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Mechanism of $HgCl_2$ Cytotoxicity in Cultured Mammalian Cells

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SUMMARY

Treatment of intact Chinese hamster ovary cells with $HgCl_2$ produced a rapid, concentration-dependent induction of DNA single-strand breaks (SSB) as revealed by alkaline elution analysis. Direct addition of $HgCl_2$ to cell lysates did not result in DNA strand breaks. $HgCl_2$ treatment of cells also caused a rapid leakage of superoxide radicals that were detected in their media by measurement of the reduction of exogenously added cytochrome *c*. There was a linear relationship between the production of radicals and the induction of DNA strand breaks, and there were also excellent temporal correlations in these parameters. Addition of oxygen radical scavengers, such as the enzymes superoxide dismutase and catalase, to the extracellular media significantly reduced the extent of DNA damage caused by $HgCl_2$ without a similar attenuation of its uptake into cells, as did the autoclaved enzymes. Similarly, addition of radical scavengers such as glycerol or ascorbate inhibited the DNA damage but also reduced the uptake of the metal by almost the same degree. Thus, because of secondary effects on uptake of the metal, the radical scavenger experiments could not address the importance of oxygen radicals in the DNA damage caused by $HgCl_2$. SSB were enhanced when cells were treated with $HgCl_2$ and diethylmaleate or diethyldithiocarbamate, agents that deplete cellular reduced glutathione or inhibit the intracellular activity of superoxide dismutase, respectively. Thus, DNA damage in cells rendered sensitive to radicals was greater when these cultures were subsequently treated with $HgCl_2$. The binding of $^{203}HgCl_2$ to the DNA of intact Chinese hamster ovary cells was also studied. These studies were made possible by the relatively high stability of Hg(II) interaction with DNA and by utilizing a gentle method of DNA isolation that minimized redistribution of intracellular Hg(II) complexes after cells were lysed. The amount of Hg(II) bound to DNA varied from approximately 7 to 35 Hg atoms per 10^4 base pairs (bp) at concentrations of $HgCl_2$ that have been previously shown to produce between 1 SSB/10^7 bp and 1 SSB/10^6 bp. The Hg(II)-DNA adducts were relatively stable complexes, since they resisted treatment with 0.1 M EDTA and 1 M NaCl and were stable to precipitation of the DNA with ethanol and trichloroacetic acid. However, the Hg(II) was released from the DNA when it was degraded enzymatically to mononucleosides, suggesting that the Hg(II)-DNA bonds formed in the cell were not truly covalent and that the strength of Hg(II) binding to DNA depended upon polynucleotide structure. In addition to the ability of $HgCl_2$ to enhance oxygen radical formation in cells and to bind with DNA, the $HgCl_2$ uptake, binding to DNA, and strand breaks were reduced by a temperature shift from 37° to 4°. At 4°, as compared with 37°, a 50% reduction in both uptake and DNA binding of Hg occurred, but the lower temperature resulted in almost complete suppression of DNA strand breaks. These results suggest that DNA lesions caused by $HgCl_2$ in intact cells were temperature-dependent, possibly involving free radicals and enzymatic systems.

INTRODUCTION

The DNA molecule is a target site of most, if not all, carcinogenic and mutagenic agents. Additionally, a number of agents are acutely cytotoxic to cells because they

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damage the DNA (1, 2). In this regard, $HgCl_2$ represents an interesting example, since it is perhaps the most potent chemical agent that causes DNA damage in cells yet it has little or no known carcinogenic or mutagenic activity. $HgCl_2$ has been shown to induce strand breaks rapidly in the DNA, and these breaks have been characterized as true single-strand breaks, and not alkaline-sensitive sites or double-strand breaks (3–5). The DNA strand breaks produced with $HgCl_2$ have been compared with those caused by X-rays in a number of ways (3, 4). However, the single-strand breaks caused by X-rays were rapidly repaired whereas those caused by $HgCl_2$ were not (4). The presence of Hg^{2+} in cells has been found to be extremely inhibitory to the repair of single-strand breaks such as those caused by X-rays (4). Therefore, although $HgCl_2$ induced DNA lesions similar to those of X-rays or other carcinogenic and mutagenic agents, the inability of cells to repair these lesions could constitute the cause of cytotoxicity.

Cellular mechanisms of $HgCl_2$-induced cytotoxicity have not been well studied. The lack of definitive studies on the mechanism of $HgCl_2$ cytotoxicity may be due to the fact that this metal interacts with many cellular sites because it is an extremely reactive soft metal ion which readily forms coordinate-covalent bonds with cellular ligands (6). Cell membrane injury has been proposed as a site of toxic injury. However, the concentrations of $HgCl_2$ that produced cell membrane injury as measured by the release of intracellular markers were equal to or above those that caused measurable DNA strand breaks. The $HgCl_2$ injury to the cell membrane was not inhibited by addition of oxygen radical scavengers (7).

The mechanism by which $HgCl_2$ produces DNA single-strand breaks is currently not known, although $HgCl_2$ has been shown to cause lipid peroxidation and to deplete cellular reserves of reduced glutathione (3, 7). Hg^{2+} also binds to the DNA, primarily to the nitrogen atoms in the thymidine base, replacing the hydrogen atom in the complementary binding of thymidine to adenine (8). This interaction increases the helix stability and its diameter to a distance approximating the difference between the atomic radius of hydrogen and mercury (8, 9). DNA single-strand breaks may arise directly from this binding of Hg^{2+} to DNA, or, alternatively, these lesions may be caused by an oxygen radical-dependent mechanism such as has been proposed for X-rays (10, 11).

In the present study, we have investigated the ability of Hg^{2+} to produce oxygen radicals in intact cells and have related this response to the induction of DNA single-strand breaks induced by treatment of cells with $HgCl_2$. We have also studied the binding of this metal to the DNA of intact cells. Our results demonstrate that $HgCl_2$ binds to DNA and enhances the production of oxygen radicals in cells. Both of these effects are important with regard to the cytotoxic effects of $HgCl_2$ at DNA target sites.

EXPERIMENTAL PROCEDURES

Materials

The radioisotopes [^{14}C]deoxythymidine (58 mCi/mmol) and $^{203}HgCl_2$ were purchased from New England Nuclear Corporation (Boston, Mass.). Cytochrome *c*, superoxide dismutase, ascorbate, α-tocopherol, sodium benzoate, catalase, mannitol, sodium dodecyl sulfate, free acid EDTA, disodium EDTA, Tris, and RNAse III were obtained from Sigma Chemical Company (St. Louis, Mo.). Tetrapropylammonium hydroxide (10% aqueous solution) was purchased from RSA Chemical Company (Ardsley, N. Y.). Polycarbonate filters were from Nucleopore (Pleasanton, Calif.). Proteinase K was purchased from NC/B Chemicals (Cincinnati, Ohio). Fetal bovine serum, Eagle's *alpha* minimal essential medium, and trypsin were purchased from GIBCO, Inc. (Grand Island, N. Y.). Liquiscint was obtained from National Diagnostics (Somerville, N. J.).

Methods

Cell culture procedure. CHO cells[6] were maintained in Eagle's *alpha* minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Confluent cultures of cells were utilized for all of the experiments reported. Prior to treatment of cells with $HgCl_2$, the stationary phase cultures of cells were placed in a minimal salts/glucose maintenance medium [Hepes buffer (50 mmol/liter at pH 7.2 containing NaCl (100 mmol/liter), KCl (5 mmol/liter), $CaCl_2$ (2 mmol/liter), and glucose (5 mmol/liter)] to minimize and control for the effect of medium or serum components on Hg^{2+} uptake into cells (12).

Alkaline elution. The alkaline elution technique for analysis of DNA strand breaks was performed as described by Kohn *et al.* (13, 14), with minor modifications. Following treatment, cells were removed with trypsin, and an aliquot containing 8.5×10^5 cells was diluted to 20 ml with ice-cold Puck's saline A (5 mM $NaHCO_3$, 6 mM glucose, 5 mM KCl, and 140 mM NaCl, pH 7.2) and deposited onto 25-mm polycarbonate filters (2-μm pore size). Filters were rinsed with 10 ml of saline A, and the cells were lysed directly on the filters by passage of 5 ml of 2% sodium dodecyl sulfate containing 0.025 M EDTA at pH 10.1. The DNA was eluted from the filter by slowly pumping (0.035 ml min^{-1}) a solution (25 ml) containing 0.02 M EDTA (free acid) plus 2% (final concentration) tetrapropylammonium hydroxide, pH 12.1. Fractions of approximately 2 ml were collected. Following the completion of the elution procedure, the filters were digested for 1 hr at 60° in 1 N HCl and counted in 10 ml of Liquiscint.

To quantitiate the extent of DNA single-strand breaks, the SSF was calculated from the alkaline elution patterns utilizing the following relationship: SSF = log A/B, where A = amount of DNA retained in the sixth fraction of untreated sample and B = DNA retained in the sixth fraction of the treated samples.

Estimation of cellular superoxide levels. Superoxide formation was measured by the extracellular reduction of exogenously added cytochrome *c* in the absence and presence of superoxide dismutase (15). Spectral differences at 550 nm were utilized to quantitate the increased reduction of cytochrome *c*.

Cellular uptake and binding of $HgCl_2$ to DNA. The uptake of $HgCl_2$ and other metal salts into cells can be influenced by the composition of the culture medium and in particular by the concentration of the metal-binding amino acids such as cysteine (12). Therefore, uptake studies were conducted in cultures maintained with a minimal salts/glucose medium [50 mM Hepes (pH 7.2) containing 0.15 M NaCl, 2 mM $CaCl_2$, 5 mM dextrose, and 5 mM KCl]. Following treatment of cells with selected concentrations of $^{203}HgCl_2$ for short time intervals (15–30 min), cells were dislodged from the dishes by trypsinization or by scraping, and the cell number was determined utilizing a Coulter particle counter or a hemocytometer. The cellular uptake of $^{203}HgCl_2$ was determined by assessing radioactivity in a Beckman gamma counter in which the counting efficiency was typically 95%. DNA was isolated from the cells by a procedure previously described (16, 17). The cells were lysed in 3 ml of 10 mM Tris-HCl (pH 8.0) containing 10

[6] The abbreviations used are: CHO cells, Chinese hamster ovary cells; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSF, strand scission factor.

mM EDTA, 10 mM NaCl, and proteinase K (50 μg/ml). The lysate was placed immediately into dialysis against the above buffer but without proteinase K and dialyzed for 20 hr at 20°. The sample was then extracted with chloroform:isoamyl alcohol (24:1), precipitated with ethanol, and digested with RNAse A (100 μg/ml) in 40 mM Tris-HCl (pH 7.2) containing 5 mM EDTA and 10 mM NaCl. The DNA was collected by ethanol precipitation; typical yields ranged between 4.5 and 6 μg for 1 million cultured cells. The quantity of DNA was determined by the absorbance at 260 nm and was also measured by reaction with diphenylamine (18). Occasional contamination of the DNA fraction with RNA occurred as determined indirectly by overestimates of the DNA by A_{260} values as compared with values obtained by the diphenylamine reaction; however, complete removal of the RNA fraction by alkaline hydrolysis at this stage of the isolation never resulted in the loss of more than 5% of the $^{203}Hg(II)$ from the DNA fraction. No protein could be detected in the DNA fraction by the micro Bio-Rad assay.

Isolation of cytoplasmic ^{203}Hg complexes. CHO cells were treated with $^{203}HgCl_2$ at 2.5 or 5 μM for 15 min. Cellular uptake was determined as described above before cultures were suspended in normal saline and disrupted by sonication (three bursts of 30 sec each). The cytoplasmic $^{203}Hg(II)$ complexes were isolated from the supernatant after centrifugation of the lysate at 3000 × *g* for 10 min.

Enzymatic degradation of DNA. The method for determination of base composition of DNA has been described previously (19). Briefly stated, DNA isolated by the proteinase K method (*vide supra*) was treated with alkaline phosphatase followed by degradation with deoxyribonuclease I and 5′-nucleotidase to yield nucleosides. The samples were applied to a Partisil 10 SAX column (Whatman, Inc., Clifton, N. J.) and eluted with 30 mM ammonium dihydrogen phosphate (pH 3.7).

RESULTS

Induction of DNA single-strand breaks following treatment of intact cells with $HgCl_2$. Exposure of intact cells to varying concentrations of $HgCl_2$ resulted in a concentration-dependent induction of DNA single-strand breaks (Fig. 1). Previous studies have definitively characterized these lesions as true single-strand breaks (5). Mixing experiments in which [^{3}H]thymidine-labeled untreated cells were combined with [^{14}C]thymidine-labeled cells treated with 10 μM $HgCl_2$ prior to their deposition on filters for alkaline elution showed no breakage of the ^{3}H-labeled DNA, indicating that the observed lesions occurred in intact cells and that the presence of intracellular $HgCl_2$ following cell lysis did not break the DNA directly. Figure 1 also shows that the induction of DNA single-strand breaks was temperature-dependent, since a considerably greater concentration of $HgCl_2$ was required to break the DNA in intact cells at 4° as compared with 37°. Differences in the amount of Hg^{2+} uptake at these two temperatures could not account for the observed differences in DNA strand breaks produced by the temperature shift.

Formation of superoxide radicals in $HgCl_2$-treated cells. Cytochrome *c* was not chemically reduced by $HgCl_2$ directly, nor did this metal alter the reduction of this cytochrome by an *in vitro* xanthine oxidase reduction system. Untreated CHO cells liberated superoxide (2 nmol of O_2^- per 30 minutes per milligram of protein) into the incubation medium (Fig. 2) as measured indirectly by the reduction of cytochrome *c*. When 15 μM $HgCl_2$ was added to the medium, there was an almost immediate increase in the basal reduction of extracellular cytochrome *c* (1–2 min) that was inhibited by the enzyme superoxide dismutase (Fig. 2). This elevated formation of superoxide reached a maximum level following 9–12 min of incubation with $HgCl_2$. There was a linear relationship between superoxide formation and the degree of DNA breakage during the initial 10-min time interval following $HgCl_2$ treatment (*inset*, Fig. 3). However, the

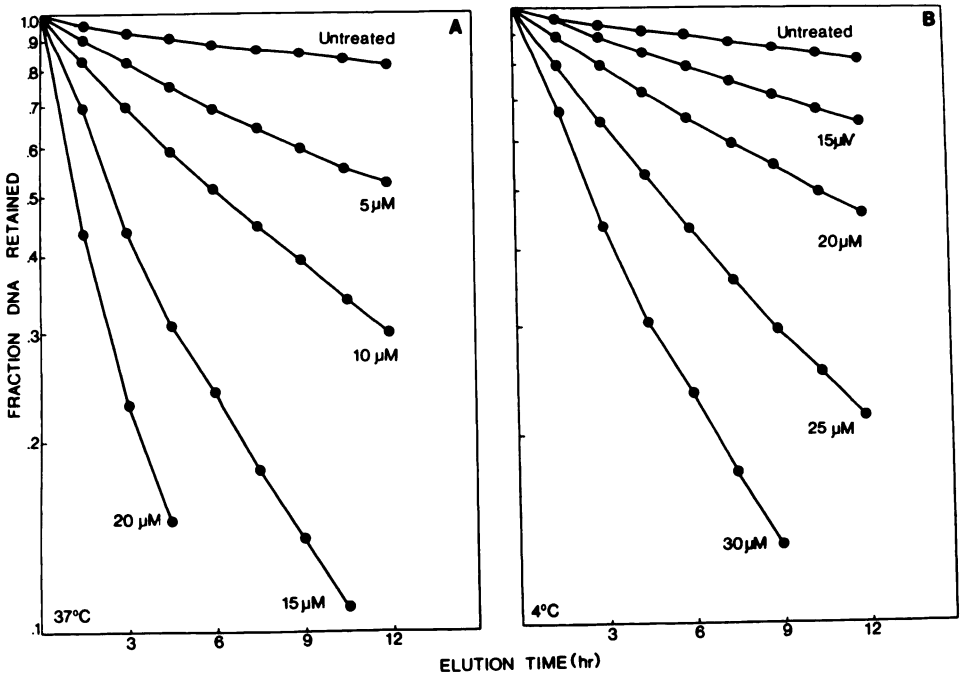

FIG. 1. *Induction of DNA single-strand breaks by $HgCl_2$*

Alkaline elution was performed as described under Methods, using cells treated for 1 hr at 37° (A) or 4° (B) with concentrations of the metal shown.

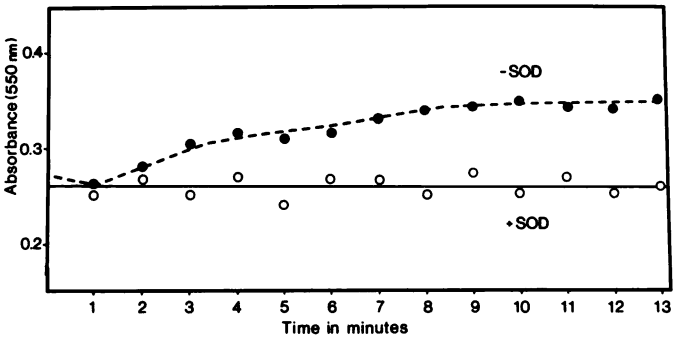

FIG. 2. *$HgCl_2$-dependent superoxide formation by CHO cells*

Cells (1×10^6) were incubated in Hanks' balanced salt solution containing 15 μM $HgCl_2$ in the presence O—O or absence ●—● of superoxide dismutase. Leakage of superoxides in the medium was measured by the reduction of exogenously added cytochrome *c* at various time intervals following addition of $HgCl_2$ (see Methods).

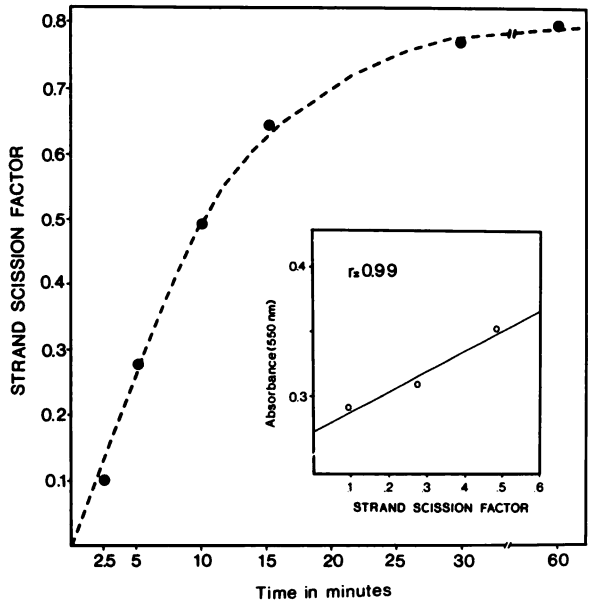

FIG. 3. *Time-dependent induction of DNA single-strand breaks by $HgCl_2$*

Following exposure of cell cultures to 15 μM $HgCl_2$ for the time intervals shown, the DNA was analyzed by the alkaline elution procedure described under Methods. The SSF was calculated according to the following relationships: SSF = $-\log A/B$, where *A* equals the amount of DNA retained in the sixth fraction of the untreated sample and *B* equals the DNA retained in the sixth fraction of the treated sample. The *inset* shows the correlations between the formation of superoxide radicals (data from Fig. 2) and the induction of single-strand breaks in the DNA (data from Fig. 3) caused by $HgCl_2$ treatment of cultured cells.

number of DNA single-strand breaks produced by the metal reached a maximal level within a 30-min time interval of $HgCl_2$ treatment (Fig. 3). There was also a linear relationship between superoxide formation and $HgCl_2$ concentration (30-min exposure) at concentrations approaching 15 μM (Figs. 3 and 4). At higher concentrations, leakage of radicals into the medium declined progressively, and at 25 μM the radical release from the cells was below basal levels (Fig. 4). The effect of $HgCl_2$ on the release of superoxide radicals from cells may explain the absence of perfect temporal correlations between radical production and DNA single-strand breaks.

Effect of radical scavengers on DNA lesions produced by $HgCl_2$. We have attempted to alter the extent of $HgCl_2$-induced DNA damage in intact cells by addition of radical scavengers at concentrations that have previously been shown to inhibit X-ray-induced DNA damage. There were some effects observed when the enzymes superoxide dismutase and catalase were added at 1×10^3 to 4×10^3 units/ml, 30 min prior to $HgCl_2$ exposure (10 μM), but essentially similar results were obtained if the enzymes were autoclaved. Addition of albumin (0.5 mg/ml) caused similar reductions in DNA damage by $HgCl_2$, suggesting that such effects were attributable to nonspecific protein interactions. Results with the other radical scavengers tested (mannitol 10 mM, sodium benzoate 50 mM, ascorbate 1 mM, α-tocopherol 0.5 mM, and glycerol 1 M) were also difficult to interpret, since in many instances substantial inhibition of $HgCl_2$ uptake occurred that was proportionally equivalent to alterations in the DNA damage. In some instances (sodium benzoate) there was no effect on either the uptake of the metal or the DNA damage.

In a related study, we investigated whether a cell rendered radical-sensitive by pretreatment with diethyldithiocarbamate, an agent known to inhibit the enzyme superoxide dismutase, or diethylmaleate, an agent that depletes the pools of reduced glutathione, would display more DNA damage when treated with $HgCl_2$. A 1 mM pretreatment with diethyldithiocarbamate alone did not produce strand breakage (data not shown), but, when these cells were subsequently treated with 5 μM $HgCl_2$ for 1 hr, DNA strand breakage was increased relative to that induced by a similar treatment with the metal alone (Fig. 5). Similar effects were observed when cells were depleted of reduced glutathione by treatment with diethylmaleate (2×10^{-4} M) (Fig. 5).

Development and validation of an assay for the measurement of the intracellular binding of $^{203}Hg(II)$ to DNA.

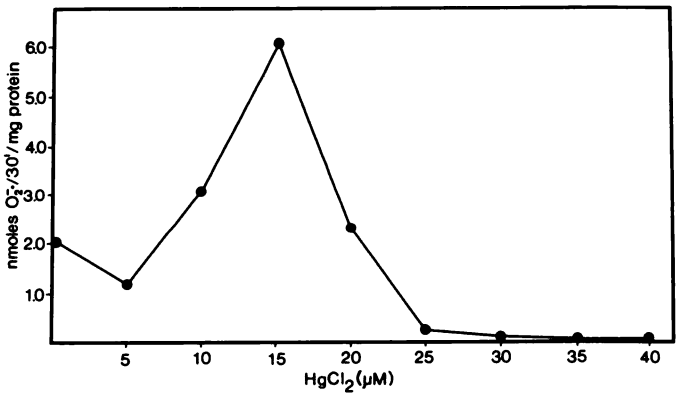

FIG. 4. *Effect of varying $HgCl_2$ concentrations on the formation of superoxide anions*

Cells were treated for 30 min with concentrations of $HgCl_2$ shown, and superoxide levels in the medium were measured by the reduction of exogenously added cytochrome *c* as described under Methods.

An assay for monitoring the amount of Hg(II) bound to DNA was devised for the purpose of studying the role of Hg(II) adducts in causing DNA damage. This method typically gave Hg(II):DNA complexes that represented from 0.5% to 1.5% of the Hg(II) that entered the cell.

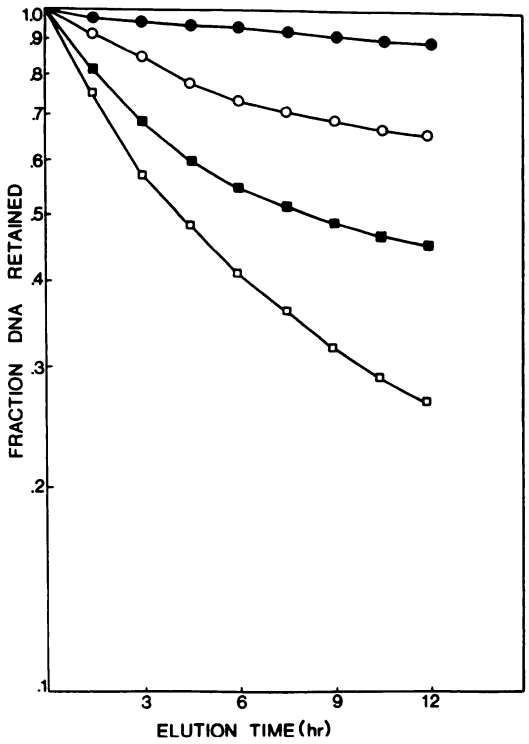

FIG. 5. *$HgCl_2$-mediated induction of DNA single-strand breaks in cells in which reduced glutathione and active superoxide dismutase were depleted*

Untreated cells (●——●) or cultures pretreated for 1 hr with solvent alone (○——○), or solvent containing 2×10^{-4} diethylmaleate (■——■) or 1 mM diethyldithiocarbamate (□——□) were then exposed to 5 μM $HgCl_2$ for 1 hr. The DNA in these cells was analyzed by the alkaline elution technique, as described under Methods.

When the cells were treated for 15 min with 1 μM $HgCl_2$ in a salts/glucose medium, 0.86 pmol of Hg per microgram of DNA remained bound in the assay. This treatment does not result in detectable levels of DNA single-strand breaks (3). Since this amount of Hg(II) bound to DNA may have been primarily due to post-lysis exchange, a series of experiments were initiated to determine the extent of post-lysis exchange in our assay. Figure 6 shows a comparison of the amounts of Hg(II) bound to DNA when (a) cells were treated in monolayer with $HgCl_2$ or (b) when cells were first lysed with proteinase K and then treated with the same amount of $HgCl_2$. In each case, the cell lysates were immediately placed in dialysis and the normal DNA isolation procedure was followed (see Methods). Fifty times more Hg(II) bound to DNA when the metal was added to disrupted cells than when it was added to intact cells (Fig. 6). This difference apparently reflected the structural constraints within the cell as Hg(II) passed through the plasma membrane, traversed the cytoplasmic milieu, and entered the nucleus to interact with the DNA. The large number of sites for Hg(II) binding within cells prevented the metal from reaching the DNA. When $HgCl_2$ was added directly to cell lysates, access of the Hg(II) to the DNA was not impeded and the extent of formation of Hg(II):DNA complexes was determined by the DNA concentration and its affinity for Hg(II) relative to that of other cellular ligands.

Since the experiment described above indicated that a large fraction of intracellular mercury was complexed to molecules other than DNA, experiments were designed to evaluate the exchange between these substrates and DNA that might occur during the binding assay (Table 1). Non-DNA, soluble, ^{203}Hg complexes were isolated from supernatants of sonicated cell samples as described under Methods and were mixed in equal proportions with lysates prepared from cells that had had no prior exposure to $HgCl_2$ or with lysates of cells that had been treated with nonradioactive $HgCl_2$ (Table 1). After each

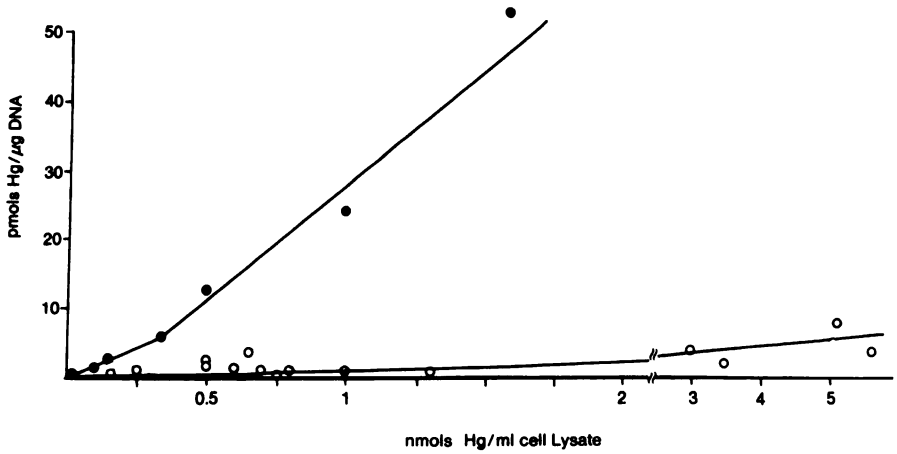

FIG. 6. *Comparison of Hg(II) binding to DNA in living cells and in cellular lysates*

The concentration of $HgCl_2$ in cellular lysates was determined from the levels of $^{203}HgCl_2$ taken up by the cultured cells. A range of $HgCl_2$ concentrations was then selected, and the $HgCl_2$ was immediately added to cellular lysates prepared as described under Methods. The amount of Hg(II) bound to DNA was then determined when the metal was added to cell lysates (●) or to whole cells (○). In replicate experiments, the standard error of the mean for ^{203}Hg binding of DNA was in the range of 25% of the mean value.

TABLE 1

Effect of ^{203}Hg complexes added to proteinase K lysates on Hg(II) binding to DNA

In the initial treatment, cell monolayers were treated for 15 min under each of the following conditions, trypsinized, counted, and mixed with ^{203}Hg complexes isolated from an equivalent number of cells that had been treated as indicated. Cell monolayers were treated for 15 min at the indicated concentrations, trypsinized, and counted. Cytoplasmic ^{203}Hg complexes were isolated in the supernatants after cells were sonicated and centrifuged; the complexes were then added to freshly prepared proteinase K lysates of cells from the series of initial treatments. The lysates were then processed by the standard binding assay (see Methods for details).

Initial treatment	Addition of cytoplasmic ^{203}Hg complexes from treated cells	Amount of Hg bound
		pmol Hg/μg DNA
A. None	$^{203}HgCl_2$, 2.5 μM	1.0
None	$^{203}HgCl_2$, 5 μM	5.6
B. $HgCl_2$, 5μM	$^{203}HgCl_2$, 2.5 μM	1.1
$HgCl_2$, 5 μM	$^{203}HgCl_2$, 5.0 μM	6.8
C. $^{203}HgCl_2$, 2.5 μM	None added	1.95
$^{203}HgCl_2$, 5 μM	None added	10.1

of these mixing experiments, the amount of ^{203}Hg per microgram of DNA was compared with the amount bound to DNA isolated from cells by the standard procedure. The amount of ^{203}Hg bound to DNA after ^{203}Hg complexes were added to lysates of untreated cells (Table 1A) was approximately 50% of the amount bound when cells were treated as monolayers (Table 1C). When ^{203}Hg complexes were added to lysates of cells pretreated as monolayers with unlabeled $HgCl_2$ (Table 1B), the amount of radiolabeled Hg-bound DNA was not significantly different from that obtained when ^{203}Hg complexes were added to lysates of untreated cells. These results suggested that the number of binding sites in DNA was not saturated by treatments of 5 or 10 μM $HgCl_2$. Collectively, these experiments indicate that there is measurable intracellular binding of ^{203}Hg to DNA, but, because of exchange reactions occurring even after gentle homogenization of cells, there was a consistent 50% overestimate of the actual ^{203}Hg bound to DNA in intact cells. The consistency of this exchange, in addition to the consistency of ^{203}Hg binding to DNA of intact cells in replicate experiments, indicated that a reliable measurement of Hg(II) binding to the DNA of intact cells could be performed.

Stability and characterization of Hg(II):DNA complexes formed in intact cells. Since covalent Hg(II) adducts can be formed using precise reaction conditions (20), it was possible that the Hg(II):DNA complexes formed in cells were covalent adducts. This was tested by degrading DNA from Hg(II)-treated cells to mononucleosides and subjecting the samples to high-pressure liquid chromatographic analysis that separated the sample into individual nucleosides. The ^{203}Hg bound to DNA was monitored after each phase of the degradation, and the extent of the loss was negligible. When the sample of nucleosides was applied to the ion exchange column, none of the ^{203}Hg was associated with any of the nucleoside peaks but rather eluted in the initial fraction, suggesting that the Hg(II) atoms complexed to the DNA depended upon the polynucleotide structure and were not truly covalently linked to individual nucleosides. The Hg(II):DNA complexes were then tested for their stability in the presence of a chelating substance (Table 2). When placed in dialysis against 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 M NaCl, DNA containing ^{203}Hg(II) retained 89% of the radioactivity after 48 hr and six changes of dialysate. Similarly, the presence of 100 mM EDTA in the dialysis solution had little effect on Hg(II):DNA complexes. Using the same dialysis procedure in the presence of unlabeled $HgCl_2$, it was found that as much a 25% of the ^{203}Hg was lost from the DNA. The ^{203}Hg bound to the DNA intracellularly was also stable to precipitation of the DNA by trichloroacetic acid and ethanol (Table 2).

The relative number of available Hg(II) binding sites in DNA was examined by adding $^{203}HgCl_2$ directly to purified DNA from CHO cells and then dialyzing against 10 mM Tris-HCl (pH 7.5) and 5 mM EDTA (Table 3). Since Tris has been shown to inhibit the formation of covalent adducts (21), this treatment should allow only less stable interactions. For each of the concentrations examined, there was nearly 10 times more Hg(II) bound for the treatments of purified DNA than for the treatments of cell monolayers. These studies suggested that the types of Hg-binding sites observed in our DNA binding assay were prevalent in DNA and that the number of available sites greatly exceeded the number of sites filled by treatments of intact cells with $HgCl_2$.

TABLE 2

Effect of various agents on the stability of ^{203}Hg:DNA complexes formed intracellularly

Each agent shown was included in a dialysis solution of the 10 mM Tris-HCl and 5 mM EDTA (unless EDTA was added). The DNA that was added for each dialysis condition at 10 μg/ml had been labeled with ^{203}Hg in intact cells and subsequently purified as described under Methods.

Agents added in dialysis	Concentration	% of ^{203}Hg remaining bound to DNA
None	—	87
NaCl	0.25 M	90
	0.5 M	91
	1.0 M	89
EDTA	0.1 M	82
	0.10 M	87
$HgCl_2$	2.5×10^{-6} M	75
	5×10^{-6} M	75
	1×10^{-5} M	75
Ethanol	70%	94
Trichloroacetic acid	2%	100
	10%	72

The stability of Hg(II):DNA complexes in intact cells was investigated by studying the loss of cellular Hg(II) following washout of the metal from the media. Figure 7 compares the loss of total cellular Hg(II) with the decline of Hg(II) bound to the DNA at hourly intervals following washout of extracellular metal. The percentage of metal bound to DNA relative to that present in the cells is depicted in Table 4. Note that during the first hour after washout of extracellular ^{203}Hg(II) there was an initial increased loss of Hg(II) bound to DNA relative to the loss in the cell, but by 2 and 4 hr the amount bound to DNA was increased relative to the metal present in the cell. The estimate for the number of Hg(II)-DNA complexes remaining 4 hr after the 5 μM treatment is 3 Hg atoms/10^4 base pairs.

Comparisons of the cellular uptake and binding of $^{203}HgCl_2$ to the DNA of intact cells. The results demonstrating an alteration in the DNA damage caused by $HgCl_2$ in intact cells at 4° as compared with 37° prompted a comparison of the binding of ^{203}Hg to DNA at these two temperatures. If the presence of intracellular Hg^{2+} alone or its binding to DNA were the only factors to consider in causing DNA strand breaks, then at 4° the level of strand breaks should be proportional to these related parameters, as it was at 37° (Fig. 8). As shown in Fig. 8, there were essentially no DNA lesions at 4° at levels of Hg in cells and bound to the DNA that produced considerable DNA lesions at 37°C. Radical production was also not detectable when cells were treated with $HgCl_2$ at 4° (data not shown).

TABLE 3

Comparison of the direct reactivity of Hg(II) for isolated DNA to its reaction with DNA in intact cells

$HgCl_2$	Hg(II) bound to DNA intracellularly[a]		Hg(II) bound to purified DNA in cell lysates[b]	
	pmol Hg/μg	No. Hg atoms/10^4 bp[c]	pmol Hg/μg	No. Hg atoms/10^4 bp
nmol/ml				
1.25	1.0	6.7	28	187.6
2.5	4.4	29.5	43	288.1
5.0	7.8	52.3	63	422.1

[a] For the intracellular measurements, the amount of $HgCl_2$ per milliliter was the concentration in the proteinase K lysate determined by cellular uptake values.

[b] Different amounts of $HgCl_2$ were added to a solution of 10 μg/ml of CHO DNA in 10 mM Tris-HCl (pH 7.5) and 5 mM EDTA for each of the measurements.

[c] bp, base pairs.

DISCUSSION

Superoxide anions have previously been shown to react with hydrogen peroxide-forming hydroxyl radicals (·OH), which potently induce single-strand breaks in the DNA by abstracting a hydrogen atom from the deoxyribose moieties, leading to unstable adjacent phosphodiester bonds (19). These radicals have been implicated in

TABLE 4

Loss of cellular and DNA-bound ^{203}Hg following removal of extracellular mercury

The numbers shown represent the percentage of total cellular ^{203}Hg that was bound to the DNA in the experiments shown in Fig. 7.

Time after removal of extracellular ^{203}Hg	% DNA-bound ^{203}Hg of total cellular Hg (initial treatment concentrations)			
	1 μM	2.5 μM	5.0 μM	10 μM
hr				
0	1.8	1.1	0.6	1.4
1	0.7	0.6	0.5	0.7
2	1.3	1.0	0.9	0.7
4	1.0	1.2	1.1	1.1

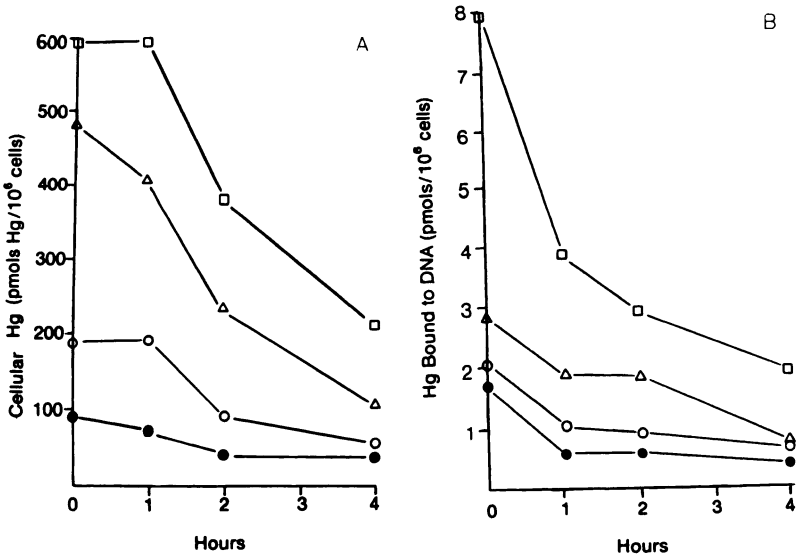

FIG. 7. *Loss of Hg(II) from the DNA or the cell following treatment and accompanied by washout of extracellular $HgCl_2$*

Cell monolayers were treated with $HgCl_2$ at 1.0 (●), 2.5 (○), 5.0 (△), and 10 μM (□). Cellular uptake (A) and the amount bound to DNA (B) were determined immediately after the 30-min treatment and at 1, 2, and 4 hr after the treatment as described under Methods.

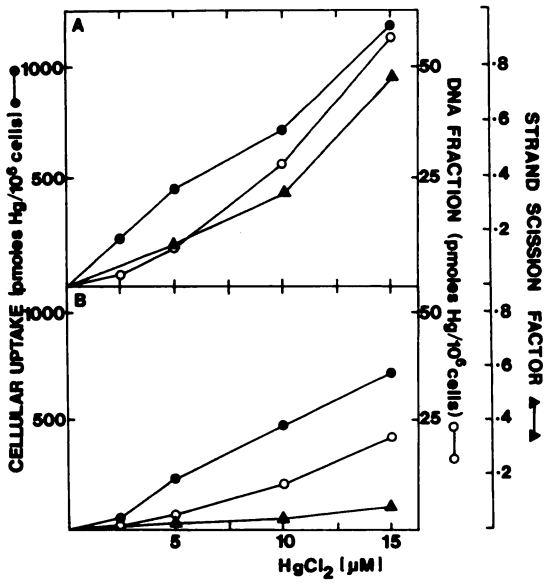

FIG. 8. *Cellular uptake and DNA binding of $HgCl_2$ in cultured cells*
Confluent cultures of cells maintained in a minimal salts/glucose medium were treated with $^{203}HgCl_2$ at the concentrations indicated. Cellular uptake (●——●) and binding of $^{203}Hg^{2+}$ to the DNA (○——○) were measured as described under Methods at either 37° (A) or 4° (B). Data showing the $HgCl_2$-induced DNA single-strand breaks (▲——▲) were taken from Fig. 1 and are expressed as the SSF (see Methods).

the production of DNA breakage by X-irradiation (22) and several antitumor antibiotics such as bleomycin (23) and Adriamycin (24). In the present study, we have suggested that radicals such as the ·OH may play a role in $HgCl_2$-induced DNA cleavage. This hypothesis is supported by the fact that Hg^{2+} inhibits the enzyme glutathione peroxidase (25), and therefore endogenously formed hydrogen peroxide may increase and react with superoxide anions, leading to an enhanced intracellular formation of ·OH radicals. Additionally, since the metal also binds to sulfhydryl groups (26), causing the depletion of intracellular radical scavengers such as glutathione (3, 7), the DNA as well as other intracellular molecules will be more susceptible to radical attack.

In the present study, we have determined that treatment of cells with $HgCl_2$ caused a rapid and concentration-dependent increase in the release of radicals from cells. At concentrations of $HgCl_2$ greater than 15 μM, the radical production was reduced; at 25 μM, it reached a level below that in untreated cells. This reduction in radical production may be due to an effect of $HgCl_2$ on the cell membrane, since Walum (21), studying the release of [^{3}H]deoxyglucose from cells, observed a similar response when $HgCl_2$ at low concentrations enhanced the release of [^{3}H]deoxyglucose while at higher concentrations the release of radioactivity was suppressed. Alternatively, at concentrations above 15–20 μM, $HgCl_2$ may be blocking cellular processes that generate oxygen radicals while at lower concentrations it may be blocking the cellular conversion of oxygen radicals to their nontoxic form. Despite the apparent effects of the metal on the release of oxygen radicals, the formation of radicals by $HgCl_2$ was for certain time intervals and concentrations temporally correlated to the induction of DNA single-strand breaks. Both effects were inhibited by the extracellular addition of selected radical scavengers. In all cases where the DNA damage was attenuated by the extracellular addition of the radical scavenger, the uptake of $HgCl_2$ into cells was also inhibited. With addition of superoxide dismutase and catalase, the metal uptake was reduced by 30–40%, whereas the DNA lesions attributable to $HgCl_2$ were absent. Since similar effects were observed with the autoclaved enzymes and with albumin, these results may be explained by a nonspecific protein interaction occurring extracellularly. Attempts were made to introduce the enzymes catalase and/or superoxide dismutase into cells by hypotonic shock or by utilizing liposome carriers, but these experiments did not protect the cell against DNA damage by $HgCl_2$ without a parallel reduction in the cellular levels of the metal. By inhibiting the intracellular activity of superoxide dismutase with diethyldithiocarbamate or by depleting reduced glutathione with diethylmaleate, damage caused by a test dose of $HgCl_2$ was greatly enhanced relative to that observed with each agent alone. These latter findings, combined with the observations that Hg(II) caused formation of radicals in cells, suggest that oxygen radicals were involved with the DNA damage caused by this metal.

Since the Hg^{2+} ion is a very reactive molecule, having a high charge to mass ratio as indicated by its low σp value, it will form coordinate covalent bonds with a variety of cellular molecules. Sulfhydryl groups will be preferentially attacked, but a number of other sites are also available for binding. We have demonstrated that Hg(II) binds to the DNA, but the significance of this reaction in terms of the rapidly induced DNA single-strand breaks is not known. Alkaline elution studies have shown that DNA interstrand cross-links are induced at later time intervals of Hg(II) treatment of cells (5). However, surprisingly no DNA-protein cross-links are caused by this metal (5), in contrast to other metals such as nickel and chromate, which potently induce this lesion in intact cells (27, 28). In contrast to these metals, however, Hg(II) has little preference for binding phosphate groups in the DNA strand and almost exclusively interacts between the thymidine and adenine bases displacing hydrogen (8). Such binding may not favor the formation of DNA-protein cross-links, but the stability of this binding would prevent denaturation of the strand, which is measured as an interstrand cross-link by alkaline elution analysis (8). The ability of Hg(II) to induce interstrand cross-links of DNA therefore is most probably related to its binding whereas the rapid X-ray-like production of single-strand breaks in the DNA may be correlated to its ability to form oxygen radicals (4). A question that remains unclear in this regard is why the interstrand cross-links take time to develop (1–5 hr) yet the measured binding of Hg^{2+} to the DNA is rapid (minutes) (5). It is known that the presence of $HgCl_2$ in the DNA inhibits the repair of single-strand breaks formed by $HgCl_2$ or X-rays (4). This effect is probably due to the binding of Hg^{2+} to repair enzymes possibly at critical sulfhydryl groups, resulting in their inactivation. Initially this may keep Hg^{2+} from interacting with the DNA in a manner that prevents the detection of DNA cross-links, but after these enzymatic sites become sat-

urated the chemical environment favors the formation of interstrand cross-links.

The interaction of Hg(II) with the DNA molecule in intact cells was investigated in part to evaluate the role of Hg:DNA cross-links (5). A role for the Hg(II):DNA complexes in strand scission seems doubtful, since comparable levels of the complexes at 4° and 37° allowed dissociation of strand scission from the binding of Hg to DNA. Thus, presumably the major toxic effect of the Hg(II):DNA adducts results from the production of DNA/DNA cross-links 4–5 hr after removal of extracellular $HgCl_2$. Since both single-strand breaks and DNA/DNA cross-links are produced at similar concentrations, it is expected that both types of DNA damage determine Hg(II) toxicity.

The binding of Hg(II) to DNA was not an unexpected phenomenon because of numerous studies relating interaction of this metal ion with purified DNA (29, 30). In fact, Hg(II), along with Ag(I), is particularly unique among metal ions in its almost exclusive binding to the bases rather than the phosphate moieties (29). The Hg(II):DNA complexes monitored by the DNA-binding assay are quite stable to both chelating agents and high salt, but these complexes do not contain covalent linkages, as described for certain *in vitro* mercuration reactions (20). The nature of the interaction of Hg(II) with the DNA in living cells appears somewhat similar to that described for binding of Hg(II) to a poly(A):poly(U) helix in which Hg(II) rapidly binds to a single uracil residue followed by the slow development of an intrastrand cross-link between two uracil residues (31). The kinetics of the formation of the cross-links in these treatments of cell cultures was compatible with the cross-links being formed during transient structural fluctuations or "breathing" of the DNA helix that occur during normal DNA metabolism. The most probable site of the initial Hg binding to DNA is expected to be the thymidine residue (29, 30), but the nature of the second base involved in the DNA/DNA cross-link is uncertain, since the extent of availability of Hg(II) binding sites in the DNA during the "breathing" response is conjectural.

A recent study has shown that a repair-deficient mutant which is more sensitive to the cytotoxic effects of $HgCl_2$ removes Hg^{2+} from the DNA at a slower rate as compared with repair-competent cells (17). These and other findings support the existence of a dynamic interaction between Hg^{2+}, DNA, and protein in the nucleus. It is difficult to study these interactions adequately by precise measurement of metal distribution in the nucleus, since exchange reaction of the metal may occur during cell fractionation or other cellular manipulation. It is therefore a necessity to study the destructive effects of the metal in the cell and to attempt to construct the most feasible course of injury based upon measurable biological consequences such as DNA damage.

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