However, in larger concentrations, like the 100 μM used in these experiments, anisomycin blocks the ability of mRNA to join the 43s complex (Lenz and Baglioni, 1979). Therefore, the initiation complexes can be formed only to the 43s step in the presence of 100 μM anisomycin. The 43s complex contains the $tRNA_{f\text{-}met}$ which is detected as it is linked to a labeled methionine. Lysates were translated in the presence and absence of added cisplatin. These lysates were resolved on a linear sucrose gradient (Fig. 33). The peak at fraction 10 represents a particle sedimenting at approximately 43s. The figure demonstrates no difference between either the peak heights or shapes. The radioactivity sediments equally in lysates translated in the absence and presence of high concentrations of added cisplatin. Thus,

Figure 32. Cisplatin does not inhibit the amino acylation of methionyl tRNA by tRNA synthetase. Assays were conducted as described in Materials and Methods. Inhibition of the charging of methionine specific tRNA was determined as a function of increasing cisplatin concentration. Each point represents 4 individual assays. Error bars represent ± 1 S.E.

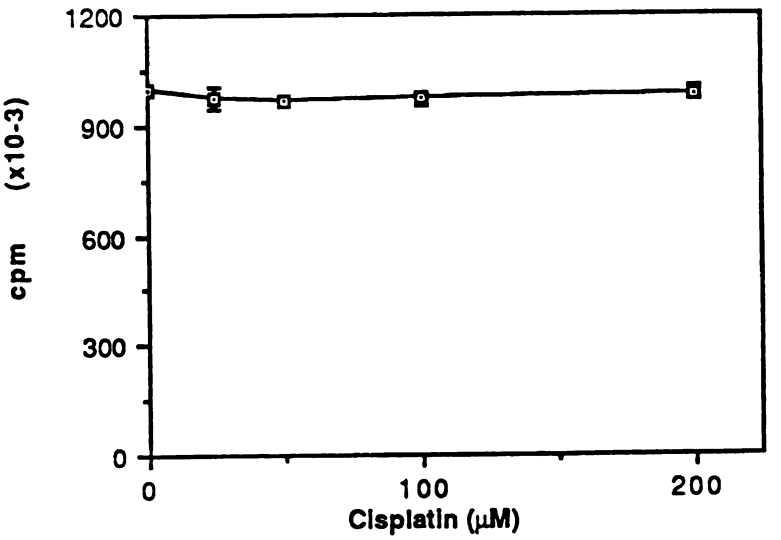

Figure 32.

Figure 33. Cisplatin does not inhibit the formation of 43s ribosomal subunits. Rabbit reticulocyte lysate reaction mixtures (150 µl) 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°C. Samples were analyzed by sucrose density gradient centrifugation (15-30% w/w linear sucrose gradients) in a Beckman SW 41 rotor for 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 43s sedimenting approximately at fraction 10. Each point represents 3 individual assays. Error bars represent ± 1 S.E.

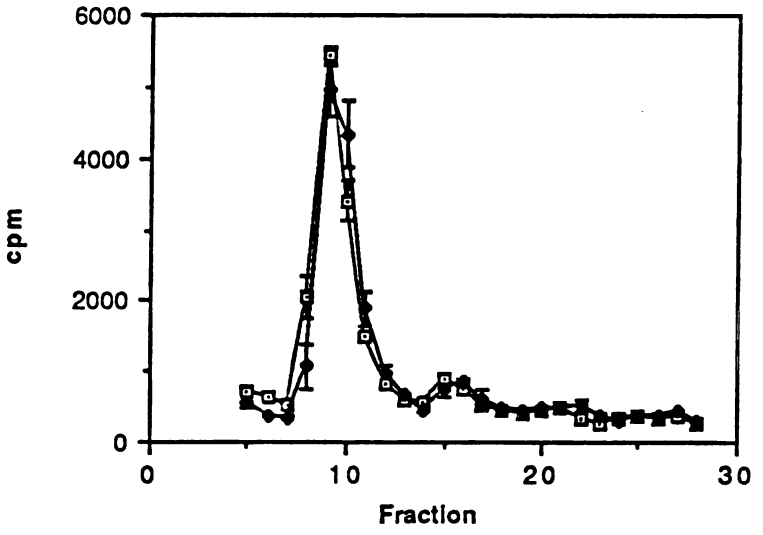

Figure 33.

cisplatin does not inhibit the formation of the ternary complex or its joining with the 40s ribosome to make a 43s initiation complex.

Inhibition of 80s Formation by Cisplatin

Following the formation of the 43s ribosomal subunit only two steps remain in the initiation of translation. These steps involve the joining of mRNA to a 43s complex and the addition of the 60s ribosome to that 48s subunit to make a complete 80s initiation complex. These final steps are assayed by resolving the initiation subunit particles on a linear sucrose gradient. As before the label is carried in the $tRNA_{f\text{-}met.}$ Actively initiating lysates demonstrate an accumulation of 43s and 80s subunits. A block in any step in the initiation process results in the build up of completed initiation subunits prior to the inhibited step. Inhibitors which prevent the joining of mRNA to the 43s subunit would be expected to accumulate 43s particles as in lysates translated in the presence of large concentrations of anisomycin (Fig. 33). Inhibitors, such as edeine or sodium fluoride, which prevent the joining of the 60s ribosome to the 48s complex demonstrate the accumulation of label in the sucrose gradient where 48s sediments (Fig. 34a). Inhibitors of elongation demonstrate a large peak at 80s in the gradient as these formed initiation complexes do not migrate along the mRNA and thus accumulate as demonstrated (Fig. 34b). Cisplatin inhibited assays were separated on linear sucrose gradients and the distribution of ribosomal subunits is shown (Fig. 34c). An accumulation of label is noted in fractions representing particles sedimenting at 48s and 75s

Figure 34. The distribution of ribosomal subunits in the presence of translation inhibitors. Rabbit reticulocyte lysate reaction mixtures (150 µl) were allowed to translate endogenous mRNA for 10 min at 30°C. Translation was carried out in the absence of inhibitors (■) or the presence (▫) of 2.5 mM sodium fluoride (a), 10 µM cycloheximide (b), 200 µM cisplatin (c), or 200 µM transplatin (d). Samples were analyzed by sucrose density gradient centrifugation (15-35% w/v linear sucrose gradients) in a Beckman SW 41 rotor for 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 43s sedimenting approximately fraction 2, 48s at fraction 5, 75s at fraction 7, and 80s at fraction 10. Each point represents 15 individual control assays, 3 sodium fluoride, 3 cycloheximide, 16 cisplatin, and 3 transplatin assays. The results from each treatment were standardized as described in the legend of Figure 30. Error bars represent ± 1 S.E.

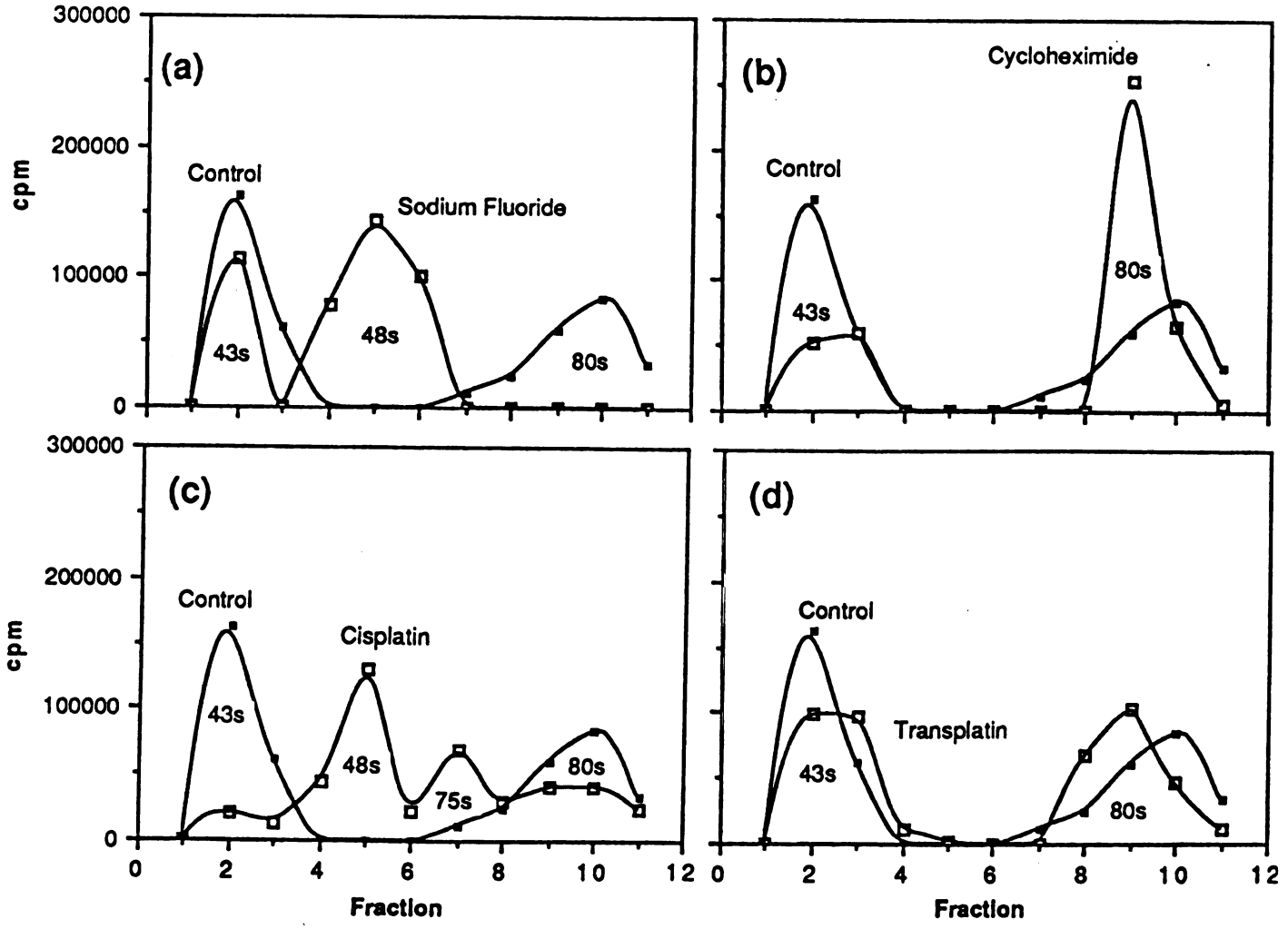
(a)
Control
Sodium Fluoride
43s
48s
80s
(b)
Cycloheximide
Control
43s
80s
(c)
Control
Cisplatin
43s
48s
75s
80s
(d)
Control
Transplatin
43s
80s
cpm
300000
200000
100000
0
0
2
4
6
8
10
12
Fraction

Figure 34.

with some subunits noted at 80s. Subunits which sediment at 75s are dimers formed by the joining of two 48s particles (Lenz and Baglioni, 1979). These results are consistent with those expected of an inhibitor which acts to block the joining of the 60s ribosome to the 48s subunit. The pattern of accumulation of ribosomal subunits following inhibition with cisplatin is similar to that found after inhibition of translation with other compounds that block 60s joining such as sodium fluoride and edeine (Fig. 34 a,c). This pattern is unlike that found following incubation of lysates with an elongation inhibitor like cycloheximide (Fig. 34 b, c). Translation assays carried out in the presence of transplatin demonstrate an accumulation of 80s ribosomal subunits consistent with an inhibition of elongation of peptide synthesis (Fig. 34 b, d) and dissimilar to the accumulation of ribosomal subunits following inhibition with cisplatin (Fig. 34 c, d).

As the joining of the 60s ribosome is inhibited when translation occurs in the presence of cisplatin, it should also be possible to demonstrate an accumulation of mRNA in the 48s sedimenting region of a sucrose gradient. Control lysates translated in the absence of added cisplatin should demonstrate the presence of message in the 80s region. To conduct this experiment RNA was labeled by the enzymatic addition of poly(A) to its 3'-end. Utilizing the enzyme poly(A) polymerase, a-^{35}S-AMP residues were incorporated onto the mRNA. Labeled message was added to lysates in the presence and absence of added cisplatin. Following translation supplemented with unlabeled methionine, $tRNA_{f\text{-}met}$, and

tRNA synthetase, the assays were resolved on linear sucrose gradients. The results indicate that cisplatin promotes the accumulation of message sedimenting at 48s with some label detected at 80s (Fig. 35). These findings differ significantly from those found in lysates translated in the absence of cisplatin. Control assays demonstrate the some 48s with the majority of label sedimenting at 80s.

Figure 35. mRNA is found in the 48s preinitiation complex following inhibition of translation by cisplatin. Labeled mRNA was translated in nuclease treated lysates (150 µl reaction mixture) for 10 min at 30°C. Translation was carried out in the absence (♦) or the presence (⊡) of 200 µM cisplatin. The isolation and labeling of mRNA was as described in Materials and Methods. Samples were analyzed by sucrose density gradient centrifugation (16-36% w/v linear sucrose gradients) in a Beckman SW 41 rotor for 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 48s sedimenting approximately fraction 13 and 80s at fraction 22. Each point represents 3 individual assays.

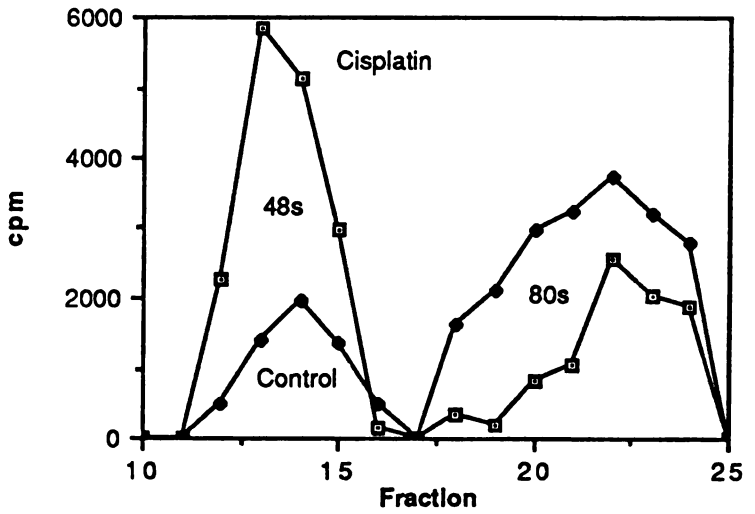
6000
4000
2000
0
cpm
Cisplatin
48s
80s
Control
10
15
20
25
Fraction

Figure 35.

DISCUSSION

Pharmacologic Concentrations Inhibit Translation

Cisplatin is a highly effective inhibitor of *in vitro* translation of mRNA. The effect occurs using translation systems prepared from either rabbit or guinea pig reticulocyte lysates. Furthermore, this effect occurs at pharmacologically relevant concentrations suggesting a possible role for the inhibition of peptide synthesis in cisplatin action or cytotoxicity. Animals undergoing treatment with therapeutic doses have been found to have a cisplatin concentration of 1.7 mM in microsomes, the site of *in vivo* translation (Choie, *et al.*, 1980; Litterst, *et al.*, 1976). Cisplatin concentrations of 33 μM have been reported in plasma from human patients undergoing treatment with this anticancer agent (Belliveau, *et al.*, 1985). Inhibition of peptide synthesis *in vitro* is found at concentrations as low as 3 μM with an IC_{50} of 39 μM. Thus, concentrations of cisplatin found to inhibit protein synthesis *in vitro* are on the same order as tissue concentrations found during its therapeutic administration. Also, the highest amount of cisplatin in a cell is found in the cytosol associated with the microsomal fraction (Choie, *et al.*, 1980). Microsomes are fragments of endoplasmic reticulum formed after the disruption of cells. Thus, the results are consistent with cisplatin having an effect on protein synthesis as

translation of mRNA into protein occurs on the cytosolic endoplasmic reticulum.

Cisplatin Activity is Related to its Structure

The stereochemistry of cisplatin is important for its ability to inhibit protein synthesis. The isomers of diamminedichloroplatinum (II), cisplatin and transplatin, demonstrate very different results in each of the comparisons made. Transplatin, an inhibitor of translation elongation (Tukalo, *et al.*, 1987; Moine, *et al.*, 1988), has a slope in the linear region of its log-dose response curve that is significantly different from that of cisplatin. Cisplatin and transplatin react differently with dithiobiuret. Transplatin has a more complete reaction with the chelator, yet still inhibits translation following their combination whereas cisplatin does not. The isomers show different degrees of response to cAMP. More specific examination of the mechanism by which these isomers inhibit translation demonstrate differences in their kinetics and in polyribosome synthesis. Finally, the distribution of ribosomal subunits in sucrose gradients shows an accumulation of 80s in transplatin inhibited assays and 48s for those with added cisplatin. Thus, the importance of stereochemistry suggests a role for drug structure in the mechanism by which cisplatin decreases protein synthesis and lessens the possibility that inhibition is due to a non-specific effect such as by a heavy metal platinum ion.

Regulation of *in vivo* Translation

The regulation of the rate of initiation has been shown to be of great importance in many cells and tissues during rapid transitions from one metabolic state to another (Ochoa, 1983). Although mechanisms by which initiation is regulated in reticulocyte lysate have been determined, it is not known to what extent these mechanisms represent general regulatory mechanisms that may be found *in vivo*.

The rate of protein synthesis in animal cells or tissues is affected by a variety of agents and metabolic conditions. In addition, qualitative regulation, resulting in alterations in the relative amounts of different proteins synthesized, occurs during hormonal induction of enzymes and changes in the patterns of gene expression (Anderson, *et al.*, 1977). Quantitative control of *in vivo* protein synthesis in a steady state situation is regulated by the number of ribosomes per cell. Qualitative control is regulated by the availability of mRNAs. During periods of transition in a cell, alterations in protein synthetic rate are achieved by changes in the rate of initiation. Changes in the rate of protein synthesis initiation can also modulate the spectrum of proteins synthesized by changing the relative efficiency with which different mRNAs are translated (Lodish, 1974; Bergmann and Lodish, 1979).

Data is available to suggest that cisplatin may be associated with decreased protein synthesis *in vivo*. Changes in serum albumin

concentrations in patients with advanced malignancy being treated with cisplatin were evaluated (Nanji, *et al.*, 1986). Patients who were considered to have a decrease in serum albumin for other reasons (nephrotic syndrome, cirrhosis, malabsorption) were excluded from the study. Serial measurements of serum albumin were performed in fifteen human patients. All patients received cisplatin in combination with other drugs. None of the other drugs were known to be hepatotoxic or decrease serum albumin. The decrease in serum albumin from the time of the first dose of cisplatin to death correlated with liver platinum levels at autopsy (Fig. 36). Thus, liver platinum concentrations were associated with a decrease in serum albumin. Serum albumin is synthesized in the cell cytosol (Rothschild, *et al.*, 1973) and quantitatively the cytosol, the location of *in vivo* protein synthesis, contains the largest amount of cisplatin (Choie, *et al.*, 1980). Thus, a correlation between liver platinum and a decrease in serum albumin suggests that cisplatin may act *in vivo* as well as *in vitro* to inhibit protein synthesis.

Regulation of *in vitro* Translation

Control of protein synthesis rates *in vitro* have been found to resemble the responses found in tissues *in vivo* in that translation is regulated by ribosome availability (Bergmann and Lodish, 1979). Thus, this allows for simpler experimental conditions for the study of the mechanisms controlling translation. Translation rates *in vitro* are determined by the rate of initiation of protein synthesis.

Figure 36. Correlation between changes in serum albumin and liver platinum (PT) in patients receiving cisplatin. Taken from Nanji, *et al.* (1986).

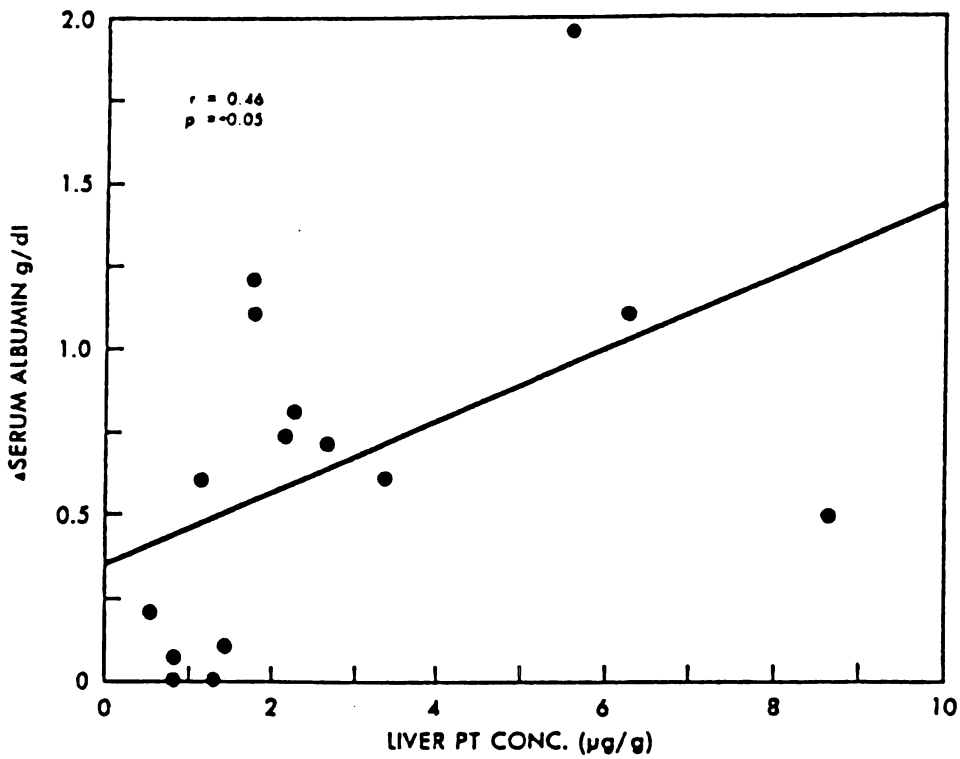
2.0
1.5
1.0
0.5
0
r = 0.46
p = <0.05
ΔSERUM ALBUMIN g/dl
0
2
4
6
8
10
LIVER PT CONC. (μg/g)

Figure 36.

More specifically, the rate limiting step in initiation is the formation of the 43s ribosomal complex and its subsequent joining with the mRNA (Ochoa, 1983). Formation of the 43s complex is controlled by eucaryotic initiation factor 2 (eIF-2). This factor is necessary for the formation of the ternary complex (eIF–2•GTP•$tRNA_{f-met}$) and its subsequent joining to 40s to make the 43s complex. In the final step of eucaryotic protein chain initiation, which involves the joining of the 48s (43s plus mRNA) and the 60s ribosomal subunits, eIF-2 is released as eIF-2•GDP (Matts and London, 1984). The recycling of eIF-2 after this step in initiation requires the replacement of GDP by GTP. This exchange is catalyzed by a reversing factor. The rate of initiation is controlled by the phosphorylation of eIF-2. Phosphorylated eIF–2 interacts with the reversing factor to form a non-functional complex (Gross, *et al.*, 1985). Since the reversing factor is present in lysates at a limiting concentration relative to eIF-2 (Matts, *et al.*, 1983), the phosphorylation of only 20-40% of lysate eIF-2 is sufficient to bind all the lysate reversing factor. This sequestration of the reversing factor prevents recycling of eIF-2•GDP. Thus, the rate of initiation is limited by the availability of ternary complex formation which is dependent upon the phosphorylation state of eIF-2. This inhibition of translation is reversed by the addition of cAMP and is enhanced by the addition of heavy metal ions which stimulate eIF-2 kinases. This does not appear to be the mechanism by which cisplatin inhibits peptide synthesis, however, as the addition of cAMP enhances cisplatin inhibition, and the formation of 43s is not affected in cisplatin supplemented assays.

Kinetics of Cisplatin Inhibition of *in vitro* Translation

Reticulocyte lysate synthesizes globin at rates approaching those observed in intact reticulocytes (Adamson, *et al.*, 1968). A linear rate of translation can continue for up to 120 min (Safer, *et al.*, 1975). In the presence of an initiation inhibitor, protein synthesis proceeds at the same rate as in its absence for approximately 6 min and then declines abruptly (Adamson, *et al.*, 1968). Cisplatin demonstrated this type of time course of translation inhibition. The length of translation proceeds at the initial rate is related to the rate of initiation before it declines (Gross and Rabinovitz, 1973). Thus, a shorter length of time prior to the decline in translation indicates a decreased rate of initiation. This suggests that a component of the system required for initiation is progressively inactivated or exhausted. Thus, the kinetics demonstrated by inhibitors of initiation, and by cisplatin, demonstrate two rates of translation activity. As sufficient quantities of initiation precursors are available in the lysate, the early rate of incorporation of amino acid label reflects the rate of elongation of peptides. The subsequent decline in translation then reflects the rate at which initiation is occurring as this is the rate limiting process in peptide synthesis. An alternate possibility to explain the two observed rates could be a time delayed inhibition of a component necessary for the elongation of the forming peptide. This possibility is without precedent in the literature and inconsistent with the data from cisplatin inhibited lysates separated on sucrose gradients. Compounds that inhibit initiation

and elongation would be expected to show an initial rate of translation less than that of an uninhibited assay followed by a further decline in the rate of synthesis. This is not observed in cisplatin inhibited assays even at large concentrations of added drug.

Cisplatin is Associated with Polysome Disaggregation

The cisplatin inhibition is also accompanied by disaggregation of polysomes. Disaggregation of polysomes with inhibitors of initiation reflects a decrease in the rate of initiation relative to elongation. In the presence of initiation inhibitors, ribosomal complexes are not formed. Thus, ribosomes formed prior to the addition of the inhibitor bind to mRNA and along with those already bound, elongate, but no new ribosomal complexes join the message. Therefore, over time, the mRNA contains fewer ribosomes. Previously bound ribosomes complete their peptide synthesis and are released from the template. In the presence of an elongation inhibitor, however, ribosomes form and bind to the mRNA but their translocation rate is significantly slowed. Thus, new ribosomes bind message but already bound complexes are not released. The result is a mRNA template containing many ribosomal complexes. Sucrose gradients show a decrease in polysomes in lysates treated with cisplatin. This finding of polysome disaggregation following the addition of cisplatin suggests that this drug acts to inhibit the initiation of translation.

Cisplatin Inhibits a mRNA Dependent Step

Cisplatin inhibition of peptide synthesis can be accounted for by an interaction with mRNA. This hypothesis is based on the following evidence. In messenger dependent assays in which cisplatin is incubated separately with either the mRNA or the lysate, the drug has comparatively little effect on the lysates. Further, in experiments antagonizing the cisplatin effect with dithiobiuret, reversal of the cisplatin effect is observed only when dithiobiuret and cisplatin are combined before the drug is exposed to mRNA. In cases in which dithiobiuret is added after template and cisplatin have been combined, inhibition is not diminished. The possibility of cisplatin binding to mRNA is not surprising as it has previously been shown to bind to nucleic acids (Eastman, 1983; Lippard, 1982; Roberts and Pascoe, 1972; Juckett and Rosenberg, 1982).

Cisplatin Alters mRNA Structure

More direct evidence of a cisplatin interaction with mRNA is demonstrated by the non-denaturing agarose gel electrophoresis. Cisplatin detectably alters the migration of mRNA in these gels. Furthermore, the migration of other species of RNA do not appear to be effected to the same degree. The ribosomal bands were not shifted in total RNA incubated with cisplatin whereas the mRNA that migrates between the ribosomal bands was. The electrophoretic pattern of RNA molecular weight markers, which are not mRNA, was

not altered by incubation with cisplatin. It can be concluded from these findings that cisplatin alters mRNA whereas the electrophoretic migration of other species of RNA is not detectably changed.

The migration of DNA in an agarose gel has been shown to be related to its secondary structure (Vinograd and Lebowitz, 1966). The same DNA travels farther in a gel when supercoiled than linear, for example. The migration of DNA following incubation with cisplatin and separation in a non-denaturing agarose gel was significantly altered (Cohen, *et al.*, 1979). Thus, the binding of cisplatin to DNA is shown to alter the structure of DNA. The change in migration of mRNA in these gels might be explained by similar cisplatin induced alterations in mRNA secondary structure or the cross-linking of various pieces of message. Alterations in secondary structure may cause message to run both faster and slower in the gel. If cisplatin causes increases in RNA secondary structure, then larger mRNAs would be more compact and migrate faster in the gel. Smaller mRNAs may travel slower in the gel if cisplatin links them to other RNAs or its binding causes them to become bulkier or have an altered ionic charge. Thus, the non-denaturing gel electrophoresis data suggest similarities between cisplatin incubated with RNA and DNA. Furthermore, apparent specificity of cisplatin for messenger RNA is noted. Ribosomal and transfer RNAs are included in the nuclease treated lysate when it is preincubated with cisplatin. Lysate treated in this manner is only minimally inhibited which further suggests a lack of cisplatin

effect on these non-messenger RNAs. This evidence disputes the assumption that selective reaction with any type of RNA does not occur to inhibit translation (Pascoe and Roberts, 1974).

Proposals for the Study of Drug Induced RNA Changes

A direct demonstration of selective cisplatin binding to RNA species would provide even more conclusive evidence for this effect. The use of radiolabeled cisplatin or atomic absorption might be utilized to measure binding following the isolation of the individual RNA species. Additional techniques exist for the examination of the structure of RNAs in solution (Ehresmann, *et al.*, 1987). These could possibly be utilized to further demonstrate and perhaps identify the alterations induced in mRNA by cisplatin. These methods test the reactivity of the nucleotides towards chemical and enzymatic probes. One approach uses an end labeled RNA and detects the location of sensitive sites by the use of different sequence specific RNases. Sequences of RNA protected by cisplatin may be recognized by their lack of RNase cleavage. Another set of RNases detect and cleave specific secondary structures. A different approach is based on primer extension by reverse transcriptase. This assay detects the stoppage of transcription at modified nucleotides. The synthesized cDNA fragments are then sized by electrophoresis. The structure of RNAs could also be determined by X-ray crystallography. To the present, however, only tRNAs have yielded crystals able to diffract at high resolution (Westhof, *et al.*, 1985).

Cisplatin Does Not Degrade mRNA

This inhibitory effect of cisplatin cannot be explained by digestion of the message by either cisplatin itself or by some contaminant of the drug solution. Inhibition of *in vitro* translation occurs in the presence of RNasin, a specific inhibitor of ribonucleases. Furthermore, denaturing agarose gel electrophoretic analysis of mRNA preincubated with cisplatin shows no degradation or other alteration in the migration of the message at concentrations which result in essentially complete inhibition of peptide synthesis. Dithiobiuret reacts with cisplatin to inactivate it. Reversal of the inhibition by dithiobiuret is evidence that the action of cisplatin is not due to a contaminant in the preparation. If a contaminant is present, it would still be added to the mRNA in the translation assay and unless it too is inactivated by dithiobiuret it should inhibit peptide synthesis. This does not occur. Furthermore, addition of cisplatin, mRNA, and dithiobiuret results in inhibition of peptide synthesis. This suggests a higher affinity of cisplatin for mRNA than for dithiobiuret.

mRNA Dependent Reactions Necessary for Translation

All phases of protein synthesis beyond the formation of the 43s ribosomal subunit involve mRNA dependent reactions. Thus, possible mechanisms of peptide synthesis inhibition that involve the mRNA template are numerous. The steps subsequent to formation of the 43s subunit require its joining to mRNA to yield a 48s particle.

This is followed by the joining of the 60s ribosome to the 48s to form the 80s initiation unit. These reactions require specific regions of the template to be available for ribosomal binding. Furthermore, the 5' region of the message must allow the 43s ribosomal complex to freely scan in a 3' direction for the initiation codon. Thus, alteration of the message such that these sites are not available to the incoming ribosomes, due to occupancy by cisplatin or altered mRNA secondary structure, for example, would inhibit translation initiation. On the other hand, in order for elongation to proceed, the mRNA secondary structure must be reduced for the ribosomal complex to properly translocate. Also, the tRNA binding sites must be unhindered where they are aligned with the mRNA. Furthermore, cisplatin can effect these processes directly by binding mRNA to prevent ribosomal joining or migration along the template or indirectly by altering the secondary structure and its stability. Additional indirect interactions with mRNA dependent steps may involve the numerous initiation and elongation factors necessary for translation. Therefore, by an interaction with mRNA cisplatin could inhibit almost any step in the complicated sequence required for normal protein synthesis.

Cisplatin Interferes With 60s Ribosomal Joining

It has been demonstrated that cisplatin inhibits the initiation of protein synthesis. The initiation of translation involves both mRNA independent and mRNA dependent steps. The addition of cisplatin to specific assays of translation initiation, however,

demonstrated results consistent with the hypothesis that cisplatin interferes with a mRNA dependent step. The mRNA independent reactions resulting in the synthesis of the 43s ribosomal subunit were not inhibited following the addition of high concentrations of cisplatin. That is, the formation of the binary complex which is eIF-2 joining to $tRNA_{f\text{-}met}$, the ternary complex, the addition of GTP to the binary complex, and the combination of the ternary complex to the 40s ribosome are all unaffected by the presence of cisplatin.

Initiation involves two sequential mRNA dependent reactions. The 43s complex first binds the 5' end of the mRNA template to make a subunit that sediments at 48s. Next, this 43s ribosomal unit scans along the message for the initiation start codon. Upon locating the start codon, the 48s unit is subsequently joined by the 60s ribosome. Either step or both may potentially be inhibited by cisplatin. Reticulocyte lysates inhibited by cisplatin clearly demonstrate the formation of labeled particles that sediment at 48s. This result is demonstrated by two methods, labeled initiator tRNAs and labeled mRNA. This indicates that 48s, as well as 43s, formation is not inhibited by the addition of large concentrations of cisplatin. Furthermore, these same gradients show greatly reduced formation of 80s in assays carried out with added cisplatin. Therefore, it is concluded that cisplatin decreases protein synthesis by inhibiting the initiation of translation due to the prevention of 60s joining to the 48s ribosomal subunit.

Cisplatin Inhibits the Synthesis of Selected Peptides

Qualitative differences occur in the peptide products whose translation is directed by cisplatin treated mRNA. SDS-polyacrylamide gels of products from inhibited *in vitro* translation assays show much greater inhibition of peptides of higher molecular weight than for the synthesis of the smaller products. This same result was observed with both guinea pig and rabbit endogenous mRNAs and exogenous viral (BMV) mRNAs exposed to cisplatin. The decrease in large translation products is related to both the length of incubation with cisplatin and the drug concentration. The electrophoretic pattern shown in the fluorograph does not have any novel bands or excessive smearing. This absence of partial products suggests that cisplatin acts to inhibit the initiation of translation. If cisplatin inhibited elongation, it might be expected that partial peptide products would be detected. These novel electrophoretic bands might result from the detection of peptides associated with ribosomes that are unable to translocate further along an altered mRNA template or from the premature release of peptides. Alternatively, partial or incomplete peptides formed may be proteolytically digested in the lysate and not visible in the SDS polyacrylamide gels. It has been previously suggested that incomplete peptide products are readily hydrolyzed (Daniels, *et al.*, 1980). It is possible that cisplatin may inhibit elongation by interfering with the movement of the ribosomal complex on the mRNA, either by being directly bound or by prevention of secondary structure melting in front of the translocating ribosome. However,

the results presented above strongly support the inhibition of initiation by cisplatin as the mechanism for blocking protein synthesis.

Proposed Mechanisms of Selective Inhibition of Synthesis

Secondary structure of mRNA has previously been shown to effect the relative production of different molecular weight peptide species. Collagen mRNAs contain significant amounts of secondary structure (Gerstenfeld, *et al.*, 1983). Collagen RNA preparations were denatured to determine whether reducing this structure would effect the production of certain novel low molecular weight peptides observed following cell free protein synthesis. The smaller products were shown immunologically to be incomplete collagen peptides. Larger peptide products were less evident in the cell free translation assay. Denaturing treatments to reduce the mRNA secondary structure greatly increased translation of the larger collagen polypeptides and the synthesis of the incomplete collagen peptides was not evident. The formation of these smaller proteins was shown to be caused by an inhibition of the elongation of collagen.

Assays performed in the absence of cisplatin synthesize discrete polypeptides that demonstrate a wide range of molecular weights. In the presence of cisplatin, however, the production of the larger peptides is inhibited. This result differs from that found with collagen as no new peptides are evident. To investigate the

possibility that the difference in peptides synthesized in the presence of cisplatin is due an alteration in their elongation, the rate of ribosomal translocation along the mRNA could be measured. Furthermore, if cisplatin consistently blocked elongation by stopping the passage of ribosomes along the template in a particular region, then discrete peptide bands would result on an electrophoretic gel. This has not been found, however. It might also be possible to identify discrete regions of mRNA where ribosomes accumulate by blocking digestion with RNase. These regions of mRNA would not be digested if they are protected by ribosomal binding.

As previously shown, translation inhibition is not due to the utilization of mRNAs degraded by cisplatin. Thus, the decrease in synthesis of large peptides is not the result of digested large mRNA templates. Proteolysis of the peptide products synthesized in the assay is also unlikely due to a lack of novel peptide bands or smearing following separation by electrophoresis. To further examine the possibility of lower molecular weight polypeptides resulting from proteolytic cleavage the effect of protease inhibitors on cell free synthesis could be determined. The addition of phenylmethyl sulfonyl fluoride or pepstatin would be expected to increase the amount of full length polypeptide chains accumulated.

Inhibition of the formation of specific peptides may explain the variability in reports on the relationship between cisplatin and protein synthesis *in vivo*. Some investigators found that cisplatin

measurably decreased peptide synthesis (Harder and Rosenberg, 1970) while others did not (Zylicz, *et al.*, 1987; Vawda and Davies, 1986). Cisplatin may inhibit the synthesis of certain peptides even though overall inhibition of translation may be undetectable. This selective inhibition may lead to toxicity in the cell.

Specific Sites of Cisplatin Interaction With mRNA

Several properties of the inhibition of translation by cisplatin have been described. These taken together can be summarized to indicate that cisplatin decreases peptide synthesis by preventing the joining of the 60s ribosome to the 48s complex in a mRNA dependent reaction and the synthesis of larger peptides is inhibited to a greater extent than smaller ones. Hypotheses to explain the mechanism of cisplatin inhibition should account for these findings. It has been reported that formation of specific intrastrand cisplatin cross-links are more likely to occur than interstrand links in DNA. Since these cross-links involve single strands of DNA there is no reason to believe they would not occur in RNA as well. It may even be easier to form these intrastrand bridges in RNA as it would not have to first melt to expose single strands as DNA would. Cisplatin binds to nucleic acids with a strong preference for guanine. Cisplatin often forms links between two guanines which are separated by any other base. A second site of preferred binding by cisplatin includes an adenine which is 5' to a guanine when separated by a third base. If such specific binding occurs to mRNA, then the initiation of peptide synthesis could be blocked by a

cisplatin linkage within the translation start codon, 5'-AUG. The greater inhibition of large peptides is not explained by such a mechanism, however.

Postulated Role of mRNA Structure Changes in Inhibition

The migration of RNA incubated with cisplatin suggests the drug alters the secondary structure of mRNA. Cisplatin induced changes in mRNA structure may involve the formation of novel secondary structures or prevention of the normal changes in secondary structure necessary for proper translation to occur. A cisplatin cross-link in a region of mRNA might interfere with initiation by preventing the 60s ribosome from gaining access to the opposite side of the mRNA where the 43s subunit is bound. A role for mRNA secondary structure in the control of translation initiation has been proposed (Hall, *et al.*, 1982). The translation of mRNA containing a mutation that increases the stability of a base paired structure including regions critical for initiation was examined. A decrease in translation resulted from this alteration of mRNA structure. In another experiment, different rates of translation were demonstrated in the same RNAs with different induced secondary structures (Gultyaev and Shestopalov, 1987).

The size of the mRNA is usually proportional to the size of the encoded protein (Kozak, 1983). If the larger peptides are synthesized from larger mRNA templates and this allows for the formation of a greater amount of secondary structure, the greater

mRNA size may allow for more cisplatin induced cross links and account for the greater inhibition of large peptides.

A correlation was found between the size of a peptide and its inhibition by cisplatin. The data also demonstrates that cisplatin inhibition of peptide synthesis is by an interaction with mRNA. This suggests that a relationship exists between the incidence of cisplatin binding to message and the length of the effected mRNA. The binding of cisplatin to DNA is greatest following reaction with certain, specific, base sequences (Rahmouni and Leng, 1987). These same base sequences are found in mRNA. It would be expected that these sequences would occur more often in larger mRNAs due to the increased numbers of bases present. Therefore, the synthesis of larger peptides may be inhibited to a greater extent due increased binding of cisplatin to their larger mRNA templates.

Cisplatin May Alter the Selection of Translated mRNAs

Cisplatin may impair the ability of certain mRNAs to compete for initiation complexes in the cell free translation assay. This effect could produce the observed selective inhibition of synthesized peptides. Certain mRNAs are initiated at a higher rate in *in vitro* translation assays (Gerstenfeld, *et al.*, 1983; Lodish, 1971). Normal hemaglobin synthesis, for example, results from competition among mRNAs (Lodish and Jacobsen, 1972). Formation of hemoglobin requires equimolar quantities of globin a- and ß-chains. The two corresponding mRNA species present in the lysate

are each translated by ribosomes with equal effectiveness. The elongation rates in polyribosomes for both these mRNAs have been shown to be identical (Lodish, 1971). The polyribosomes synthesizing ß-chains, however, contain more ribosomes than those synthesizing a-chains, although the lengths of these two mRNA species are similar. Initiation on mRNA coding for the ß-chain takes place more effectively compared to the mRNA coding for the a-chain. To compensate for this difference and to produce equimolar quantities of the a- and ß-chains, the cell lysate contains a correspondingly higher amount of mRNA for the a-chain than for the ß-chain. Many other examples of selective discrimination of mRNAs in translation exist (Chevrier, *et al.*, 1988; Palmiter, 1978; Svitkin, *et al.*, 1978). It is though that the 5'-terminal sequence mRNA has some structural characteristics determining its higher affinity for initiation (Lodish, 1971). Cisplatin may induce structural alterations in certain mRNAs such that these are preferentially inhibited.

In order to test this hypothesis, the relative efficiency of translation of specific mRNAs in the presence and absence of added cisplatin could be determined. Saturating concentrations of at least two mRNAs would be added to a translation assay at the same time. Better initiating mRNAs would be evident by their ability to compete more favorably in a cell free system for the limited amounts of initiation factors. Using this assay, mRNAs could be compared to determine the relationship between different known properties of

the message (length, sequence, or structure, for example) and efficiency of initiation in the presence of cisplatin.

Initiation Rates are Inversely Related to Concentration

In contrast to prokaryotic mRNA, eucaryotic mRNA is metabolically stable; there is no rapid degradation of mRNA accompanied by re-synthesis (Spirin, 1966). A large fraction of mRNA does not take part in translation at a given time (Gross, 1967). Newly initiating ribosomes normally bind to mRNA molecules that are already being translated by other ribosomes (Pain, 1986). When protein synthesis is stimulated, these extra mRNAs are initiated and actively translated. Thus, when maximally stimulated, such as by the addition of cAMP, the translation system has a reserve to utilize in order to increase its peptide synthesis. Translation increases in two ways. First, by reducing the phosphorylation of eIF-2 to stimulate the formation of ribosomal initiation complexes and, second, to make more mRNA available for these complexes to bind and initiate.

The data presented demonstrates that cisplatin inhibits protein synthesis *in vitro* by an interaction with mRNA. The log dose response relationship between cisplatin concentration and translation inhibition suggests the binding of cisplatin to mRNA is proportionate to its concentration. Thus, more mRNAs in the lysate are effected with increases in cisplatin concentration. At lower concentrations of the drug, a smaller amount of the mRNA is bound

by cisplatin and unavailable to bind a 60s ribosome than at higher concentrations. As 48s complexes are less stable than 80s (Pain, 1986), a 43s complex can dissociate from a cisplatin effected mRNA and eventually reassociate with an unaffected mRNA to initiate translation. Thus, it would be expected that initiation of peptide synthesis could occur in the presence of cisplatin, but the rate would be slower than in its absence. Increases in the cisplatin concentration would slow the rate further as the probability of a 43s preinitiation complex finding an unaffected mRNA would lessen. The data fits this hypothesis. The kinetics of translation following the addition of increasing concentrations of cisplatin show initial rates of synthesis that are the same as uninhibited assays. The rate of translation then decreases in proportion to the concentration of added cisplatin. As the rate of translation is limited by the rate of initiation, this reflects decreasing rates of initiation with increasing drug concentration. This finding, therefore, may be interpreted to suggest that the rate of initiation is inversely proportional to the amount of cisplatin affected mRNA.

Stimulated Translation is More Susceptible to Inhibition

The addition of cAMP has been shown to increase inhibition of protein synthesis by cisplatin. If cAMP maximally stimulates translation activity, then most of the mRNA in the lysate would be required for translation and little would be held in reserve. Thus, inhibition of translation would be increased as a 43s preinitiation complex that finds a cisplatin bound mRNA would not have the option

of finding another non-affected, reserve, mRNA as in a non-stimulated assay. Each cisplatin inhibited mRNA would act to inhibit initiation as each is required in a maximally stimulated translation assay. This hypothesis suggests that the addition of cAMP would enhance the potency of cisplatin in its inhibition of *in vitro* translation. This is what is found as the log dose response curve of cisplatin inhibition of peptide synthesis is parallel shifted to the left following the addition of cAMP.

SUMMARY AND CONCLUSIONS

The current hypotheses for the mechanism of cisplatin cytotoxicity and anticancer effect have been discussed. Further, the difficulties that exist in the ability of these suggested mechanisms to explain the observed cytotoxic and chemotherapeutic actions of cisplatin were noted. The studies which were undertaken dispute some of the assumptions made in proposing DNA as the target for the cytotoxic and antitumor action of cisplatin.

Cisplatin was found to inhibit protein synthesis in isolated, cell free, preparations derived from different species. Furthermore, this inhibition occurs at pharmacologically relevant concentrations. Cisplatin appears to inhibit *in vitro* translation by causing an alteration in the structure of mRNA such that the 60s ribosome is unable to bind to the 48s preinitiation complex. The mechanism by which this acts to cause cell toxicity or inhibit tumor growth is unknown at this time. The drug may be toxic to tumor cells by inhibiting the translation of the most metabolically active cells or by selectively inhibiting the synthesis of specific peptides necessary for their growth.

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