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Correlations of DNA Strand Breaks and Their Repair with Cell Survival following Acute Exposure to Mercury(II) and X-Rays

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SUMMARY

Alkaline elution analysis demonstrates that both $HgCl_2$ and X-rays result in a rapid induction of DNA single-strand breaks at acutely cytotoxic doses ($HgCl_2$, 25–100 μM for 60 min; X-rays, 150–600 rads) in cultured Chinese hamster ovary cells. Cytotoxicity, as measured by cell-plating efficiency, correlates linearly with the level of DNA breakage induced by both agents ($HgCl_2$, $r = 0.97$; X-rays, $r = 0.99$), although a substantial difference in axis intercepts of the two linear regression lines indicates that a higher level of DNA damage was required by X-rays as compared with $HgCl_2$ to produce an equivalent level of cell killing. DNA damage induced by X-rays was rapidly repaired such that within 1 hr following treatment the elution rate of DNA from treated cells resembled that obtained in untreated cultures. In contrast, DNA damage after Hg^{2+} insult was not repaired, and further damage was evident following a similar 1-hr recovery period. Addition of noncytotoxic, non-DNA-damaging concentrations of $HgCl_2$ (10 μM) to cells 15–45 min following treatment with X-rays greatly inhibited the repair of the DNA strand breaks. Thus, although both $HgCl_2$ and X-rays induce rapid and striking single-strand breaks in the DNA, persistence of Hg^{2+} in the cell can inhibit the repair of these breaks. The inhibition of DNA repair by $HgCl_2$ may explain why this agent is not severely mutagenic or carcinogenic despite its ability to induce an X-ray-like DNA damage and why a lower level of mercury-induced DNA damage, compared with that induced by X-rays, was required to produce an equivalent level of cell death.

INTRODUCTION

Mercury compounds (inorganic, elemental, and organic) are serious environmental contaminants and produce highly toxic effects on selected organ systems *in vivo* (1). Organic and elemental mercury, by virtue of their lipid solubility, distribute to the central nervous system and produce neurological disorders (2), while inorganic mercury primarily injures the kidney (3). Elemental mercury is oxidized intracellularly by the catalase-peroxidase system to ionic mercury, and thus its intracellular effects should in theory resemble those of inorganic mercury (4). The mechanism of Hg^{2+} toxicity at the cellular level, however, is currently not well understood. Inhibition of sulfhydryl enzymes, particularly those in the cell membrane, such as Na^+/K^+-ATPase, may be involved in the mechanism of its cellular injury (5). The high degree of chemical reactivity of Hg^{2+}, an extremely soft metal ion (6), suggests that its interaction with critical cellular macromolecules may not be easily reversible. An important aspect of its cytotoxic effects is the possibility that Hg^{2+} indirectly leads to cell injury by depletion of reduced glutathione (7), generation of oxygen radicals (8), and lipid peroxidation (7, 9). In fact, agents with antioxidant activity are able to decrease the toxicity of Hg^{2+} both *in vivo* and *in vitro* (10, 11). Thus Hg^{2+} may directly injure critical components in the cell by combining with macromolecules having an affinity for mercury ions or may indirectly cause cell injury by the mechanism discussed above.

We previously demonstrated a relationship between the acute cytotoxicity of mercury(II) and X-rays in that both agents rapidly cause the induction of single-strand breaks in DNA, and we proposed that these effects were indirectly mediated by oxygen radical generation, a fact widely accepted for X-rays and suggested for $HgCl_2$ (8, 12). Relatively little information is available on the effects of mercury(II) on the DNA of intact cells, but the induction of DNA strand breaks by X-rays is thought to be the basis of its cytotoxic action (13).

Hg^{2+} has been shown to possess weak mutagenic activity in bacterial and mammalian systems (14, 15) and to induce a low level of chromosomal aberrations (16), whereas it did not significantly enhance the incidence of sister chromatid exchanges (17). Mercury is not at

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present considered to be a carcinogen, although $HgCl_2$ was shown to enhance viral transformation of cells at a concentration of 50 μM (18), and, in another study, metallic mercury was able to induce tumors in animals (19), an effect ascribed to the phenomenon of "solid state" carcinogenesis. Further studies are required to assess definitively the carcinogenic potential of mercury, but X-rays are well established as a carcinogenic agent since they have been shown to cause cancer in humans (20) and in experimental animals (21), and are able to induce transformation in tissue culture (22).

The similarities of the DNA damage induced by X-rays and $HgCl_2$ would suggest that $HgCl_2$ possesses more mutagenic and carcinogenic activity than has been reported, assuming the existence of a good correlation between DNA damage and mutation or carcinogenesis as has been suggested for X-rays (23) and other established carcinogenic agents (24). An obvious difference between Hg^{2+} and X-rays is that following damage with X-rays the cell does not have to contend with the continuous presence of Hg^{2+} and is therefore able to recover more rapidly. We have therefore compared the ability of cells to repair DNA damage induced by both of these agents and found that X-ray-induced damage is rapidly repaired (within 1 hr), in contrast to that produced with $HgCl_2$, which actually increases in extent during a similar 1-hr repair period following removal of extracellular mercury. We report herein that a much lower level of DNA damage is required to produce a given cytotoxic response with $HgCl_2$ as compared with that produced by X-rays. Finally, we have demonstrated that the addition of $HgCl_2$ at non-cytotoxic and non-DNA-damaging concentrations was capable of inhibiting the repair of the single strand-breaks caused by X-rays. Since unfaithful repair of DNA has been highlighted as a critical element in the potency of X-rays in inducing mutations and transformations (25), our results suggest that further work in understanding the low mutagenic potential of mercury, despite its potent DNA-damaging activity, should be directed toward a study of its effect on repair enzymes activated following DNA damage by X-rays and other chemical agents.

EXPERIMENTAL PROCEDURES

Materials

The radioisotope, [^{14}C]deoxythymidine (58 mCi/mmole), was purchased from New England Nuclear Corporation (Boston, Mass.). Free acid EDTA, disodium EDTA, and sodium dodecyl sulfate were obtained from Sigma Chemical Company (St. Louis, Mo.). Tetrapropylammonium hydroxide (10% aqueous solution) was purchased from RSA Chemical Company (Ardsley, N. Y.), and polycarbonate filters were from Nucleopore (Pleasanton, Calif.). Bovine serum, McCoy's 5A medium, and trypsin were purchased from Gibco, Inc. (Grand Island, New York). Liquiscint was obtained from National Diagonistic (Somerville, N. J.).

Methods

Cell culture. CHO[1] cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. All experiments were carried out with asynchronous cultures in the logarithmic growth phase with a generation time of 14–16 hr.

Colony-forming assay. Following irradiation or exposure to selected $HgCl_2$ concentrations for 1 hr, the cell monolayers were rinsed once with Saline A and trypsinized. Appropriate numbers of cells were then plated into 60-mm dishes and allowed to grow until distinct colonies were formed (8–9 days). The cells were then fixed with 95% ethanol and stained with a 0.2% crystal violet solution; the number of surviving colonies (with more than 50 cells) was determined in each plate and expressed as a function of the number of cells plated.

Alkaline elution assay. The alkaline elution was carried out by a procedure virtually identical with that described by Kohn *et al.* (26). Briefly stated, 1×10^6 cells were plated in 100-mm dishes and incubated for 24 hr in the presence of [^{14}C]deoxythymidine (0.02 μCi/ml). The medium was then removed and replaced with 10 ml of medium without isotope, and the cells were allowed to incubate at 37° overnight. Following treatment, cells were removed by trypsinization, and an aliquot containing 8.5×10^5 cells was diluted to 20 ml with ice-cold Saline A 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, 0.025 M EDTA (pH 10.1). Cell lysates on the filter were rinsed with 5 ml of 0.02 M EDTA. DNA was eluted with 25 ml of a solution containing 0.025 M EDTA (free acid) plus 2% (final concentration) tetrapropylammonium hydroxide (pH 12.15) at a flow rate of 0.035 ml/min. Fractions of approximately 3 ml were collected and counted in 7 ml of Liquiscint containing 0.7 ml of glacial acetic acid. The filters were digested for 1 hr at 60° in 0.4 ml of 1 N HCl and counted in 7 ml of Liquiscint containing 0.7 ml of glacial acetic acid and 2.5 ml of 0.4 N NaOH.

RESULTS

Clonogenic survival of cells exposed to $HgCl_2$ or X-rays. The colony-forming ability of CHO cells exposed to increasing doses of $HgCl_2$ (1 hr) or X-rays is shown in Fig. 1. Little cell killing was produced at $HgCl_2$ concentrations below 10 μM and with doses of X-rays less than 50 rads. At higher levels, both $HgCl_2$ and X-rays decreased the cell survival rate in a dose-dependent manner. At 31.6 μM $HgCl_2$ and 425 rads of X-rays, the survival rate of treated cells was decreased to 50% of the survival rate in untreated cultures. Exposure of CHO cells to $HgCl_2$ for 1 hr at concentrations up to 50 μM did not decrease trypan blue exclusion as compared with untreated cells, indicating that at this time interval the cell membrane was not sufficiently damaged so as not to exclude this high molecular weight dye. However, following longer incubation periods (5 hr), after a 1-hr insult with 50 μM $HgCl_2$ cells exhibited a decreased exclusion of trypan blue relative to untreated cells.

DNA damage induced by $HgCl_2$ or X-rays and its relationship to cytotoxicity. The alkaline elution assay was employed to study the relative number of single-strand breaks produced in the DNA of CHO cells exposed to $HgCl_2$ and ionizing radiation. The SSF, calculated from alkaline elution profiles of the DNA from appropriately treated cells, indicated that both the $HgCl_2$ and X-rays induce a dose-dependent level of single-strand breaks in the DNA of intact cells (Table 1) (see Discussion in reference to previous work characterizing the nature of DNA lesions caused by $HgCl_2$). Note that the threshold for induction of single-strand breaks by $HgCl_2$ was greater than 10 μM when McCoy's 5A culture medium containing 10% fetal bovine serum was used. In order to assess the relationships between the degree of DNA damage and cytotoxicity, the data from Fig. 1 (plating efficiency) were plotted against the negative

[1] The abbreviations used are: CHO, Chinese hamster ovary; SSF, strand scission factor.

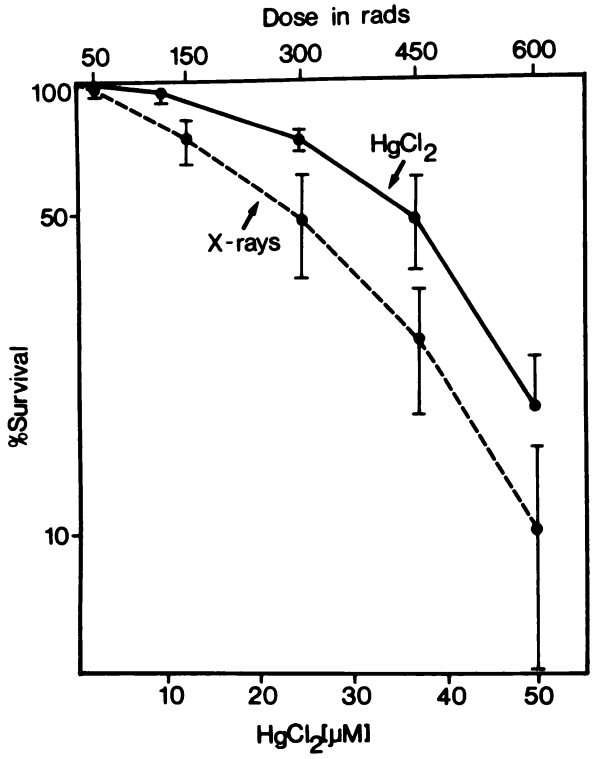

FIG. 1. *Effect of $HgCl_2$ and X-rays on cell-plating efficiency*

Colony-forming assays were performed as described under Methods. Cells were exposed to the indicated concentrations of $HgCl_2$ for 1 hr. Data are plotted as percentage of cloning efficiency compared with untreated controls, and each point represents the mean ± standard error of triplicate values in a single experiment.

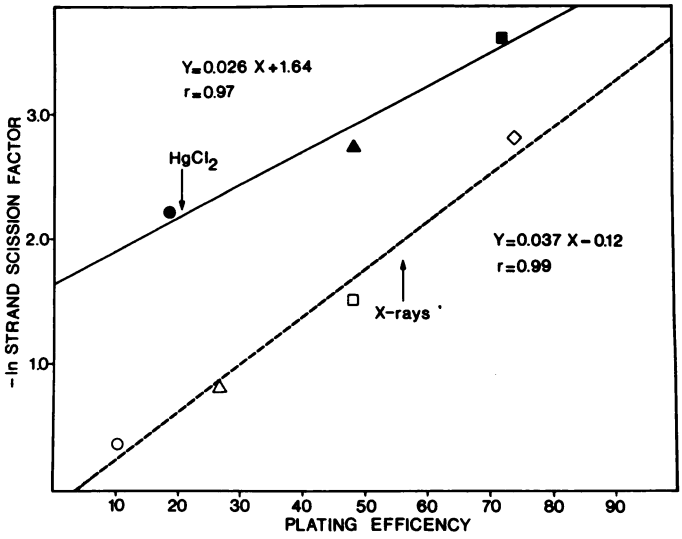

FIG. 2. *Correlation between cytotoxicity and induction of DNA single-strand breaks by $HgCl_2$ and X-rays*

The effect of $HgCl_2$ and X-rays on cell-plating efficiency and SSF was determined as described under Methods. Each point represents the results obtained at a single metal concentration (*closed symbols*), or radiation dose (*open symbols*) with the following treatment: $HgCl_2$, ■ 25 μM, ▲ 37.5 μM, or ● 50 μM; or X-ray, ◇ 150 rads, □ 300 rads, △ 450 rads, or ○ 600 rads. All $HgCl_2$ treatments were carried out for 1 hr.

natural log of SSF values (Fig. 2). An excellent inverse correlation was observed with either $HgCl_2$ ($r = 0.97$) or X-rays ($r = 0.99$): the higher the level of DNA breakage, the lower the cloning efficiency. Differences in the ordinate intercept indicate that equitoxic levels of the two agents induce a different degree of DNA breakage. A 1.4-fold difference in the slope of the lines obtained with $HgCl_2$ ($s = 0.026$) and X-rays ($s = 0.037$) is indicative of differences in the kinetics of DNA damage and cytotoxicity. Experiments were therefore performed to investigate whether the higher cell sensitivity to the $HgCl_2$-induced DNA damage could possibly be related to a difference in the kinetics of rejoining of the DNA fragments formed following $HgCl_2$ and X-ray exposure.

TABLE 1

Induction of DNA single-strand breaks by X-rays and $HgCl_2$

Alkaline elution analysis of DNA was performed as described under Methods, using cells treated as detailed in the table below. Each number represents the mean of four separate elutions.

Dose	SSF[a]
$HgCl_2$ (μM)	
10	0
25	0.027
37.5	0.068
50	0.110
100	0.498
X-rays (rads)	
50	0.016
150	0.061
300	0.220
450	0.441
600	0.681

[a] SSF was calculated from the alkaline elution patterns by the following relationship: SSF = −log A/B, where A = amount of DNA retained in the sixth fraction of the untreated sample and B = DNA retained in the sixth fraction of the treated sample.

Inhibition of DNA repair by $HgCl_2$. As shown in Fig. 3A, DNA damage induced by X-rays was rapidly repaired such that within 60 min after exposure the DNA alkaline elution profile resembled that of the untreated samples. In contrast, the DNA damage induced by $HgCl_2$ was not repaired following removal of extracellular mercury during a similar 1-hr recovery period (Fig. 3B). In fact, during this recovery period more strand breaks were generated in cells exposed to $HgCl_2$ (Fig. 3B). Several other experiments of this type with varied $HgCl_2$ concentrations and longer time intervals of repair revealed essentially similar findings, except that at 4 hr following a 1-hr exposure to $HgCl_2$ (100 μM) DNA-DNA cross-links were observed (see Discussion). Since DNA damage induced by $HgCl_2$ was similar to that caused by X-rays, whereas the repair of damage in the case of $HgCl_2$ was severely inhibited, we investigated whether $HgCl_2$ would inhibit the repair of DNA damage induced by X-rays. As shown in Fig. 4, a non-DNA-damaging, non-cytotoxic level of $HgCl_2$ (10 μM; see Table 1) severely impaired rejoining of X-ray-induced single-strand breaks.

DISCUSSION

The mechanism by which Hg^{2+} induces cell injury and death is currently not well understood. The chemical reactivity (i.e., softness) of this divalent metal ion, as quantitated by its σ_p value, indicates a high propensity to react with various anions (6). We have previously demonstrated that the uptake of $HgCl_2$ was extremely rapid

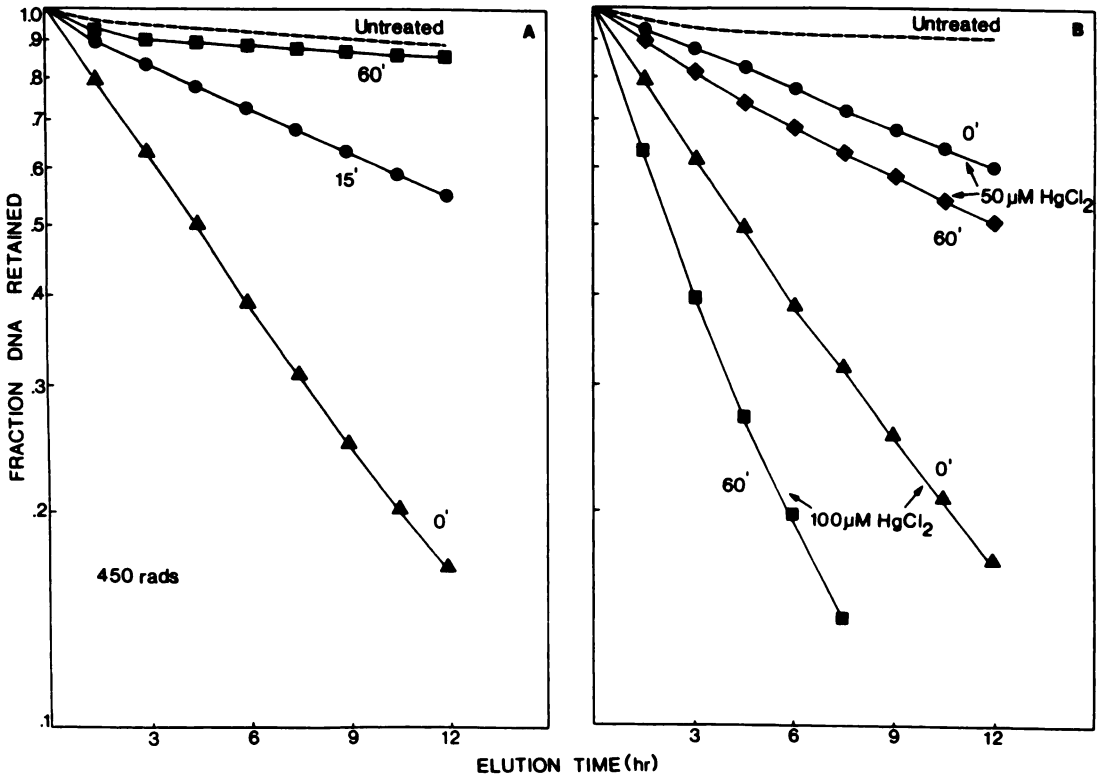

FIG. 3. *Induction of DNA single-strand breaks and their repair following treatment with $HgCl_2$ and X-ray*

Cells were treated with $HgCl_2$ for 1 hr (A) or with X-rays (B), and their DNA was analyzed with alkaline elution immediately following treatment or after incubation of the cultures at 37° for an additional 15–60 min in fresh media. Alkaline elution analysis was performed as described under Methods.

and greater than the entry of a number of other toxicologically relevant metal ions such as $CdCl_2$ and $CuSO_4$ (12). This high uptake may be due to its chemical reactivity, since Hg^{2+} may readily form lipid-soluble complexes that facilitate its cellular entry. The rapid uptake of $HgCl_2$, combined with its chemical reactivity, may lead to cellular injury by its direct interaction with critical macromolecules or it may indirectly injure cells by generating oxygen radicals (8), leading to depletion of reduced glutathione (7, 12) and lipid peroxidation (7). We have previously demonstrated that the acute cytotoxic effects of $HgCl_2$ mechanistically resembled those produced by X-rays, since $HgCl_2$ caused a marked depletion of reduced glutathione that was temporally correlated with a rapid and striking degree of single-strand breaks in the DNA of intact cultured cells (12). In the present study, we demonstrated that the induction of DNA damage by $HgCl_2$ and X-rays correlates linearly with cell killing as measured by clonogenic survival of cells. The induction of DNA breakage as a mechanism of cell injury by $HgCl_2$ represents a relatively new concept in understanding its potential site of action at the cellular level. In isolated hepatocytes, where clonogenic survival cannot be assessed, inhibition of Hg^{2+}-induced lipid peroxidation by antioxidants did not prevent the release of lactic dehydrogenase, suggesting that lipid peroxidation induced with $HgCl_2$ was not responsible for the observed cell membrane injury (7). Not inconsistent with this finding, we propose in the present study that the indirect oxygen radical-dependent toxicity of $HgCl_2$ is related to its ability to induce DNA damage. These conclusions are supported by the high degree of correlation between cell killing and DNA damage induced with $HgCl_2$ and its similarity to the cellular perturbations produced with X-rays. Recent studies have demonstrated an enhanced sensitivity of CHO DNA repair mutants to $HgCl_2$ as compared with wild-type CHO cells (27); these repair mutants also displayed an enhanced cytotoxic response to X-rays as well (27). The fact that the cytotoxic response to $HgCl_2$ may resemble that caused by X-rays is further supported by the alteration of $HgCl_2$-induced DNA damage by antioxidants such as ascorbic acid and mannitol (8).

Less DNA damage appeared to be required to produce the same level of cell killing with $HgCl_2$ as compared with X-rays. This result was investigated further by examining the repair of the DNA strand breaks following $HgCl_2$ or X-ray treatment. In the latter instance, the single-strand breaks were rapidly rejoined such that 1 hr following treatment the alkaline elution pattern of DNA resembled that of untreated cells whereas strand breaks induced with $HgCl_2$ were not readily repaired (Fig. 3B). The observations that cells are unable to repair the single-strand breaks induced with $HgCl_2$ and that low concentrations of $HgCl_2$ (10 μM) inhibit the rejoining of single-strand breaks induced by X-rays suggest that $HgCl_2$ may act on DNA homeostasis by inhibiting DNA repair processes. Thus, in contrast to X-rays, DNA damage induced by $HgCl_2$ was not readily repaired and may represent an irreversible injury that leads to cell death.

Although single-strand breaks in the DNA were the primary lesion induced by $HgCl_2$, they were not the only

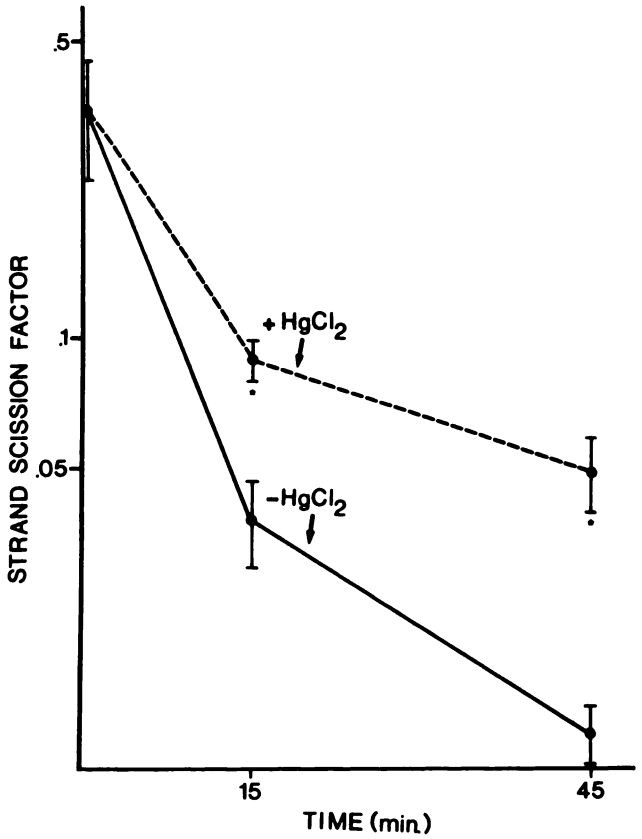

FIG. 4. *Effect of noncytotoxic concentrations of $HgCl_2$ on the rejoining of DNA single-strand breaks induced by X-rays*

Following exposure to 450 rads, cells were allowed to repair at 37° for 15–45 min in the absence or presence of 10 μM $HgCl_2$. Alkaline elution was performed on these cultures as described under Methods. DNA single-strand breaks were expressed as SSF, calculated from the alkaline elution patterns by the following relationship: SSF = $-\log A/B$, where A = amount of DNA retained in the sixth fraction of the untreated sample and B = DNA retained in the sixth fraction of the treated sample. *Asterisks* adjacent to $HgCl_2$-treated cell points indicate statistically significant differences from untreated cultures ($p < 0.05$, Student's t-test).

lesion caused by this agent. DNA-DNA cross-links develop with time following exposure to $HgCl_2$, probably resulting from its ability to interact directly with the DNA bases (28). However, $HgCl_2$ does not induce detectable DNA-protein cross-links as determined by alkaline elution analysis (28). DNA-protein cross-links are lesions induced by relatively non-toxic levels of other metal compounds of known carcinogenic activity (28). Additionally, DNA repair activity is considerable following exposure to these carcinogenic metal compounds as assessed by CsCl density gradient sedimentation (29). Following $HgCl_2$ treatment, DNA repair activity as assessed with the same methodology (28) or by observing changes in alkaline elution rates (Fig. 3B) was not enhanced despite the induction of DNA strand breaks. Thus the low mutagenic activity of $HgCl_2$ despite its active ability to induce DNA strand breaks may be due to the inhibition of DNA repair, an event considered essential for the induction of mutations by X-rays (25) and other mutagenic/carcinogenic agents (24) or to its lack of induction of DNA-protein cross-links. The strand breaks induced by $HgCl_2$ represented true single-strand breaks and not double-strand scission or production of alkaline-sensitive sites (28). These results are supported by the absence of breaks in DNA examined with agarose gel electrophoresis and neutral sucrose gradients at $HgCl_2$ concentrations as high as 300 μM for 1 hr (28). However, $HgCl_2$ did produce strand breaks as analyzed by nucleoid sedimentation in neutral sucrose gradient (28). Since nucleoids were analyzed under neutral conditions, and the level of breaks seen with $HgCl_2$ on this system was comparable to those observed with technique that use alkaline conditions, it was considered that $HgCl_2$ does not induce alkaline-labile sites in the DNA (28). Recent studies have demonstrated that $HgCl_2$ as well as a number of other toxicologically important metals produce an S-phase-specific cell cycle blockade, suggesting that mercury specifically interferes with events involved in DNA replication (30). These results provide further evidence of the importance of the effects of $HgCl_2$ on DNA metabolism. Future studies directed at understanding the low mutagenic potential of mercury despite its potent DNA-damaging activity should be directed at studying its effects on the regulation of DNA homeostasis.

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