ABSTRACT

Intracellular nickel ions (Ni²⁺) have been shown to cause single-strand breaks in DNA, that were rapidly repaired, and DNA-protein cross-links, that persisted for at least 24 h following removal of extracellular nickel ion. In this study, we have used the techniques of alkaline elution, chromatin fractionation, and sodium dodecyl sulfate:polyacrylamide gel electrophoresis to examine the DNA-protein cross-linking induced by NiCl₂ in Chinese hamster ovary cells. Continuous treatment of logarithmically growing Chinese hamster ovary cells with 2.5 mM NiCl₂ in complete medium resulted in DNA single-strand breaks within 1 h, followed by a time-dependent increase in the induction of DNA-protein cross-links at 2, 3, and 6 h. Since the entry of nickel into cells was maximal within 2 h of exposure, the time delay for the formation of DNA-protein cross-links was not limited by metabolic uptake. The nickel-induced DNA-protein cross-linking appeared to require active cell cycling, since single-strand breaks but no cross-linking could be detected in confluent cells treated with 1, 2.5, or 5 mM NiCl₂ for 3 h. DNA-protein cross-linking induced by nickel occurred in late S phase of the cell cycle. High-molecular-weight nonhistone chromatin proteins and possibly histone H1 migrating at the Mr 30,000 range became cross-linked to DNA and were not overtly lethal to these cells, the onset of this DNA lesion appeared to cause the cross-linking of proteins that normally reside in close association with DNA. Alterations of the normal association of these proteins with DNA by nickel may be an early event in the nickel transformation process.

INTRODUCTION

Epidemiological studies have provided evidence that a large proportion of human cancer is environmental in origin (1). At least 20 chemicals or industrial processes have been associated with an increased incidence of human cancer, and approximately 100 others were described as "reasonably anticipated to be carcinogens" (2). It is generally thought that some form of unrepaired DNA damage is a necessary initiation step in chemical carcinogenesis, and it has been shown that the frequency of mutation and transformation induced by certain chemicals can be related to the degree of DNA damage present during DNA replication (3). Thus, persistent DNA lesions, i.e., those which are not easily repaired, would have the greatest propensity to result in "fixed" alterations in the DNA molecule. DNA-protein cross-linking is one such DNA lesion, since a number of documented or suspected human carcinogens have been shown to induce DNA-protein cross-links that persist through several DNA replication cycles. This DNA lesion warrants further study.

Among the industrial processes associated with human cancer is nickel refining. Exposure to nickel compounds has been clearly associated with increased incidence of respiratory cancers in occupationally exposed workers, and nickel compounds are also potent inducers of tumors in experimental animals (for a review, see Ref. 4). Several investigators have reported that both water-insoluble crystalline compounds, such as Ni₃S₂, and water-soluble nickel compounds, such as NiSO₄ and NiCl₂, induced transformation of cells grown in culture. The water-insoluble nickel compounds were more potent transforming agents than the soluble compounds, and this differential activity was ascribed to the mode of delivery of nickel ions into the nucleus (for reviews, see Refs. 5 and 6). Carcinogenic nickel sulfide particles, which were phagocytized by transformable cells, aggregated around the nucleus where they were dissolved by lysosome-facilitated acidification. Thus, the observed phagocytosis of nickel sulfide particles was thought to represent an efficient means of attaining high intranuclear concentrations of nickel ions.

Considerable evidence currently exists to demonstrate that nickel ions can interact with and damage DNA. Administration of nickel carbonate i.p. to rats induced single-strand breaks, DNA-DNA cross-links, and DNA-protein cross-links in tissues that had accumulated the highest intranuclear concentrations of nickel ion (7–9). Treatment of cultured CHO cells with NiCl₂, NiSO₄, or NiCO₃ induced single-strand breaks that were repaired quickly and DNA-protein cross-links that persisted up to 24 h after removal of the nickel (10). Although the DNA-protein cross-links were not overtly lethal to these cells, the onset of this DNA lesion correlated with impaired cell reproduction. In this paper, we have further characterized the DNA-protein cross-links induced by NiCl₂. These results demonstrate that the DNA-protein cross-links induced by NiCl₂ were both concentration and time dependent and preferentially occurred in cells at the late S phase of the cell cycle. The nickel cross-linked proteins were predominately associated with magnesium-insoluble regions of fractionated chromatin and include nonhistone chromatin proteins, nonhistone DNA-binding proteins, and a Mr 30,000 protein(s) that comigrates electrophoretically with histone H1.

MATERIALS AND METHODS

Chemicals. Nickel chloride and MgCl₂ were purchased from Alfa Inorganics (Danvers, MA) and were of the highest grade of purity. Cesium carbonate

1 This work was supported by Grant CA24581 from the National Cancer Institute and by Contract DE AS05-81-ER600-16 from the United States Department of Energy.

2 Recipient of Rosalie B. Hite fellowship in cancer research.

3 To whom requests for reprints should be addressed, at Department of Pharmacology, University of Texas Medical School at Houston, P. O. Box 20708, Houston, TX 77025.

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sulfate, N-laurylsarcosine, deoxyribonuclease, free acid EDTA, disodium EDTA, 2-mercaptoethanol, Nonidet P-40, and Tris (Trizma base and Trizma hydrochloride) were purchased from Sigma Chemical Company (St. Louis, MO). Fisher Scientific Company (Fairlawn, NJ) was the source of NaCl and urea. Polyvinylchlorinated filters were purchased from Millipore Corp. (Bedford, MA). Tetrapropylammonium hydroxide (10% aqueous solution) was obtained from RSA Chemical Company (Ardsley, NY). Proteinase K was purchased from NC/B Chemicals (Cincinnati, OH). Eagle's α-minimal essential medium and trypsin were purchased from Gibco, Inc. (Grand Island, NY), and Liquisint was purchased from National Diagnostics (Somerville, NJ). New England Nuclear Corporation (Boston, MA) was the source of [3H]thymidine (58 mCi/mmol), [35S]methionine (1166 Ci/mmol). Electrophoresis-grade Tris, glycine, sodium dodecyl sulfate, acrylamide, bisacrylamide, TEMED, and ammonium persulfate were all purchased from Bio-Rad (Richmond, CA).

Cell Culture. CHO cells were grown as monolayer cultures in α-MEM supplemented with 10% fetal bovine serum (Rehatuin F.S.; Armour Pharmaceutical Corp., Kankakee, IL) and 1% of a standard solution of penicillin/streptomycin/Fungizone (GIBCO, Inc., Grand Island, NY) in a humidified atmosphere of 95% air-5% CO2 at 37°C. For experiments in which synchronized cells were utilized, CHO cells were subcultured at 75% confluency using trypsin and replated at a density of 8 × 106 cells per 150- x 15-mm tissue culture dish (Miles Laboratories, Naperville, IL). Twenty-four h after seeding, Colcemid (0.02 μg/ml) (Calbiochem, LaJolla, CA) was added. After 6 h of Colcemid treatment, metaphase cells were dislodged by gentle pipeting of the overlying medium. Mitotic cells were collected by centrifugation and chilled to 4°C. This procedure was repeated at 10-min intervals for 1 h in order to obtain sufficient numbers of synchronized cells. The resulting mitotic index was routinely greater than 85%. Mitotic block was released by washing and replating cells in fresh medium at 37°C. By measuring [3H]thymidine incorporation into DNA and the time interval of mitosis, cells found to traverse the cell cycle as follows: 4 h, G1; 7 h, S; and 3 h, G2.

Alkaline Elution. Alkaline elution of DNA was performed using a modification of the technique described by Kohn et al. (11). CHO cells were seeded into 100-mm-diameter tissue culture dishes (Corning Glassworks, Corning, NY) and incubated for 24 h with [3H]thymidine (0.02 μCi/ml). For experiments on logarithmically growing or density-inhibited cells, cultures were washed and incubated in complete medium without FBS. The monolayer of cells was removed from the dishes by gentle scraping with a rubber policeman into ice-cold Puck's Saline A (5 mwi NaCl:90 mM NaHCO3:6 mw glucose:5 mw KCI:140 HIMNaCI, pH 7.2). After 6 h of Colcemid treatment, metaphase cells were dislodged by gentle pipeting of the overlying medium. Mitotic cells were collected by centrifugation and chilled to 4°C. This procedure was repeated at 10-min intervals for 1 h to obtain sufficient numbers of synchronized cells. The resulting mitotic index was routinely greater than 85%. Mitotic block was released by washing and replating cells in fresh medium at 37°C. By measuring [3H]thymidine incorporation into DNA and the time interval of mitosis, cells found to traverse the cell cycle as follows: 4 h, G1; 7 h, S; and 3 h, G2.

Fractionation of Chromatin. CHO cell chromatin was fractionated into putative euchromatin, heterochromatin, and pelleted heterochromatin using a modification of the method of Gottesfeld, et al. (12). For these experiments, cells in logarithmic growth phase, which had been prelabeled with 5 μCi of [35S]methionine per ml for 48 h, were treated with 2.5 mM NiCl2 for 5 h. All subsequent steps to be described were carried out at 4°C. In the presence of 1 mM pheylmethylsulfonyl fluoride, a protease inhibitor. Nuclei were isolated by Potter-Elvehjem homogenization of the cells in the presence of 1% Triton X-100 followed by low-speed centrifugation. This procedure was repeated twice with Triton X-100 and twice in the same buffer without Triton X-100. Nuclei in the final pellet were swelled in a hypotonic 10 mM Tris (pH 7.2) solution and lysed by Dounce homogenization. Chromatin was purified by centrifugation through a discontinuous (1.3–1.6 M) sucrose step gradient. The gelatinous pellet was resuspended into either a solution of 8% glycerol:10 mM Tris (pH 8) or normal saline and disrupted by six separate sonications of 10-s duration utilizing a Biosonik IV (Bronwill, Rochester, NY) at 70% maximal power. The fraction referred to as pelleted heterochromatin (Pt) was collected by centrifugation at 25,000 g for 90 min. The supernatant was slowly titrated to 2 mM MgCl2 and stirred for 30 min, and the magnesium-insoluble heterochromatin (Ps) was pelleted in the same manner as Pt. The magnesium-soluble DNA (Ss) remaining in the second supernatant fraction has been shown to be euchromatin (13). In some experiments, these fractions were homogenized into 1 ml of urea extraction buffer containing 9 M urea, 2% 2-mercaptoethanol, and 4% Nonidet P-40. The sample volume was increased to 4 ml and stirred for 4 h, and the DNA was pelleted by centrifugation at 110,000 × g for 48 h at 25°C.

Isopycnic Cs2SO4 Gradient Centrifugation. Equilibrium density centrifugation was accomplished using the Cs2SO4 method of Monahan and Hall (14). This method was selected because it maximized the separation of chromatin RNA, DNA, and protein. It was also selected because the salt and detergent components of the gradients [0.2% sodium Sarkosyl, 10% dimethyl sulfoxide, 2.65 g Cs2SO4, 1 mM NaCl, 10 mM EDTA, 10 mM Tris (pH 7.1)] resemble those used in the alkaline elution buffer. Gradients (5 ml) containing the chromatin samples were centrifuged at 30,000 rpm for 50 h in a SW 50.1 rotor. The polycrystalline tubes containing the gradients (Beckman Instruments, Palo Alto, CA) were punctured at the bottom, and 6 or 7 drop fractions were collected. Absorbance (A260) was monitored from each fraction. Three distinct peaks were found and shown to consist of RNA, DNA, and protein in independent experiments where nuclei from cells that had been pulse labeled with either [3H]uridine, [3H]thymidine, or [35S]methionine were examined. The DNA peak was routinely collected into 5 or 6 fractions that were pooled in such a manner as to yield high and low density regions of the main DNA band. The fractions representing the two regions were dialyzed overnight against 0.1 mM Tris (pH 7.0), lyophilized, and resuspended into 0.5 ml of DNase buffer [50 mM Tris (pH 7.6):10 mM NaCl:3 mM MgCl2:5 mM CaCl2]. Aliquots of this fraction were utilized to quantitate DNA (A260/280, 1 unit = 50 μg/ml) and to measure associated radioactivity by liquid scintillation counting.

Electrophoresis. DNA samples prepared by isopycnic centrifugation were digested with DNase I (100 μg/ml, 37°C, 1 h), lyophilized, and
resuspended into electrophoresis buffer containing 6.25 mM Tris-HCl (pH 6 to 8), 5% β-mercaptoethanol, 10% glycerol, 2.3% sodium dodecyl sulfate, and 0.002% bromophenol blue. Electrophoresis was performed by the method of Laemmli (15) using 7.5% polyacrylamide. Molecular weight markers (Bio-Rad) were lysozyme (M, 14,400), soybean trypsin inhibitor (M, 21,500), carbonic anhydrase (M, 31,000), ovalbumin (M, 45,000), bovine serum albumin (M, 66,200), and phosphorylase b (M, 92,500). Following electrophoresis, the gels were fixed in 40% methanol:10% acetic acid and silver stained. Photographs of gels were taken using a Polaroid Land Film 4 x 5 positive/negative type 55 film (Polaroid Corp., Cambridge, MA).

Colonies per plate expressed as a function of the original number of cells of undisturbed growth. The number of surviving colonies was determined after the plates were fixed with ethanol and stained with crystal violet. The percentage of survival was calculated as the number of surviving colonies per plate expressed as a function of the original number of cells plated.

RESULTS

Alkaline Elution Analysis of DNA Single-Strand Breakage and DNA-Protein Cross-Linking by NiCl₂. We have previously demonstrated that treatment of CHO cells with NiCl₂ for 3 h in α-MEM with 10% FBS could induce single-strand breaks at 1 mM NiCl₂ and both single-strand breaks and DNA-protein cross-links at 2.5 mM NiCl₂. Single-strand breaks but no DNA-protein cross-links were observed at 5 mM NiCl₂; however, this concentration of nickel was cytotoxic following a 3-h time interval of exposure. DNA-protein cross-links induced by 2.5 mM NiCl₂ persisted up to 24 h after removal of the nickel, and DNA-protein cross-links accumulated to measurable levels 24 h after treatment with 1 mM NiCl₂. Few single-strand breaks remained after this time period (10). To further characterize the onset and degree of DNA-protein cross-linking induced by NiCl₂, we treated CHO cells with 2.5 mM NiCl₂ and assessed DNA damage by alkaline elution at various times after treatment. Chart 1 demonstrates that this amount of NiCl₂ induced some single-strand breaks that were detectable within 1 h following addition of the NiCl₂. DNA-protein cross-links and single-strand breaks that were masked by cross-linked protein became apparent within 2 h of treatment and were increased after 3 and 6 h of continuous treatment. A quantitative summary of DNA-protein cross-linking induced by NiCl₂ in logarithmically growing cells has been compiled in Table 1.

In order to relate the time course of DNA damage to the amount of nickel in cells, the cellular uptake of nickel ions was measured using the radioisotope ⁶⁵NiCl₂. Table 2 shows that the amount of intracellular Ni²⁺ increases in a concentration-dependent manner; however, at a given concentration of NiCl₂, 90% of the maximum possible uptake was attained within a 2-h time interval after addition of NiCl₂.

The data on nickel uptake indicated that the entry of nickel into cells was not temporally consistent with the formation of DNA-protein cross-links. We then tested the hypothesis that active cell cycling was required for DNA-protein cross-linking to occur by examining DNA damage in cells whose growth was slowed due to contact inhibition. Cells were treated with 1.0, 2.5, or 5 mM NiCl₂, and DNA damage was assessed either immediately after treatment (Chart 2) or 24 h following removal of NiCl₂ (data not shown). Only single-strand breaks were induced in confluent cells utilizing nickel exposure conditions that induced DNA-protein cross-links in cycling cells.

Cell Cycle-dependent Effects of NiCl₂. To determine if a particular phase of the cell cycle rendered cells more susceptible to DNA-protein cross-linking, cells were synchronized by mitotic selection. Cells were released from mitotic block by extensive washing in the presence of 20% FBS at 37°C and replated at high density (60 to 70% of the density at confluency). One h after replating, these cells had adhered to the substratum, spread, and begun to traverse the cell cycle. Cells were treated with 2.5 mM NiCl₂ for 3 h beginning at 1, 4, 7, or 10 h after release from mitotic block. Chart 3 demonstrates that NiCl₂ induced single-strand breaks in cells in the G₁, the early S, the
late S, and the G2 phases of the cell cycle. Significant DNA-protein cross-links, however, were found only in cells that were treated with NiCl₂ during the late S phase. Some DNA-protein cross-linking was detected in cells in G2; however, this observation may be due to the dispersion of synchrony at these late time intervals after mitotic synchronization.

Because the induction of DNA-protein cross-linking by NiCl₂ occurred predominately in late S phase of the cell cycle, we investigated the effect of NiCl₂ treatment on the colony-forming ability of CHO cells at each phase of the cell cycle. Cells in the indicated stage of growth were treated with 2.5 mM NiCl₂ for 3 h in complete medium, incubated 24 h in the absence of NiCl₂, trypsinized, and replated in the absence of metal to assess colony formation. Table 3 demonstrates that the colony-forming ability of noncycling cells was not altered by NiCl₂; in contrast, cells in the late S phase of the cell cycle displayed striking sensitivity to NiCl₂. Cells in log-phase growth, or cells synchronized in G₀, or G₂, showed intermediate sensitivity to the growth-inhibitory action of NiCl₂.

**Characterization of Proteins in Chromatin Fractions Involved with Nickel-induced Cross-Linking.** When sheared chromatin was subjected to equilibrium density centrifugation, the resulting DNA was separated into high and low density regions. When DNA from untreated cells was purified in this manner, very little protein remained bound to DNA. The little residual protein bound to DNA was evenly distributed between high and low density DNA with more being associated with the low density region (Chart 4A). This may be due to nonspecific contamination of protein present in the gradient. When the DNA was rebanded in fresh Cs₂SO₄ gradients, the contaminating protein was removed (Chart 4B). Note that Chart 4B represents an enlarged view of the region of the gradient where the DNA sediments. Note the scale of [³⁵S]methionine radioactivity is greatly reduced in B while the scale of [³H]thymidine remains the same. When DNA was purified from nickel-treated cells, however, there was a 5-fold increase in radioactivity associated with the high density region compared with the same DNA region from untreated cells (Chart 4B). No increase in the low density region could be detected. The DNA fractions from each region were pooled and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography as described in "Materials and Methods." The proteins released from high density DNA of nickel-treated cells by DNase digestion are shown in Fig. 1. Proteins cross-linked by nickel in the high density region exhibit molecular weights of 20,000, 31,000, 35,000, 48,000, 52,000, 55,000, 70,000, and 95,000. Fig. 1 compares protein distribution in stationary-phase cultures of CHO cells. Confluent cultures of CHO cells were treated as indicated in each quadrant for 3 h in complete medium and assayed for DNA damage immediately after treatment. Cells were irradiated (as labeled) and lysed in the presence of DNAse I or absence of proteinase K.

**Table 1** Comparison of DNA-protein cross-linking induced by NiCl₂ in logarithmically growing CHO cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
<th>Time (h)</th>
<th>Cross-linking factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>1.0</td>
<td>3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>NiCl₂</td>
<td>1.0</td>
<td>3 + 24</td>
<td>1.7 ± 0.3</td>
</tr>
<tr>
<td>2.5</td>
<td>1</td>
<td>1.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>1.5 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>3</td>
<td>2.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>3 + 24</td>
<td>3.7 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td>3.8 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>3</td>
<td>1.3 ± 0.1</td>
<td></td>
</tr>
</tbody>
</table>

* Cross-linking factor equals the percentage of retained DNA after 9-h elution in the treated cells expressed as a function of the DNA retained following 9 h of elution in the untreated population of cells.

* Mean ± SE.

* Cells were treated for 3 h, and DNA damage was analyzed 24 h after removal of NiCl₂.

* P < 0.05, NiCl₂-treated versus untreated.

* Standard error from two experiments.

**Table 2** Uptake of Ni²⁺ by CHO cells treated with NiCl₂

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>7</th>
<th>Concentration/ cell (μM)²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.113</td>
<td>0.283</td>
<td>0.287</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>0.147</td>
<td>0.286</td>
<td>0.287</td>
<td>520</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>0.292</td>
<td>0.286</td>
<td>0.287</td>
<td>720</td>
<td></td>
</tr>
</tbody>
</table>

* The volume of a CHO cell was estimated to be 400 μl.

* Mean ± SE.

* P < 0.05, NiCl₂-treated versus untreated.

* Standard error from two experiments.

**Table 3** Characterization of Proteins in Chromatin Fractions Involved with Nickel-induced Cross-Linking

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Time (h)</th>
<th>Cross-linking factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>1</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>2.5</td>
<td>2</td>
<td>1.5 ± 0.0</td>
</tr>
<tr>
<td>2.5</td>
<td>3</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>2.5</td>
<td>3 + 24</td>
<td>3.7 ± 1.9</td>
</tr>
<tr>
<td>2.5</td>
<td>6</td>
<td>3.8 ± 2.3</td>
</tr>
<tr>
<td>5.0</td>
<td>3</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

* Cross-linking factor equals the percentage of retained DNA after 9-h elution in the treated cells expressed as a function of the DNA retained following 9 h of elution in the untreated population of cells.

* Mean ± SE.

* Cells were treated for 3 h, and DNA damage was analyzed 24 h after removal of NiCl₂.

* P < 0.05, NiCl₂-treated versus untreated.

* Standard error from two experiments.
Nickel-induced DNA-protein cross-linking in heterochromatin

Early S

Table 3
Survival of CHO cells following treatment with NiCl₂ during various stages of their growth

Cells were treated for 3 h with 2.5 mM NiCl₂ in α-MEM with 10% FBS; 24 h after removal of nickel the cells were trypsinized and replated for a colony-forming assay. See “Materials and Methods” for synchronization procedures.

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>% of survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confluent monolayer</td>
<td>94</td>
</tr>
<tr>
<td>Log-phase growth</td>
<td>81</td>
</tr>
<tr>
<td>G₁ (early S)</td>
<td>67.0</td>
</tr>
<tr>
<td>Late S</td>
<td>13.5</td>
</tr>
<tr>
<td>G₂</td>
<td>61.0</td>
</tr>
</tbody>
</table>

Values normalized to 100% survival of untreated cells in the appropriate growth phase.

Treated with NiCl₂ suggested that the measured DNA-protein cross-link may be asymmetrically distributed in the genome. The late-S-phase-specific induction of the cross-link with nickel indicated that this reaction may occur in heterochromatin, since it replicates during the late S phase while euchromatin is known to replicate during the early part of the S phase. Chromatin was isolated and fractionated into putative euchromatin, heterochromatin, and pelleted heterochromatin fractions based upon sonication in a non-ionic solution and its solubility properties in the presence of 2 mM MgCl₂. Table 4 shows that total chromatin or fractionated chromatin isolated from nickel-treated cells in the absence of detergent with low ionic strength, and under neutral conditions, does not contain significantly more protein than that present in chromatin from untreated cells. However, when either total chromatin or fractionated chromatin was extracted with detergent and high ionic strength, such as is present in the isopyknic centrifugation in Cs₂SO₄ containing 0.2% Sarkosyl, there were significant differences in the amount of protein that remained bound to DNA. Table 4 demonstrates that proteins bound to DNA that were stable to such conditions increased 2-fold and 4-fold in the insoluble and magnesium-precipitable fractions, respectively, when cells were treated with NiCl₂. There were no similar increases in the amount of protein bound to DNA in the magnesium-soluble fraction of nickel-treated cells. Table 5 shows that, when chromatin is sonicated in normal saline instead of a non-ionic solution, the distribution of DNA in the insoluble and magnesium-insoluble fractions was altered. The presence of salt condensed the DNA in such a way that 80 to 90% of the DNA exhibited fast sedimentation, while only 5% remained selectively precipitable by MgCl₂. However, the percentage of total DNA in the magnesium-soluble fraction did not change. Protein concentration was measured in these fractions, and the magnesium-precipitated fraction was found to contain an increased proportion of the nickel-cross-linked proteins (i.e., 11.3-fold increase compared with a 3.7-fold increase reported in Table 4). The proteins associated with these fractions, that were resistant...
NICKEL-INDUCED DNA-PROTEIN CROSS-LINKING IN HETEROCHROMATIN

**DISCUSSION**

Previous studies have demonstrated that treatment of cultured mammalian cells with crystalline NiS or various water-soluble nickel salts resulted in DNA strand breaks that were repaired (16, 17) and DNA-protein cross-links that resisted repair (10). These results were consistent with the in vivo experiments of Ciccarelli and Wetterhahn (6) who reported that the induction of organ-specific DNA single-strand breaks, DNA-interstrand cross-links, and DNA-protein cross-links correlated with the nuclear concentration of nickel and that the DNA-protein cross-links persisted up to 48 h after treatment. Here we report that the induction of DNA-protein cross-linking by treatment of cells with NiCl₂ was dependent on active cell cycling, occurred predominantly in late S phase of the cell cycle, and was concentrated in heterochromatin. Single-strand breaks could be detected within 1 h in cells at any state of growth. This was consistent with a mechanism of direct strand breakage by nickel ions. DNA-protein cross-linking, however, was detectable only after 2 to 3 h of treatment. This was true even of cells synchronized in late S phase, suggesting that DNA-protein cross-linking was concentration dependent as well as being dependent upon the cell cycle. The DNA-protein cross-links did not appear to be related to NiCl₂ cytotoxicity, since this lesion was absent at toxic levels of the metal and present at nontoxic concentrations.

The fact that nickel inhibits DNA replication has been known for some time (18, 19). It is likely that a bulky adduct such as a DNA-protein cross-link that is resistant to the activity of repair enzymes may also physically impair DNA replication; however, it is unlikely that this lesion alone is responsible for complete inhibition of DNA replication, since the inhibition of replication was more dose related and more readily reversible than the DNA-protein cross-link. It is possible that DNA-protein cross-links may also affect the cycle of chromosome decondensation. Borochov et al. (20) have recently reported that nickel ions facilitate precipitation of chromatin by increasing the degree of compaction of the molecule. Nickel ions may also directly interfere with the enzymatic or even cytoskeletal functions that are essential for replication to occur (21, 22).

The biochemical interactions of nickel with DNA and protein have been well characterized and may be central to understanding the induction of DNA-protein cross-links. Nickel ions (Ni²⁺) are electrophilic and of intermediate chemical softness, indicating that they are capable of coordination with nitrogen, oxygen, and sulfur ligands of biological macromolecules. Several investigators have reported the existence of organ- and organelle-specific cystolic nickel binding proteins (23, 24), and others have examined the binding of nickel to DNA and RNA (8, 25, 26). Although nickel ions bind to DNA and protein avidly, they do not bind to either DNA or protein with high affinity, with the possible exception of one renal glycoprotein (27). The binary complexes between nickel and protein or nickel and DNA are unstable to salt, detergent, EDTA, or even precipitation of the macromolecule with ethanol or polyethelene glycol. However, ternary complexes of DNA, nickel, and protein exhibit greatly enhanced stability to these treatments. Ciccarelli and Wetterhahn (6) have recently reported that the amount and distribution of nickel ions bound to chromatin fractions from rat liver and kidney were dependent upon the protein:DNA mass ratio. The levels of nonhistone protein associated with chromatin appeared to influence the amount of nickel that had access to the DNA. Since the DNA and proteins that comprise chromatin are organized in a nonrandom fashion, it follows that the DNA lesions that were induced by nickel would also be distributed in a nonrandom fashion. Thus, in lieu of examining the binding of nickel to DNA and protein, we have taken the alternative approach of characterizing the region(s) of DNA and type of proteins that become tightly

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**Table 5**

Distribution of [³⁵S]methionine radioactivity in fractionated chromatin

<table>
<thead>
<tr>
<th>Chromatin fraction</th>
<th>% of total a</th>
<th>Untreated</th>
<th>Nickel treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>100</td>
<td>4485</td>
<td>5267 (1.2)</td>
</tr>
<tr>
<td>Euchromatin</td>
<td>10 ± 0.7</td>
<td>4666</td>
<td>5525 (1.2)</td>
</tr>
<tr>
<td>Heterochromatin</td>
<td>44 ± 3.5</td>
<td>6644</td>
<td>6408 (1.0)</td>
</tr>
<tr>
<td>Pelleted heterochromatin</td>
<td>47 ± 3.5</td>
<td>2575</td>
<td>3755 (1.5)</td>
</tr>
</tbody>
</table>

a Values represent the percentage of total DNA averaged between untreated and treated samples.

---

**Table 4**

Distribution of [³⁵S]methionine radioactivity in fractionated chromatin

<table>
<thead>
<tr>
<th>Chromatin fraction</th>
<th>% of total a</th>
<th>Untreated</th>
<th>Nickel treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfractionated</td>
<td>100</td>
<td>4485</td>
<td>5267 (1.2)</td>
</tr>
<tr>
<td>Euchromatin</td>
<td>10 ± 0.7</td>
<td>4666</td>
<td>5525 (1.2)</td>
</tr>
<tr>
<td>Heterochromatin</td>
<td>44 ± 3.5</td>
<td>6644</td>
<td>6408 (1.0)</td>
</tr>
<tr>
<td>Pelleted heterochromatin</td>
<td>47 ± 3.5</td>
<td>2575</td>
<td>3755 (1.5)</td>
</tr>
</tbody>
</table>

a Values represent the percentage of total DNA averaged between untreated and treated samples.
NICKEL-INDUCED DNA-PROTEIN CROSS-LINKING IN HETEROCROMATIN

Associated with the DNA in the presence of nickel.

Heterochromatin has classically been defined as late-replicating, nontranscriptionally active DNA that remains condensed during interphase. In most cells, 80 to 90% of chromatin is thought to be transcriptionally inactive and has a more condensed conformation than the euchromatic (transcriptionally active) DNA. Cytogeneticists have applied the term “heterochromatin” to a special class of highly condensed transcriptionally inactive chromatin that stains darkly by banding techniques, and others have characterized a region termed “satellite DNA” that consists of highly repetitive, G-C- or A-T-rich sequences. However, chromatin can also be fractionated biochemically using several different techniques into regions that differ in their physicochemical properties as well as in their probable function. Actively transcribed DNA can be separated from bulk DNA on the basis of hypersensitivity to mechanical or enzymatic (DNase II) shearing and separated according to its greater solubility in the presence of divalent cations. Gottesfeld et al. (12) have characterized this DNA fraction with respect to a number of criteria including template activity for RNA synthesis, DNA sequence complexity, and DNA sequence homology with cellular RNA, and they concluded that it was euchromatic in origin. Chromatin can be fractionated into additional components including (a) a fast-sedimenting component and (b) a region of DNA that is precipitable by addition of 2 mM MgCl₂.

The relationship of these fractions to cytogenetically defined heterochromatin or satellite DNA is unclear at the present time; however, they are classified as heterochromatic primarily due to the lack of transcriptional activity (12, 13). This was supported by several biochemical and physical parameters including the complexity of repetitive sequences, the degree of condensation of the DNA, and the protein make-up of the fractions (12, 13). The data presented in this report regarding the degree of condensation of the DNA and protein make-up of the various fractions are consistent with this classification.

Furthermore, application of biochemically fractionated or whole chromatin to Cs₂SO₄ gradients that lacked 0.2% Sarkosyl yielded results that support the preferential cross-linking of protein by nickel to heterochromatin DNA. This procedure fractionates chromatin into a visible flocculent band of DNA in the high density region of the gradient (80% of total DNA), a medium density DNA fraction that trails from this high density main band (15%), and a small DNA aggregate near the top of the gradient (5%). Kaufman et al. (28) have used this technique to demonstrate that the high density DNA was late replicating. Thus, this DNA was likely to be heterochromatic. When chromatin was biochemically fractionated first and then applied to Cs₂SO₄ gradients, the fractions termed P₁, P₂, and S₁ migrated to the high, medium, and low density regions of the gradient, respectively. Unfortunately, deletion of Sarkosyl from these gradients resulted in extensive protein degradation. Thus, the samples used to identify the proteins that were cross-linked to DNA were taken from the gradients containing Sarkosyl.

It is interesting that the region that was sensitive to MgCl₂ was also the region most affected by the DNA-protein cross-linking induced by nickel; however, the significance of this observation is unknown. Nickel appeared to primarily affect the DNA association of nonhistone proteins and a M. 30,000 protein(s) that is likely to be histone 1. Some of the nonhistone proteins fall into the classification of DNA-binding proteins, since they were tightly associated with the DNA even in the untreated cells. However, this affect did not occur in all regions of DNA, suggesting that there were preexisting structural differences in the DNA regions that influence nickel binding and subsequent cross-linking. These observations may be related to the data of Ono et al. (29) who reported that nickel ions were retained in the nuclei of rat liver cells and that nucleoli contained 18 times more nickel than nuclei. They suggest that the cross-linked proteins may include proteins associated with the nuclear matrix and chromosomal-scaffolding proteins.

Although the importance of DNA-protein cross-linking to the transforming effect of nickel is unknown, the consequences of this lesion in terms of DNA replication may contribute to our understanding. As already discussed, it has been shown that the frequency of mutation and transformation induced by certain chemicals can be related to the degree of DNA damage present when cells attempt to replicate their DNA. Previous studies describing the chemical carcinogenesis process have included a period of decreased cell division followed by “escape,” i.e., spontaneous replication (30). Nickel is particularly interesting in this regard, since it damaged DNA during replication, and therefore the damage may not lend itself to active repair. Furthermore, since the damage was specific to late S phase, it occurred predominately in cells that were already fully committed to DNA replication. This may facilitate the cell’s ability to escape the inhibition and replicate over the cross-link. Thus cells treated with compounds that induce this type of damage may have a greater propensity to “fix” a mutation in their DNA. Interestingly nickel compounds are not potent mutagens in either bacterial or mammalian single gene mutation assays (31). However, this may be due to the lack of significant DNA-protein cross-linking in euchromatin which is the DNA region where the selectable mutated genes would most likely reside. Finally, there may be other important consequences of replication over this type of DNA damage. Sen and Costa (32) have shown that treatment of cells with crystalline NiS or appropriately delivered nickel ions resulted in chromosomal aberrations that included gaps, breaks, and exchanges in the heterochromatic centromeric region of the autosomes and shattering of the heterochromatic long arm of the X-chromosome. These findings as well as the present results suggest that a primary site of nickel action is in heterochromatin DNA. The significance of these results in terms of the carcinogenic action of nickel is not known, but these findings may help explain why nickel compounds do not exhibit potent mutagenicity in mammalian systems.

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REFERENCES

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Fig. 1. Electrophoresis of proteins released from low and high density regions of CHO cell DNA by DNase I digestion. Logarithmically growing CHO cells in which cellular proteins had been intrinsically tagged with 35S-methionine were treated with 2.5 mM NiCl2 for 6 h in complete medium. DNA from both untreated and NiCl2-treated cells was fractionated by isopyknic centrifugation of whole chromatin into regions designated as high density (HD) or low density (LD) DNA (see "Materials and Methods"). Samples normalized to 50 μg of DNA were applied to each lane.

Fig. 2. Photograph of silver-stained SDS-polyacrylamide gel electrophoresis of proteins released from different regions of fractionated chromatin by boiling in electrophoresis sample buffer. Logarithmically growing CHO cells were treated with 2.5 mM NiCl2 for 6 h in complete medium. Sonicated chromatin from untreated (Lanes 1, 3, 5) and treated cells (Lanes 2, 4, 6) was fractionated by differential centrifugation in the absence or presence of 2 mM MgCl2 into regions designated euchromatin (Sp, Lanes 1 and 2), pellet heterochromatin (P, Lanes 3 and 4), and heterochromatin (P, Lanes 5 and 6). Each sample was extracted with a solution containing 9 mM urea, 2% 2-mercaptoethanol, and 4% Nonidet P-40 prior to boiling in electrophoresis sample buffer. Samples normalized to 5 μg of DNA were applied to each lane.

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