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Characterization of DNA Lesions Induced by $CaCrO_4$ in Synchronous and Asynchronous Cultured Mammalian Cells

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SUMMARY

Alkaline elution studies demonstrated $CaCrO_4$-induced DNA single strand breaks and DNA-protein crosslinks. DNA single strand breaks increased following treatment with 10–400 μM $CaCrO_4$ in Chinese hamster ovary cells maintained with a minimal salts/glucose medium. DNA single strand breaks were rapidly repaired when extracellular $CaCrO_4$ was removed even following exposure levels of $CaCrO_4$ (200 μM for 2 hr) which reduced survival to 0.6%. Under these exposure conditions the trypan blue exclusion was greater than 80%, whereas cell growth was inhibited by 46% within 24 hr. The DNA-protein crosslinks induced by 10 μM $CaCrO_4$ were repaired in the absence of metal within 24 hr. In contrast, the amount of DNA-protein crosslinks measured 24 hr after a 2-hr treatment with 50, 100, and 200 μM $CaCrO_4$ remained unchanged at the 50 μM level or increased at the two higher concentrations. Thus, at concentrations of 50 μM or greater there was no repair of the DNA protein crosslinks, and this may have been due to cytotoxicity of the metal. $CaCrO_4$ at 10 or 25 μM exposure for 6 hr also induced DNA-protein crosslinking in Chinese hamster ovary cells maintained in normal tissue culture growth media. The lack of repair of DNA-protein crosslinks at the 25 μM level, which did not substantially reduce cell survival, indicated the persistence of these lesions in a noncytotoxic form. Uptake of $CaCrO_4$ was linear with all of the concentrations tested. Analysis of the cell cycle sensitivity to $CaCrO_4$ revealed that cells in early S phase were the most sensitive to the cytotoxic and strand breaking activity of $CaCrO_4$. Compared with other phases of the cell cycle, there was also an elevated level of DNA-protein crosslinks when cells were treated in early S phase and incubated 24 hr without $CaCrO_4$. These results implicate the DNA-protein crosslink as an important lesion that may be responsible for the cytotoxic and carcinogenic properties of chromate.

Chromium (VI) compounds are potent toxic and carcinogenic agents (1). Epidemiologic studies have shown that industrial workers exposed to chromium (VI) had a higher incidence of lung cancer than the normal population (2, 3). Renal and hepatic toxicity has also been reported in workers exposed to chromium (VI) (4). Chromium (VI) compounds induce mutation in bacteria (5) and transform mammalian cells *in vitro* (6).

Hexavalant chromium (VI) is thought to be more toxic and carcinogenic than the trivalent form because it readily enters the cells by the sulfate transport system (7, 8). Since hexavalent chromium (VI) is reduced to the trivalent form (III) intracellularly, the trivalent form is thought to be the ultimate toxic and carcinogenic ion (7, 8). Chromium (VI) compounds induced DNA single strand breaks and DNA protein crosslinks *in vivo* (9) and in cultured mammalian cells (10–12). However, the role of these DNA lesions in the toxicity of chromium compounds is currently not understood. A number of metals and their compounds block cell cycle progression in S phase (7). However, the DNA lesions induced during various phases of the cell cycle have not been studied and it is not clear whether S phase is associated with the production of differing DNA lesions or just a greater magnitude of the same lesion. Not all DNA lesions induced by metals such as chromate may have similar consequences, not only in their effects on cell cycle progression, but also in their cytotoxic and carcinogenic action.

In the present study we have shown that $CaCrO_4$ induced DNA single strand breaks and DNA-protein crosslinks in intact cells. The breaks occurred rapidly and were easily repaired. In contrast, the DNA-protein crosslinks developed slowly and were not as easily repaired. Both single strand breaks and DNA-protein crosslinks were induced during all phases of the cell cycle. The breaks were more prevalent during S phase than during the other phases of the cell cycle. DNA-protein crosslinks initially did not exhibt a cell cycle-specific pattern, but

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ABBREVIATIONS: CHO, Chinese hamster ovary; SGM, salts/glucose medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSF, strand scission factor; CLF, crosslink factor.

incubation of cells for 24 hr following an initial treatment of synchronized cells also indicated that the highest levels of these crosslinks were eventually produced when cells had been treated with $CaCrO_4$ in early S phase.

Experimental Procedures

Materials

The radioisotopes [^{14}C]deoxythymidine (58 mCi/mmol) and $Na_2Cr^{51}O_4$ were obtained from New England Nuclear Corp. (Boston, MA) and ICN Chemical Co. (Irvine, CA), respectively. Free acid EDTA and sodium dodecyl sulfate were acquired from Sigma Chemical Co. (St. Louis, MO). Tetrapropyl ammonium hydroxide (10% aqueous solution) was procured from RSA Chemical Co. (Ardsley, NY). Polycarbonate filters were from Nucleopore (Pleasanton, CA), while Polyvinyl chloride filters were from Millipore Corp. (Bedford, MA). Proteinase K was obtained from EM Laboratories, Inc. (Elmsford, NY). Bovine serum, α-minimal essential medium, and trypsin were obtained from Gibco, Inc. (Grand Island, NY). Liquiscint was from National Diagnostic (Sommerville, NJ).

Methods

Cell culture. The AA8 CHO cells were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37° in α-minimal essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin/Fungizone (Gibco, Inc.). In some instances synchronized or log phase cultures of cells were placed in SGM (50 mmol/liter of Hepes buffer at pH 7.2 containing 100 mmol/liter of NaCl, 5 mmol/liter of KCl, 2 mmol/liter of $CaCl_2$, and 5 mmol/liter of glucose) prior to treatment of cells with a freshly prepared solution of $CaCrO_4$. Treatment of cells with metals in this SGM prevented the binding of the metal to components present in culture medium which could have decreased metal uptake into cells. In other instances cells were treated with $CaCrO_4$ in complete tissue culture medium. CHO cells were plated at 2×10^6 cells/150-mm tissue culture dish in order to assure a larger proportion of cycling cells. Forty-eight hours after plating, Colcemid (0.03 μg/ml) was added for 6 hr and metaphase cells were dislodged by gently pipetting the overlying medium. Mitotic cells were collected by centrifugation and then washed twice with ice-cold medium. The resulting mitotic index was more than 85%. Mitotic cells were plated into 100-mm-diameter culture dishes and treated with $CaCrO_4$ for 2 hr in SGM beginning at 2, 6, 10, and 14 hr after the initial plating, to analyze DNA lesions and cytotoxicity during the G_1, early S, late S and G_2 phases of the cell cycle, respectively (13, 14).

Alkaline elution. Analysis of DNA lesions by alkaline elution was performed, as described by Kohn *et al.* (15) with minor modifications. Cells were seeded into 100-mm tissue culture dishes and incubated for 48 hr with [^{14}C]thymidine (0.02 μCi/ml). For experiments on logarithmically growing cells, cultures were washed and incubated in complete medium without the radioactivity for 2 hr prior to treatment with $CaCrO_4$. For cell cycle experiments, [^{14}C]thymidine-labeled cells were synchronized as described above. Following treatment, cells were washed twice and removed by scraping with a rubber policeman into ice-cold Puck's saline A (5 mM $NaHCO_3$, 6 mM glucose, 5 mM KCl, and 140 mM NaCl, pH 7.2). In the analysis of strand breaks a volume containing 1.0×10^6 cells was deposited onto 25-mm polycarbonate filters or polyvinyl chloride filters when DNA-protein crosslinks were analyzed. Filters were rinsed with cold saline A and cells were lysed directly on the filters by passage of 5 ml of 2% sodium dodecyl sulfate containing 0.025 M EDTA. DNA was eluted with 25 ml of a solution containing 0.025 M EDTA plus 2% (final concentration) tetrapropylammonium hydroxide (pH 12.15) at a flow rate of 0.028 ml/min. Fractions of approximately 2.5 ml were collected and radioactivity was determined by scintillation counting 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 neutralized with 2.5 ml of 0.4 N NaOH at 25° for 1 hr. The radioactivity was determined by scintillation counting in 10 ml of Liquiscint containing 1.0 ml of glacial acetic acid. The degree and nature of DNA crosslinks were assessed by utilizing a test dose of X-rays (600 rads) and proteinase K as previously described (15). To quantitate the extent of DNA single strand breaks and DNA-protein crosslinks, the SSF and CLF were calculated from the alkaline elution patterns utilizing the following relationship: SSF = log A/B, CLF = [log A−log AX]/[log B−log BX], were A = amount of DNA retained in the sixth fraction of untreated sample and B = DNA retained in the sixth fraction of the $CaCrO_4$-treated samples, AX = amount of DNA retained in the sixth fraction of untreated X-irradiated and BX = amount of DNA retained in the sixth fraction of $CaCrO_4$-treated X-irradiated sample.

Colony-forming assay. Following treatment with $CaCrO_4$, cells were rinsed twice with SGM and subsequently trypsinized. The cell number was determined with a Coulter particle counter or hemocytometer. An appropriate number of cells (200–4000 cells) was then plated into 60-mm dishes and allowed to form distinct colonies (8–10 days). Cells were then fixed with 95% ethanol and stained with a 0.2% crystal violet solution. The number of surviving colonies (>50 cells) was determined in each plate and the percentage of survival was calculated by expressing this as a function of the total number of cells plated.

Cellular uptake of ^{51}Cr. CHO cells were incubated with 20 μCi of ^{51}Cr at various concentrations for 2 hr in SGM. Following treatment, cells were washed twice with SGM and dislodged from the dishes by scraping, and the cell number was determined as described above. The cellular uptake of ^{51}Cr was determined by assessing radioactivity in a Beckman gamma counter in which the counting efficiency was 95%.

Results

Asynchronous CHO cells were treated with $CaCrO_4$ for 2 hr in SGM and the DNA damage was measured by alkaline elution. As shown in Fig. 1, $CaCrO_4$ produced a time- and concentration-dependent induction of DNA single strand breaks. Some of the DNA single strand breaks may have originated from alkali-labile sites since the elution curves of DNA from $CaCrO_4$-treated cells were nonlinear and exhibited a concave kinetic pattern when compared to the elution curves of DNA from X-irradiated cells. Additionally, previous studies from our laboratory have demonstrated that chromium induces alkali-labile sites (12).

Fig. 2 shows that treatment of cells with $CaCrO_4$ for 2 hr in SGM resulted in DNA-protein crosslinks because of the increased retention of DNA from irradiated cells treated with the metal. There was an absence of DNA interstrand crosslinks, because essentially all of the crosslinks were eliminated by the addition of proteinase K in the lysis solution. As shown in Fig. 3, the CLF induced by this metal increased 1.21, 2.21, and 2.65 in cells treated with 10, 50, and 100 μM $CaCrO_4$, respectively, for 2 hr. However, the CLF decreased from 2.65 to 1.98 in cells treated with 100 and 200 μM $CaCrO_4$, respectively. Cells were incubated for 2 hr in SGM with varying concentrations of $CaCrO_4$ (10–200 μM) containing the radioisotope ^{51}Cr and the intracellular level of this metal was estimated by assessing the radioactivity found in 10^6 cells. Fig. 3 shows the relationship between the cellular uptake of chromium (VI) and the induction of DNA lesions. Chromium (VI) uptake increased in a linear proportion with concentration. The concentration-dependent uptake of chromium (VI) was consistent with the induction of DNA single strand breaks which also depended on the metal levels.

To examine further the relationship between the induction of DNA lesions by $CaCrO_4$ and the cytotoxicity induced by this compound, cells were exposed to various concentrations of

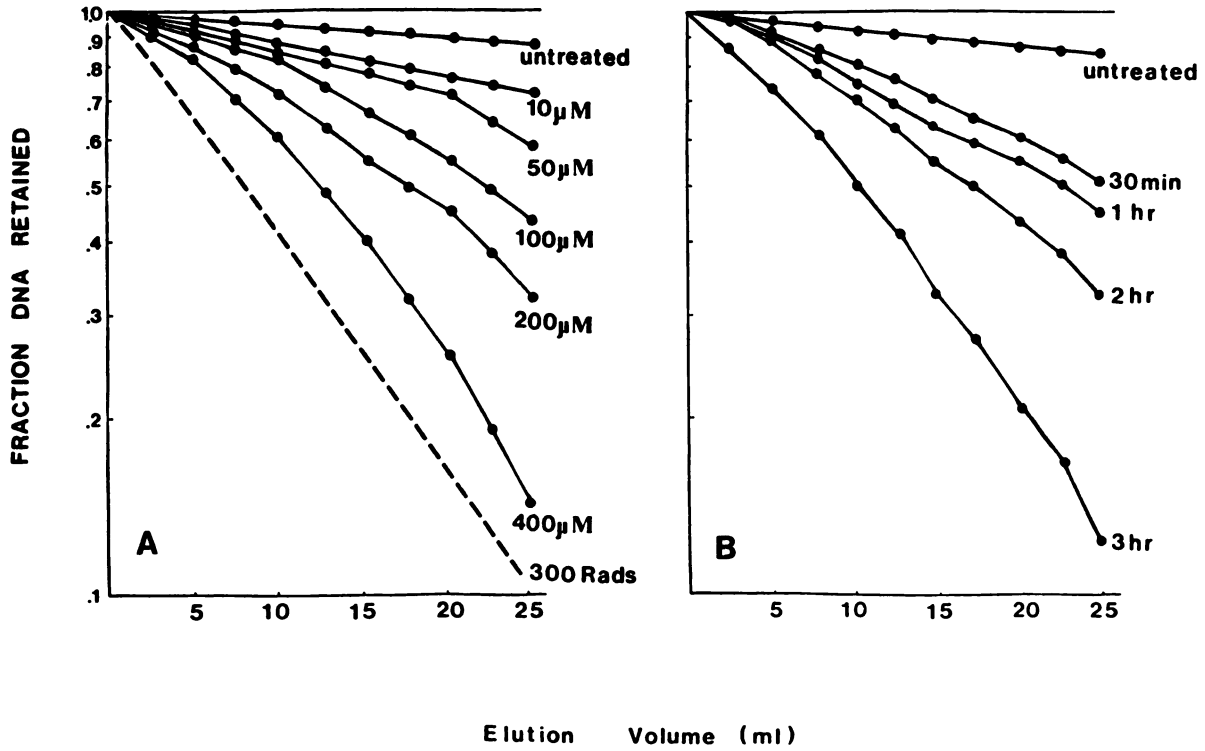

Fig. 1. Alkaline elution analysis of DNA single strand breaks in CHO cells treated with $CaCrO_4$. Cells were treated for 2 hr in SGM with varying levels of $CaCrO_4$ (A) or with a fixed concentration of $CaCrO_4$ (200 μM) for selected time intervals (B). Following this treatment, cellular DNA was analyzed by the alkaline elution method. Prior to elution, cell lysates were incubated with proteinase K to eliminate the retention of DNA caused by the induction of DNA-protein crosslinks.

$CaCrO_4$ for 2 hr in SGM, and their ability to form colonies was determined (Table 1). There was no loss of cell survival in chromium-treated cells at concentrations up to 10 μM. However, the survival decreased to 27.3, 6.8, and 0.6% at 50, 100, and 200 μM $CaCrO_4$, respectively. When the effect of $CaCrO_4$ on the growth of CHO cells was examined at 24 hr after its removal, percentage of growth decreased to 60, 48, and 46% at 50, 100, and 200 μM $CaCrO_4$, respectively. When trypan blue exclusion was utilized as a test of cytotoxicity, more than 80% of the cells were viable following 2 hr treatment with $CaCrO_4$ at 200 μM in SGM followed by a 24-hr incubation in complete growth medium.

To determine whether the DNA lesions induced by $CaCrO_4$ were repaired, cells were treated with $CaCrO_4$ for 2 hr in SGM and then incubated in metal-free complete growth medium for various time intervals. Fig. 4 shows that DNA single strand breaks induced by 200 μM $CaCrO_4$ were about 50% repaired within 1 hr, and completely repaired when $CaCrO_4$ was removed for 24 hr. Fig. 5 indicates that DNA-protein crosslinks induced by 10 μM $CaCrO_4$ were repaired within 4 hr after metal removal. However, treatment with concentrations of $CaCrO_4$ in excess of 50 μM induced DNA-protein crosslinks that remained at the same level (50 μM) or increased to a higher level (100 and 200 μM), 24 hr after metal removal (Fig. 5). The persistence of this lesion may be due in part to the cytotoxicity of $CaCrO_4$, although single strand breaks were repaired at these high doses. However, Table 2 compares the initial levels of strand breaks and DNA-protein crosslinks with those present 18 hr after the metal was removed, and then relates these DNA lesions to cell survival. At 25 μM exposure to $CaCrO_4$ for 6 hr there was essentially no decrease in cell survival, and the single strand breaks were repaired 18 hr later, but at this time there was essentially no repair of DNA-protein crosslinks. Although the lack of repair of DNA-protein crosslinks at higher concentrations of $CaCrO_4$ may be due to cytotoxicity, the results of the 25 μM exposure level (Table 2) illustrated that the DNA-protein crosslink was a poorly repaired lesion, when most of the cells did not die.

To study whether there was any cell cycle-specific sensitivity in the formation of DNA lesions or in the cytotoxicity of $CaCrO_4$, cells were synchronized in mitosis and then treated with 100 μM $CaCrO_4$ for 2 hr in SGM beginning at 2 (G1 phase), 6 (early S phase), 10 (late phase), and 14 (G2 phase) hr following their release from mitosis. Table 3 shows that $CaCrO_4$ induced DNA single strand breaks in cells during all phases of the cell cycle immediately after treatment. During early S phase, there was a significant increase in SSF when compared to the G1 phase ($p < 0.05$) or the G2 phase ($p < 0.01$) of the cell cycle. The level of SSF in late S phase increased significantly when compared to the G2 phase ($p < 0.01$), but this increase was not significantly different from that in G1 or early S phase. However, DNA single strand breaks induced by $CaCrO_4$ in early S phase were repaired within 24 hr after treatment even though the cells in early S phase were the most sensitive to DNA single strand breaks.

Table 4 shows that $CaCrO_4$ rapidly induced DNA-protein crosslinks in cells during all phases of the cell cycle. Initially, there was no significant difference in CLF during each of the cell cycle phases examined. Since the level of DNA-protein crosslinks at higher concentrations of $CaCrO_4$ (100 or 200 μM) exhibited a striking increase with time, we examined the amount of this lesion remaining 24 hr after an initial 2-hr

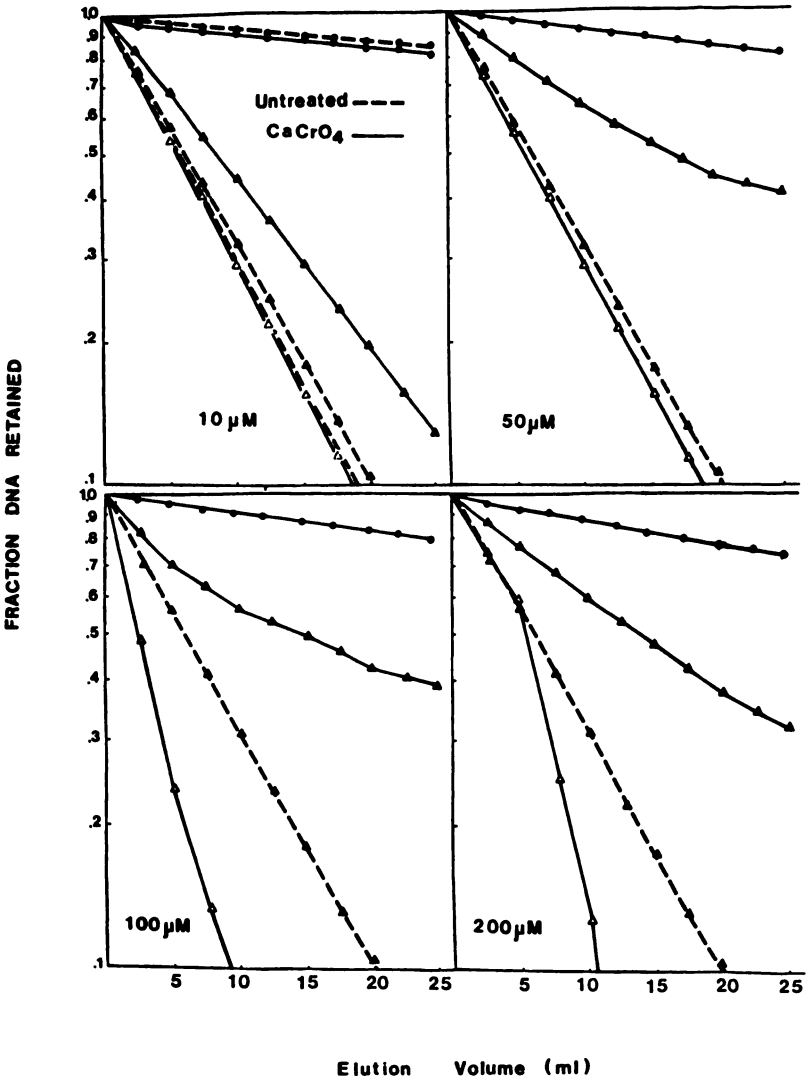

Fig. 2. Measurement of DNA-protein crosslinks by alkaline elution in CHO cells treated with $CaCrO_4$. Cells were treated in SGM for 2 hr with selected concentrations of $CaCrO_4$ as indicated in each quadrant. Following treatment, cellular DNA was analyzed by the alkaline elution method. Cells were either lysed directly (●) or exposed to X-ray (600 rads) and then lysed in the absence (▲) or presence (△) of proteinase K.

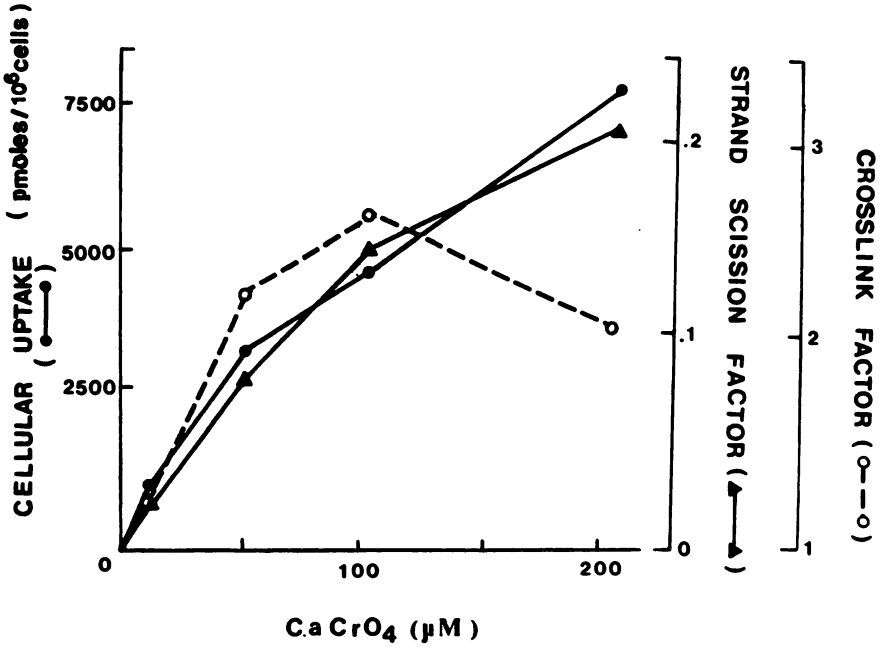

Fig. 3. Comparison in CHO cells of the uptake of ^{51}Cr, with the incidence of DNA single strand breaks and DNA-protein crosslinks. Cells were treated in SGM for 2 hr with various concentrations of $CaCrO_4$. The cellular uptake of chromium was estimated by utilization of the isotope ^{51}Cr (●——●) (Experimental Procedures). DNA single strand breaks and DNA-protein crosslinks were expressed as SSF (▲——▲) and CLF (○– –○), respectively.

TABLE 1

Effect of $CaCrO_4$ on plating efficiency and cell growth of CHO cells

$CaCrO_4$ concentration	Percentage of survival (% of control)[a]	Percentage of cell growth (% of control)[b]
μM		
1	105 ± 14.2	ND[c]
10	97.6 ± 6.4	ND
50	27.3 ± 4.8	60
100	6.8 ± 2.2	48
200	0.6 ± 0.2	46

[a] Logarithmic CHO cells were incubated for 2 hr in SGM containing various concentrations of $CaCrO_4$. Following this treatment, cells were plated to form colonies for 8–10 days as described in Experimental Procedures. Each value shown in the table represents the mean ± SD.

[b] Following treatment with $CaCrO_4$ for 2 hr in SGM, cells were washed with SGM and incubated in $CaCrO_4$-free complete growth medium for 24 hr. The cell number of each plate was determined with a Coulter counter particle analyzer or a hemocytometer. The number of cells in each plate was normalized to 100% growth of untreated cells. Each value shown in the table represents the average of two experiments.

[c] ND, not determined.

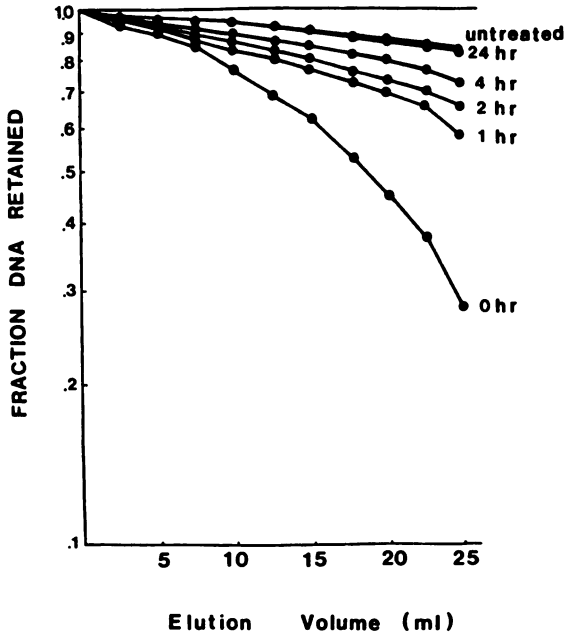

Fig. 4. Alkaline elution analysis of the repair of DNA single strand breaks following treatment with $CaCrO_4$. Cells were treated for 2 hr with 200 μM $CaCrO_4$ in SGM and cellular DNA was analyzed by alkaline elution either immediately after treatment or after incubation of the cultures in fresh medium for time intervals varying from 4 to 24 hr. Prior to elution, the cell lysate was incubated with proteinase K to eliminate the retention of DNA caused by the induction of DNA-protein crosslinks.

treatment of syncronized cells. As shown in Table 4, DNA-protein crosslinks induced at all phases of cell cycle were not repaired. In fact, in some instances the extent of DNA-protein crosslinks increased with time. The CLF in early S phase was significantly higher than that in late S phase ($p < 0.05$). The percentage increase in CLF from 2 hr to 24 hr after the metal was less in cells exposed during early S (39%) than during G2 (56%) phase. However, since cells were synchronized in mitosis it was difficult to obtain a good G2 population of cells due to the dispersion of synchrony with time. These results illustrate that single strand breaks induced by $CaCrO_4$ exhibit cell cycle specificity during S phase. Cells in G2 and early S phase were more readily fixed into DNA-protein crosslinks than those at other phases of the cell cycle.

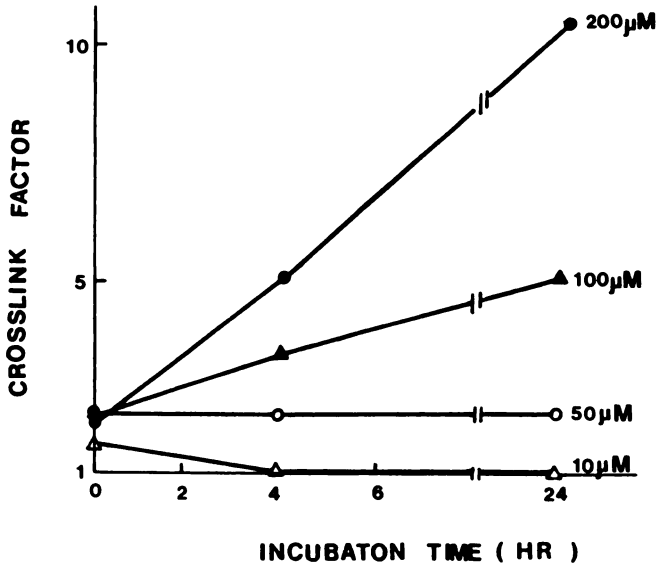

Fig. 5. Formation and repair of DNA protein crosslinks following $CaCrO_4$ treatment. Cells were treated for 2 hr with various concentrations of $CaCrO_4$ in SGM and the cellular DNA was analyzed by alkaline elution either immediately after treatment or after cultures were incubatedd for additional time intervals in fresh medium (4, 24 hr). DNA-protein crosslinks were expressed as CLF (Experimental Procedures).

Since DNA strand breaks were cell cycle specific, we examined whether the colony formation of cells was affected by $CaCrO_4$ in a cell cycle-specific manner. Table 5 demonstrated that the survival of cells in the early S phase was significantly decreased after 100 μM $CaCrO_4$ relative to the cell survival at each of the other cell cycle phases. When trypan blue exclusion was utilized as a test of cytotoxicity, more than 82% of cells were viable after 2 or 24 hr even when they had been treated with a high level of $CaCrO_4$ (100 μM). However, there was a lack of proportionality in correlations made among the strand breaks, crosslinks, and cytotoxicity. Cells in early S phase were 2.5-, 1.8-, and 2.2-fold more sensitive to the effects of $CaCrO_4$ on cell survival than cells in G1, late S, and G2 phase, respectively. DNA single strand breaks or DNA-protein crosslinks in early S phase increased only 1.4-, 1.1- and 2-fold (SSF) or 1.2-, 1.5-, and 1.26-fold (CLF) when compared to G1, late S, and G2 phases, respectively.

Discussion

Previous studies demonstrated that chromium (VI) compounds induced DNA-protein crosslinks, DNA single strand breaks, and alkali-labile sites in cultured mammalian cells (10–12). DNA interstrand crosslinks were also observed *in vivo* but have not been reported in cultured mammalian cells (9, 10). In both of these systems, the DNA single strand breaks were repaired quickly, whereas the DNA-protein crosslinks were not as readily repaired (9, 10). Cytotoxicity was thought more likely to correlate with the DNA-protein crosslinks because single strand breaks were not observed until cells were exposed to substantially toxic levels of chromate (16, 17). In the present study, $CaCrO_4$ induced DNA single strand breaks that were rapidly repaired even at highly cytotoxic levels of $CaCrO_4$ (200 μM). The DNA single strand breaks increased in a dose-dependent manner but did not correlate with the logarithmic decrease in cell survival. Cells synchronized in S phase, particularly those in early S phase, exhibited, in the presence of

TABLE 2

Comparison of the incidence of strand breaks and DNA protein crosslinks with cell survival following treatment with $CaCrO_4$

$CaCrO_4$ concentration	Percentage of survival (% of control)[a]	DNA single strand breaks (SSF)		DNA protein crosslinks (CLF)	
		6-hr Treatment	18 hr after Initial response	6-hr Treatment	18 hr after Initial response
μM					
10	98.3 ± 2.5	ND[b]	ND	1.17 ± 0.24	ND
25	79.7 ± 8.7	0.036 ± 0.011	ND	2.33 ± 0.25	1.72 ± 0.24
50	32.0 ± 3.3	0.064 ± 0.007	0.004	3.25 ± 0.58	2.57 ± 0.49

[a] CHO cells in the logarithmic stage of cell growth were incubated for 6 hr with $CaCrO_4$ in complete culture medium. Following treatment, cells were plated to form colonies as described in Experimental Procedures. Additionally, cellular DNA was analyzed by alkaline elution immediately after 6 hr treatment or following an 18-hr incubation in $CaCrO_4$-free fresh medium. DNA single strand breaks and DNA protein crosslinks were expressed as SSF and CLF, respectively. Each value shown in the table is the mean ± SD for at least three separate experiments.

[b] ND, not determined.

TABLE 3

Induction and repair of DNA single strand breaks by $CaCrO_4$ during various stages of the cell cycle

Cells were synchronized in metaphase as described in Experimental Procedures. The mitotic block was released by washing and replating the cultures in fresh medium. Cells were incubated for 2 hr with $CaCrO_4$ in SGM. DNA was analyzed by alkaline elution immediately after 2 hr treatment or following an additional 24-hr incubation in $CaCrO_4$-free medium as described in Experimental Procedures. DNA single strand breaks were expressed as SSF. Each value shown in the table is the mean ± SE for at least four determinations.

Cell cycle position	Concentration	2-hr Treatment (SSF)	24 hr after Initial treatment (SSF)
	μM		
G1 (2–4 hr)	100	0.095 ± 0.010	ND[a]
Early S (6–8 hr)	100	0.133 ± 0.010[b]	0.005
Late S (10–12 hr)	100	0.120 ± 0.012	ND
G2 (14–16 hr)	100	0.066 ± 0.010	ND

[a] ND, not determined.

[b] $p < 0.05$ compared to the G1 phase (Student's t test).

TABLE 4

Induction and Repair of DNA-protein crosslinks following treatment with $CaCrO_4$ in cells at different phases of their cell cycle

Cells were synchronized in mitosis as described in Experimental Procedures. The mitotic block was released by washing and replating the cultures in fresh medium. Cells in each phase of the cell cycle were incubated for 2 hr with 100 μM $CaCrO_4$ in SGM. Cellular DNA was analyzed by alkaline elution after incubation of the cultures for an additional time interval (24 hr) in fresh medium. The DNA protein crosslinks were quantitated as described in Experimental Procedures. Each value shown in the table is the mean ± SE for at least four determinations.

Cell cycle position	Concentration	2-hr Treatment (CLF)	24 hr after Initial treatment (CLF)	Increase
	μM			
G1 (2–4 hr)	100	3.41 ± 0.59	3.85 ± 0.22	13
Early S (6–8 hr)	100	3.40 ± 1.19	4.71 ± 0.49[a]	39
Late S (10–12 hr)	100	2.97 ± 0.54	3.13 ± 0.17	5
G2 (14–16 hr)	100	2.41 ± 0.34	3.75 ± 0.46	56

[a] $p < 0.05$ compared to Late S phase (Student's t test).

$CaCrO_4$, the least cell survival and the greatest amount of single strand breaks; however, there was a lack of proportionality among these two parameters. In logarithmically growing cells, cytotoxicity correlated with the onset of repair-resistant DNA-protein crosslinks. The synchronized S phase CHO cells were significantly more sensitive to the cytotoxicity of $CaCrO_4$ and to the buildup of DNA-protein crosslinks. However, neither the DNA single strand breaks nor the DNA-protein crosslink induced in each phase of the cell cycle correlated quantitatively with the cytotoxicity. These results suggest that the DNA damage induced by $CaCrO_4$ may contribute to the cytotoxicity but apparently cannot totally account for the cell death induced

TABLE 5

Survival of CHO cells following treatment with $CaCrO_4$ during various stages of the cell cycle

Cells were treated for 2 hr with 100 μM $CaCrO_4$ in SGM. Following treatment, appropriate numbers of cells were plated and allowed to form colonies for 8–10 days as described in Experimental Procedures. Each value shown in the table is the mean ± SD.

Cell cycle position	Concentration	Percentage of survival (% of controls)
	μM	
G1 (2–4 hr)	100	9.11 ± 3.75
Early S (6–8 hr)	100	3.59 ± 0.81[a]
Late S (10–12 hr)	100	6.46 ± 1.44[b]
G2 (14–16 hr)	100	7.78 ± 3.03

[a] $p < 0.01$ compared to G1, late S, and G2 (Student's t test).

[b] $p < 0.05$ compared to G1, (Student's t test).

by this compound. Cell growth at high concentrations of $CaCrO_4$ (100 and 200 μM) was approximately one-half that of untreated cells, whereas percentage of survival measured by colony formation in cells treated with 50, 100, and 200 μM $CaCrO_4$ decreased logarithmically to 27, 6.8, and 0.6%, respectively. At 24 hr after treatment, the trypan blue viability of cells exposed to $CaCrO_4$ was more than 80% even at the highest concentration tested (200 μM). These results indicate that there are large differences in results obtained between the trypan blue exlusion assay and the colony-forming assay, and also suggest that the effect of $CaCrO_4$ on the cytotoxicity in cells may require considerable time to take effect.

The DNA-protein crosslinks measured immediately after a 2-hr exposure to $CaCrO_4$ increased with concentration up to 100 μM but did not exhibit a further elevation at the higher concentrations tested. This lack of concentration-dependent DNA-protein crosslinks at 100 and 200 μM $CaCrO_4$ was not due either to limited cellular uptake of the metal or to the loss of cells containing these lesions since cells treated with $CaCrO_4$ exhibited linear uptake of $CaCrO_4$ and the trypan blue exclusion assay indicated that greater than 80% of cells survived these treatments, even at the highest concentration of $CaCrO_4$ (200 μM). When cells were incubated for 4 hr in fresh complete medium after the $CaCrO_4$ treatment, the DNA-protein crosslinks induced by 10 μM $CaCrO_4$ were repaired. However, the amount of DNA-protein crosslinks induced by 50, 100, and 200 μM $CaCrO_4$ either remained at the same level (50 μM) or increased approximately 2- or 5-fold (100 μM or 200 μM, respectively) 24 hr after removal of $CaCrO_4$. In experiments conducted by exposure of cells to $CaCrO_4$ in complete growth medium (Table 2), at the 25 μM $CaCrO_4$ exposure level, there

was not a significant decrease in cell survival, yet there was a high degree of DNA-protein crosslinks which were not repaired. These findings illustrate that the DNA-protein crosslinks lesion resists repair even at noncytotoxic levels of $CaCrO_4$. It is possible that at higher concentrations of $CaCrO_4$ the rate of formation of DNA-protein crosslinks exceeded their rate of repair. Alternatively, the high concentrations of $CaCrO_4$ may have directly interfered with enzymic processes involved with this repair. An inhibition of such repair by $CaCrO_4$ would be consistent with enhancement of cytotoxicity at higher metal levels. However, DNA single strand breaks were repaired even at highly cytotoxic concentrations of $CaCrO_4$. Enzymes involved in the repair of single strand breaks may differ from those that repair DNA-protein crosslinks. Alternatively, several studies have shown that chromium (VI) must be reduced to chromium (III) before it can form a stable ternary complex with DNA and protein (8, 10). The conversion of chromium (VI) to chromium (III) may be rate limiting in the formation of DNA-protein crosslinks but not limiting in the induction of strand breaks. The DNA single strand breaks induced by $CaCrO_4$ were apparent after only 30 min of treatment and were related directly to the concentration and cellular uptake of chromium (VI). These results suggest that strand breaks may be induced directly by chromium (VI), whereas DNA-protein crosslinks may require time-dependent metabolism. This is consistent with the observation that the hexavalent but not the trivalent form of chromium-induced DNA single strand breaks in isolated nuclei (10).

More strand breaks were induced in early S phase than in other phases of the cell cycle. In contrast, DNA-protein crosslinks were not initially cell cycle specific, but 24 hr after the initial chromate treatment these lesions also exhibited a similar cell cycle-specific pattern as the strand breaks. During the S phase, cells may be more susceptible to DNA damage by chromium because the unfolded DNA becomes accessible to binding of the metal with phosphate groups and bases (12). When cells in each cell cycle phase were incubated in fresh media for 24 hr following the chromate treatment, the CLF in early S phase increased significantly. The strand breaks induced in this phase were all repaired. Twenty-four hours after treatment the CLF in early S and G2 phase increased 39 and 56%, respectively, when compared to those present at 2 hr following treatment. However, the CLF in G1 and late S phase only increased to 13 and 5%, respectively. It is not clear what effect there may be on the passage of cells through the cell cycle during this 24-hr incubation period. However, this increase in CLF in early S and G2 phase may be due to either an interference with repair mechanism or a requirement for reductive metabolism (see above). It should be noted that synchronization of cells in the G_2 phase of the cell cycle is difficult, and there is considerable dispersion of synchrony at this stage since mitotic selection was utilized.

Several agents damage DNA in a cell cycle-specific manner (13, 14, 18, 19). Ultraviolet radiation induced DNA-protein crosslinks during early S phase (20). In contrast, $NiCl_2$ induced DNA-protein crosslinks in the late S phase (14). Cells in late S phase were also more sensitive to the cytotoxicity of $NiCl_2$. Interestingly, ultraviolet light and chromium (VI) compounds are potent mutagens, whereas nickel compounds are not mutagenic (21). The difference in mutagenicity may be related to the fact that the maximum mutation frequency was universally observed in cells treated during the G1/early S phase of the cell cycle (22–28). Since genetically active euchromatin is replicated during early S phase, mutations are very likely to occur. Thus, ultraviolet light and chromium compounds may be more mutagenic than nickel compounds because they induce DNA damage in early S phase, whereas nickel damages DNA during the late S phase. This hypothesis is supported by observation that nickel compounds are not mutagenic in mammalian and bacterial systems (29), and they induce chromosomal aberrations including gaps, breaks, and exchanges preferentially in the heterochromatic centromeric regions of autosomes and the heterochromatic long arm of the X-chromosome (13). $CaCrO_4$ induced the same types of chromosomal aberrations, but these lesions were distributed randomly throughout the chromosomes.[4]

It is known that the frequency of transformation is related to the degree of DNA damage present when cells attempt to replicate their DNA (30). Since the DNA single strand breaks induced by $CaCrO_4$ were repaired even at the highly cytotoxic treatments, whereas DNA-protein crosslinks were resistant to repair, the DNA-protein crosslinks have a high propensity to be present during DNA replication. Alterations of DNA structure and function, as well as the effect of DNA-protein crosslinks on replication, may be related to the carcinogenicity of chromium compounds.

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References

1. Leonard, A., and R. R. Lauwerys. Carcinogencity and mutagenicity of chromium. *Mutat. Res.* **76**:227–239 (1980).
2. Doll, R. Problems of epidemiological evidence. *Environ. Health Perspect.* **40**:11–20 (1981).
3. Furst, A., and R. T. Haro. A survey of metal carcinogenesis. *Prog. Exp. Tumor Res.* **12**:102–133 (1969).
4. National Institute of Occupational Safety and Health. Biological effects of exposure, in *Criteria for a Recommended Standard: Occupational Exposure to Chromium (VI).* U. S. Department of Health, Education, and Welfare, Washington, D. C., 23–121 (1975).
5. Nishioka, H. Mutagenic activities of metal compounds in bacteria. *Mutat. Res.* **31**:185–189 (1975).
6. Fradkin, A., A. Janoff, B. P. Lane, and M. Kuschner. *In vitro* transformation of BHK 21 cells grown in the presence of calcium chromate. *Cancer Res.* **35**:1058–1063 (1975).
7. Costa, M., A. J. Kraker, and S. R. Patierno. Toxicity and carcinogenicity of essential and non-essential metals. *Prog. Clin. Biochem.* **1**:1–49 (1984).
8. Jennette, K. W. Chromate metabolism in liver microsomes. *Biol. Trace. Element Res.* **1**:55–62 (1979).
9. Tsapakos, M. J., T. H. Hampton, and K. E. Wetterhahn. Chromium (IV)-induced DNA lessions and chromium distribution in rat kidney, liver, and lung. *Cancer Res.* **43**:5662–5667 (1983).
10. Fornace, A. J., D. S. Seres, J. F. Lechner, and C. C. Harris. DNA-protein cross-linking by chromium salts. *Chem.-Biol. Interact.* **36**:345–354 (1981).
11. Christie, N. T., O. Cantoni, R. M. Evans, R. E. Meyn, and M. Costa. Use of mammalian DNA repair deficient mutants to assess the effects of toxic metal compound on DNA. *Biochem. Pharmacol.* **33**: 1661–1670 (1984).
12. Cantoni, O., and M. Costa. Analysis of the induction of alkali sensitive sites in the DNA by chromate and other agents that induce single strand breaks. *Carcinogenesis* **5**:1207–1209 (1984).
13. Sen, P., and M. Costa. Induction of chromosomal damage in Chinese hamster ovary cells by soluble and particulate nickel compounds: preferential fragmentation of the heterochromatic long arm of the x-chromosome by carcinogenic crystalline NiS particles. *Cancer Res.* **45**:2320–2325 (1985).
14. Patierno, S. R., M. Sugiyama, J. P. Basilion, and M. Costa. Preferential DNA-protein crosslinking by $NiCl_2$ magnesium-insoluble regions of fractionated CHO cell chromatin. *Cancer Res.*, in press (1985).
15. Kohn, K. W., R. A. G. Ewig, L. C. Erickson, and L. A. Zwelling. Measurement of strand breaks and cross-links in DNA by alkaline elution, in *DNA repair: A Laboratory Manual of Research Procedures* (E. C. Friedberg and P. C. Hanawalt, eds.). Mercel Dekker, New York, 379–401 (1981).
16. Douglas, G. R., R. D. L. Bell, C. E. Grant, J. M. Wytsma, and K. C. Bora.

[4] P. Sen and M. Costa, unpublished observations.

Effect of lead chromate on chromosome aberration, sister-chromatid exchange and DNA damage in mammalian cells *in vitro*. *Mutat. Res.* **77**:157–163 (1980).
17. Whiting, R. F., H. F. Stich, and D. J. Koropatnick. DNA damage and DNA repair in cultured human cells exposed to chromate. *Chem.-Biol. Interact.* **26**:267–280 (1979).
18. Fraval, H. N. A., and J. J. Roberts. G^1 phase Chinese hamster V79-379A cells are inherently more sensitive to platinum bound to their DNA than mid S phase or asynchronously treated cells. *Biochem. Pharmacol.* **28**:1575–1580 (1979).
19. Gupta, P., and T. Sharma. Differential sensitivity of munjac lymphocyte chromosome to mitomycin C, bromodeoxyuridine and hydroxylamine at different cell cycle stages. *Mutat. Res.* **93**:161–174 (1982).
20. Han, A., M. Korbelik, and J. Ban. DNA to protein crosslinking in synchronized HeLa cells exposed to ultraviolet light. *Int. J. Radiat. Biol.* **27**:63–74 (1975).
21. Burki, H. J., C.-K. Lam, and R. D. Wood. UV-light-induced mutations in synchronous CHO cells. *Mutat. Res.* **69**:347–356 (1980).
22. Carver, J. H., W. C. Dewey, and L. E. Hopwood. X-ray induced mutants resistant to 8-azaguanin. II. Cell cycle dose response. *Mutat. Res.* **34**:465–480 (1976).
23. Jostes, R. F., K. M. Bushnell, and W. C. Dewey. X-ray induction of 8-azaguanine resistant mutants in synchronous Chinese hamster ovary cells. *Mutat. Res.* **83**:146–161 (1980).
24. Goth-Goldstein, R., and H. J. Burki. Ethylnitrosourea induced mutagenesis in asynchronous and synchronous Chinese hamster ovary cells. *Mutat. Res.* **69**:127–137 (1980).
25. Aebersold, P. M., and H. J. Burki. 5-Bromodeoxyuridine mutagenesis in synchronous hamster cells. *Mutat. Res.* **40**:63–66 (1976).
26. Aebersold, P. M. Mutation induction by 5-fluorodeoxyuridine in synchronous Chinese hamster cells. *Cancer Res.* **39**:808–810 (1979).
27. Tsutsui, T., J. C. Barrett, and P. O. P. Ts'o. Induction of 6-thioguanine and ouabain resistant mutations in synchronized Syrian hamster cell cultures during different periods of the S phase. *Mutat. Res.* **52**:255–264 (1978).
28. Orkin, S. H., and J. W. Littlefield. Nitrosoguanidine mutagenesis in synchronized hamster cells. *Exp. Cell Res.* **66**:69–74 (1971).
29. Heck, J. D., and M. Costa. A review of the *in vitro* assessment of the toxicity of metal compound II mutagenesis. *Biol. Trace Element Res.* **4**:319–330 (1983).
30. Cerutti, P. Persistence of carcinogen-DNA adducts in cultured mammalian cells, in *Mechanisms of Chemical Carcinogenesis* (C. C. Harris and P. A. Cerutti, Eds). Alan R. Liss, Inc., New York, 419–427 (1981).

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