DNA Damage and Repair in Mouse Leukemia L1210 Cells Treated with Nitrogen Mustard, 1,3-Bis(2-chloroethyl)-1-nitrosourea, and Other Nitrosoureas

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SUMMARY

The technique of alkaline elution was applied to studies of DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard (HN2) and nitrosoureas. DNA cross-linking was measured in terms of the reduction in the effect of X-ray on the kinetics of DNA elution and was observed in cells treated with HN2 and three chloroethylnitrosoureas: 1,3-bis(2-chloroethyl)-1-nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, and chlorozotocin. Evidence was obtained that cross-links are of two types, DNA interstrand cross-links and DNA-protein cross-links, distinguishable on the basis of sensitivity to proteinase-K. HN2 and 1,3-bis(2-chloroethyl)-1-nitrosourea each produced both types of cross-links. In most of the studies reported, the combined effect of the two types of cross-links was measured. With HN2, cross-linking was observed at subtoxic doses, and the cross-links were fully repaired by 24 hr. At higher HN2 doses, which reduced colony-forming ability, cross-links were not fully repaired by 24 hr. Cross-linking by chloroethylnitrosoureas differed from that by HN2 in that the effect increased for about 6 hr after removal of the drug (treatment times were 0.5 hr for HN2 and 1 hr for nitrosoureas). The chloroethylnitrosoureas, but not HN2, also produce DNA single-strand breaks or lesions, which were converted to single-strand breaks in alkali. Methylnitrosourea produced little or no cross-linking for at least 6 hr after treatment but did produce extensive DNA damage of the alkali-labile type; this damage was repairable and had little effect on colony-forming ability. Fluoroethylnitrosourea, the fluoro analog of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, was much less effective than 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in producing cross-links. The findings on the kinetics and structure dependence of cross-linking are similar to the previously reported findings on interstrand cross-linking of purified DNA and support a two-step reaction mechanism involving a chloroethyalted intermediate.

INTRODUCTION

DNA is considered to be the probable target for the cytotoxic and antitumor actions of bifunctional alkylating agents. Since bifunctionality greatly increases the cytotoxicity and antitumor activity of alkylating agents, it is reasonable to suppose that the essential reaction is a cross-link (38). Cross-linking has been reported to occur between opposite DNA strands (27, 29), between guanine residues in the same DNA strand (41), and between DNA and protein (23).

The production of DNA interstrand cross-links in mammalian cells treated with bifunctional alkylating agents has been detected with several methods, each of which, however, has its own limitations (1, 19, 20, 32, 47). Methods that require DNA isolation prior to cross-link assay (1, 19, 32, 47) subject the DNA to extensive and usually uncontrolled fragmentation, with consequent impairment of sensitivity and quantitation. The density-shift method used by Bali and Roberts (1) gives the most unequivocal demonstration of interstrand cross-linking but requires DNA isolation, as well as treatment of cells with bromodeoxyuridine, which could alter responses to other drugs.

Jolley and Ormerod (20) demonstrated interstrand cross-linking in HN2-treated cells by analysis of the DNA in cell lysates with denaturation and centrifugation techniques without prior isolation. Quantitation was aided by using X-ray to induce known frequencies of single-strand breaks in the cell DNA prior to lysis. These methods, however, are relatively cumbersome and appear not to have been further developed or applied.

The repair of interstrand cross-links has been reported in both bacteria (28) and mammalian cells (19, 32, 47). All of those studies utilized methods involving DNA isolation, except for 1 of the 2 methods used by Harrap and Gascoigne (19), in which cells were lysed directly on alkaline sucrose gradients, which, however, were uncalibrated. The recent findings of Harrap and Gascoigne (19) suggest that important differences may exist between sensitive and resistant tumors in the kinetics of cross-link formation and repair and emphasize the need for improved methodology in this area.

Although it has long been suspected that bifunctional alkylating agents produce cross-links between DNA and protein, as evidenced by the reduced extractability of DNA from treated cells (3, 18, 34), there have been few studies of this phenomenon because of the unavailability of adequately sensitive quantitative methods (23). There have

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been no reports relating to the question of repair of this type of cross-link.

The 1-(2-chloroethyl)-nitrosoureas are not bifunctional alkylating agents in the usual chemical sense, since they have only a single alkylating chloroethyl group. These compounds nevertheless react with purified DNA to produce interstrand cross-links. This occurs in a 2-step reaction, of which the 2nd step is slow and occurs in the absence of drug (24). The mechanism may involve chloroethylation of a DNA base followed by a slow 2nd reaction of the bound chloroethyl group with a base on the opposite strand. Whether these compounds cross-link DNA in cells has not previously been determined.

Nitrosoureas also produce DNA single-strand breaks or lesions that are converted to single-strand breaks under alkaline conditions. This is especially prominent with MNU, as well as other methylating agents (8), and has also been demonstrated with BCNU, although to a much lower extent compared to equitoxic treatments with MNU.2

In addition to their alkylating activities, many nitrosoureas have carbamoylating activity, which, however, affects proteins but not DNA (5). The carbamoylating activity produces a variety of biochemical effects (7, 21, 22) but seems not to be a major source of cytotoxicity or antitumor effect (46). The role of carbamoylation in producing various effects can be tested by using chlorozotocin, a chloroethynitrosourea that lacks carbamoylating activity (15, 30, 35, 45).

We have recently developed a new technique for measuring DNA single-strand breaks, DNA interstrand cross-links, and DNA-protein links (25). The technique is based on measurements of the alkaline elution kinetics of DNA from cells lysed on filters and has the advantages of high sensitivity, reproducible quantitation, and a degree of simplicity that facilitates its use in routine assay procedures. We have previously shown how this technique can be used to give a highly sensitive measurement of DNA cross-linking by HN2 (13, 25). Cross-linking by UV was completely reversed by proteolytic treatment, suggesting that these are DNA-protein cross-links (13, 14).

In the current work, we apply the alkaline elution technique to examine the formation and repair of DNA lesions in L1210 cells treated with pharmacologically reasonable doses of HN2 and nitrosoureas. The major new findings include evidence that: (a) L1210 cells can repair and fully recover from large numbers of DNA interstrand and DNA-protein cross-links produced by HN2 treatment; (b) chloroethynitrosoureas also produce both interstrand and DNA-protein cross-links, but the kinetics differs from the case of HN2; and (c) the kinetics and structure dependence of cross-linking by chloroethynitrosoureas in L1210 cells are similar to those previously found in reactions with purified DNA.

An abstract describing part of this work has appeared (11).

**MATERIALS AND METHODS**

**Cell Growth and Labeling.** Stock cultures of mouse leukemia L1210 cells (31) were propagated in Roswell Park Memorial Institute Medium 1630, containing 20% fetal bovine serum without antibiotics. Cultures to be used for experiments were initiated weekly from stock cultures, pencilin and streptomycin were added, and the cells grew exponentially with a doubling time of 12 hr. Mature DNA was labeled by a 20-hr incubation with [2-14C]thymidine (53 mCi/mmol, 0.01 μCi/ml; Schwarz/Mann, Orangeburg, N. Y.) or with [methyl-3H]thymidine (20 Ci/mmol, 0.1 μCi/ml; New England Nuclear, Boston, Mass.; diluted with unlabeled thymidine to give a concentration of 10-4 μ in the culture medium).

**Drug Treatments.** Drugs were obtained from the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute.

14C-Labeled L1210 cells were centrifuged at 900 rpm for 3 min at 37°, resuspended at 8 to 10 × 10⁵ cells/ml in fresh medium at pH 7.1, and incubated for 1 hr prior to treatment. The nitrosoureas, other than chlorozotocin, were dissolved in 95% ethanol immediately before use, and stock concentrations were such that the ethanol concentration in the medium never exceeded 0.4%; this concentration of ethanol by itself produced no observable effect. Chlorozotocin was dissolved in 0.01 M sodium citrate, pH 4.5, just before use. HN2 stock solutions of 0.01 M HN2 in 0.01 N HCl were stored frozen. In some experiments, the serum concentration in the treatment medium was reduced to 1%. For removal of drug, cells were centrifuged, washed, and resuspended in fresh medium.

**Survival Studies.** L1210 cells were exposed to drugs for the indicated times and then centrifuged, washed, and resuspended in fresh medium. Colony-forming ability was determined by the soft-agar technique described by Chu and Fischer (6). Colonies were counted 11 and 21 days after cloning, and there were no significant differences between the counts at these 2 times.

**Alkaline Elution Assays.** The factors governing DNA alkaline elution kinetics and the procedures used in the assays have been previously described (13, 14, 25).

Approximately 5 × 10⁵ 14C-labeled experimental cells plus a similar number of 3H-labeled reference cells (see below) were diluted in 20 ml of cold phosphate-buffered saline (0.15 M NaCl, 0.7 mM KH₂PO₄, 4.3 mM K₂HPO₄) and collected on a polyvinylchloride filter (pore size, 2 μm; diameter, 25 mm) (Millipore Corp., Bedford, Mass.). The cells were washed with cold phosphate-buffered saline and lysed on the filter with 5 ml of 0.2% Sarkosyl (Ciba-Geigy), 2 M NaCl, and 0.04 M EDTA (pH 10) at room temperature (22–24°C). The lysis solution was allowed to flow through by gravity, and the filter was washed with 3 ml of 0.02 M EDTA, pH 10. No suction was applied during or after lysis. Elution was carried out in the dark at a flow rate of 0.035 to 0.045 ml/min; the eluting solution consisted of 0.02 M EDTA (acid form) plus tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, N. Y.; 10% solution in water) added in the amount required to give a pH of 12.1 to 12.2. Eluted fractions were collected at 90-min intervals and mixed with 3.3 volumes of Aquasol (New England Nuclear), containing 0.75% acetic acid, for scintillation counting. Radioactivity remaining on filters was determined by treating filters with 0.4 ml of 1 N HCl at 65° for 1 hr followed by 2.5 ml of 0.4 N NaOH at room temperature for 1 hr and then adding 10 ml of...
Aquasol. Radioactivity remaining in the funnel was recovered by 5 washes with 2.5 ml of 0.4 N NaOH, and the counts were added to those remaining on the filter.

The quantitative reproducibility of the assay was greatly improved by the inclusion of an internal standard in each sample (14). As internal standard, we use 3H-labeled L1210 cells irradiated with 150 R at 0°. Such reference cells have the advantage that the elution kinetics following this dose of X-ray is nearly 1st order with respect to time, so that the semilogarithmic elution plot is nearly linear. The elution of [14C]DNA from experimental cells, therefore, can be plotted against the elution of [3H]DNA from reference cells without appreciably changing the shapes of the elution curves. The half-times for elution of 150 R-irradiated reference cells varied in different experiments from 8 to 14 hr, depending on filter lot and unidentified experimental factors. Within a given experiment, however, the reference elution half-times were usually within a range of ±10%. The normalization relative to internal standards gave quantitatively consistent data in replicate experiments, independent of differences in the elution rates of the reference DNA. An internal standard was used in all assays, except those of Chart 7. The essential findings were qualitatively the same whether analyzed with or without normalization.

For assay of both DNA breaks and cross-links, cells were eluted both directly and after exposure to 300 R of X-ray at 0°. After X-ray exposure, the cells were kept at 0° to avoid repair of DNA breaks. The purpose of the X-ray dose is to introduce a controlled number of strand breaks in the DNA. Cross-linking is manifested by an inhibition of the effect of the X-ray dose on the elution kinetics (13, 25).

Proteolytic Digestion of Lysates. In the experiments to distinguish DNA-protein links from DNA interstrand cross-links, proteinase-K (0.5 mg/ml) (EM Laboratories, Inc., Elmsford, N. Y.) was included in the lysis solution (0.2% Sarkosyl, 2 m NaCl, 0.04 m EDTA; pH 10), and the lysate on the filter was kept in contact with this solution for 1 hr at room temperature. The inclusion of proteinase reduced the extent of protein retention ([3H]leucine label) from 10-20% to 1-3%. Repeated treatments with proteinase caused no further changes in elution of DNA.

X-ray. Cells were chilled in an ice bath and X-irradiated while suspended in their growth medium in glass tubes packed in ice. X-ray was delivered by 2 vertically opposed Westinghouse Quadrocondex X-ray units operated at 200 kV, 15 ma (0.25-mm copper plus 0.55-mm aluminum filter). The dose exposure rate was 140 R/min.

RESULTS

Cell Survival. For comparison of drug effects on DNA with cytotoxicity, L1210 cells were exposed to various drug concentrations, and their ability to form colonies in soft agar was determined. Colony survival curves were obtained for 0.5-hr exposures to HN2 (Chart 1) and for 1-hr exposures to BCNU (Chart 2). HN2 produced exponential loss of colony-forming ability, whereas BCNU exhibited a shoulder in the survival curve.

Cross-linking by HN2. Treatment of L1210 cells with HN2 produced changes in alkaline elution that we attribute to DNA cross-linking (Chart 3) (13, 25). The alkaline elution measurement is based on the observation that the rate of elution of DNA single strands from cell lysates depends on strand length (25). DNA elution from untreated cells is slow.
DNA Damage and Repair: Nitrosoureas and HN2

Chart 3. Cross-linking of DNA in L1210 cells by HN2. Cells were labeled for 20 hr with [14C]thymidine, treated with 0.2 or 0.7 µM HN2 for 0.5 hr, and then incubated in the absence of drug for the indicated times. Cells were then analyzed by alkaline elution with or without X-irradiation. Open symbols, no X-ray; closed symbols, cells exposed to 300 R at 0° before assay; dashed lines, no drug.

and the rate is greatly increased when single-strand breaks are introduced by subjecting the cells to X-ray before assay (Chart 3A, dashed lines). Cross-linking of DNA inhibits the increase in elution rate produced by a standard X-ray dose (13, 25). This effect of HN2 is shown in Chart 3 by the reduced separation between curves representing irradiated (closed symbols) and unirradiated samples (corresponding open symbols), compared with the separation between the irradiated and unirradiated controls (dashed lines). Cross-linking by HN2 is thus shown by the reduction of the effect of a standard X-ray dose on elution.

The unirradiated HN2-treated samples elute more slowly than the control. The elution of the control is probably due to the slow production of strand breaks in alkali (25), and HN2 treatment apparently hides the expression of these breaks in the same way that it inhibits the effect of breaks caused by X-ray.

Cross-linking was readily discerned even after treatments with low concentrations of HN2, which produced little or no loss of viability. Treatments with 0.2 or 0.7 µM HN2 for 0.5 hr, which gave colony survivals of approximately 40 and 1%, respectively, produced large cross-linking effects immediately after drug exposure (Chart 3, 0-hr curves). At a concentration of 0.05 µM HN2, alkaline elution still disclosed substantial cross-linking (data not shown), although colony survival was 85 to 100%. Most of the cross-links, therefore, either are not lethal or are repaired before their lethal potential is expressed.

For determination of whether HN2 cross-links are repaired, treated cells were incubated at 37° for various periods after drug removal, and the extent of cross-linking was then measured. Chart 3 shows that the effect on alkaline elution that we attribute to DNA cross-linking is partially or totally reversed during posttreatment incubation. In cells treated with 0.2 µM HN2 for 0.5 hr, repair was nearly complete after 24 hr (Chart 3A). Treatment with 0.7 µM HN2 caused much greater cross-linking, and recovery was incomplete after 24 hr (Chart 3B).

The effect of postincubation following HN2 treatments is shown in Chart 4. The extent of elution from irradiated (closed symbols) or unirradiated (open symbols) cells is quantified by the fraction of the DNA retained on the filter when 50% of the internal standard DNA has eluted; this quantity is called "relative retention." The mean and range of relative retention values for irradiated and unirradiated control cells are shown at the right of the chart. Cross-linking by HN2 reduces the separation between the values for irradiated and unirradiated cells. This effect was maximal shortly after treatment and then gradually reversed over a period of many hours. [More detailed studies of the kinetics of the HN2 effect have since shown that cross-linking initially increases and peaks at about 1.5 hr after removal of drug (33).] After 24 to 25 hr, there was a dose-dependent residual cross-linking effect. Whether the residual cross-linking approaches a limit at longer repair times has not been determined.

The HN2 treatments did not produce overt cellular damage during the time periods examined following treatment. At the higher doses, there was significant cell enlargement but phase microscopy revealed no loss of refractility and no blebbing or other indication of severe cell damage. The recovery of total DNA radioactivity in the elution assays was constant for at least 24 hr following treatment, and there

Chart 4. Effect of HN2 treatment and postincubation in L1210 cells. Experimental cells were exposed to the indicated concentrations of HN2 for 0.5 hr, washed, and incubated for various times in fresh medium. Relative retention is the fraction of the [14C]DNA of experimental cells remaining on the filter when 50% of the internal standard (150-R X-irradiated 3H-labeled L1210 cells) has eluted. Closed symbols, cells irradiated with 300 R at 0° and analyzed by alkaline elution; open symbols, same treatment, no X-ray. HN2 concentration (µM): A, 0.2; O, 0.7; □, 1.4. Irradiated (•) and unirradiated (O) controls represent 6 independent experiments. Limit lines, S.D. of 3 or more independent experiments (for data representing less than 3 experiments, the individual values are shown).
was no radioactivity released into the medium. Hence, there was no substantial cell disruption with loss of DNA during this time.

**Cross-linking by BCNU.** BCNU also produced cross-linking effects, but, when examined immediately after drug treatment, the effects were much smaller compared with equitoxic doses of HN2. Chart 5 shows the effects of treatments with 32 and 64 μM BCNU for 1 hr, which produced colony survivals of approximately 1 and 0.01%, respectively. Although the extents of cross-linking were initially small, cross-linking increased for at least 6 hr after removal of the drug (Charts 5 and 6). This is in contrast to the results with HN2. Also contrasting with HN2 is the appearance of DNA strand breaks in cells treated with BCNU. This is shown by a dose-dependent increase in the direct elution of DNA from cells, assayed without prior X-ray (Chart 5, open circles). This effect was slight after 32 μM BCNU (Chart 5A) but substantial after 64 μM BCNU (Chart 5B). The strand breakage effect of BCNU is underestimated, however, because it is partially hidden by the cross-linking effect. The cross-link effect as measured by the separation between elution curves of irradiated and unirradiated samples is not altered by strand breakage (13).

The extents of DNA elution after various BCNU doses and posttreatment incubations are depicted in Chart 6, as assayed either directly without X-ray (open symbols) or with 300 R (closed symbols). The magnitude of cross-linking can be gauged in Chart 6 by the separation between closed and open symbols. The increased elution in the direct assays (no X-ray) returns to normal within a few hours, indicating repair of single-strand breaks, a sufficient increase in cross-linking to mask completely the breaks, or a combination of both processes. The major result shown in Chart 6, however, is that, unlike HN2-treated cells, which repair cross-links during posttreatment incubation, BCNU-treated cells exhibit increasing cross-linking for at least 6 hr after treatment. Whether cross-link repair occurs at later times after BCNU treatments is uncertain, especially because secondary processes must eventually intervene in dying cells.

**Fate of DNA Synthesized after BCNU Treatment.** During exposure of cells to BCNU, thymidine incorporation into DNA is inhibited (17, 36, 42, 43). After removal of the drug, however, treated cells are able to synthesize an approximately full complement of DNA and become blocked in or near the ensuing G2 phase (39). In accord with these reports, we have found that, during treatment of L1210 cells with 50 μM BCNU for 1 hr, thymidine incorporation is inhibited about 80%. After removal of the drug, thymidine incorporation recovers after a lag of 1 to 2 hr and reaches a nearly normal level.

The question arises of whether the DNA synthesized after treatment is in some way abnormal and thus responsible for the inability to carry out steps in preparation for mitosis. For testing of this hypothesis, a comparison was made between the fate of DNA synthesized prior to BCNU treatment and the fate of DNA synthesized immediately after removal of BCNU. The 2 classes of DNA were labeled with different isotopes in the same cell cultures and were analyzed by alkaline elution. The details of the experiment are described in Chart 7. Chart 7, A and C, shows the usual production of single-strand breaks and cross-links in the prelabeled [14C]DNA. Chart 7, B and D, shows the effect of BCNU pretreatment on DNA labeled with [3H]thymidine during the 1-hr period following drug removal. The cells were analyzed either 2 hr (Chart 7B) or 24 hr (Chart 7D) after drug removal. At 2 hr, control cells showed the increased elution rate expected for DNA strands in the process of replication, as these strands have not yet reached their full length (open circles) (26). The increase in elution of the newly synthesized DNA produced by 300 R was similar in magnitude to the effect of this X-ray dose on mature DNA molecules; this is expected from the

![Chart 5. Cross-linking of DNA in L1210 cells by BCNU. Experimental conditions and symbols are the same as for Chart 3, except the treatment was 32 or 64 μM BCNU for 1 hr.](chart)

![Chart 6. Effect of BCNU treatment and postincubation or DNA in L1210 cells. Cells were exposed to the indicated concentrations of BCNU for 1 hr, washed, and incubated for various times in fresh medium. Relative retention was determined as in Chart 4. Closed symbols, cells irradiated with 300 R at 0° and analyzed by alkaline elution; open symbols, same treatment, no X-ray. BCNU concentration: â). 32 μM; •, 100 μM. Controls of irradiated (8) and unirradiated (O) cells represent the mean and S.D. of 12 independent experiments.](chart)
nearly linear relation between the logarithm of DNA retention and single-strand break frequency (25). The DNA from BCNU-treated cells (solid circles, no X-ray) eluted slightly more rapidly than the control; this could be due to BCNU-induced strand breaks and/or retarded replication. The major result, however, is the reduction of the difference between irradiated and unirradiated assays (closed triangles versus closed circles). This difference was 46% of control in the postlabeled DNA (Chart 7B), compared with 50% of control in the prelabeled DNA (Chart 7A). At 24 hr after treatment, these differences were 38% of control in the postlabeled DNA (Chart 7D) and 34% of control in the prelabeled DNA (Chart 7C). Thus, there was no significant difference in cross-linking between prelabeled and postlabeled DNA, and the cross-linking of both increased between 2 and 24 hr after treatment. The results fail to disclose any differences in cross-linking or strand integrity between DNA synthesized after BCNU treatment and preexisting DNA.

Effects of Other Nitrosoureas. Several nitrosoureas were selected for comparison, based on chemical differences relating to alkylating or carbamoylating functions. The compounds were tested by treatments of L1210 cells for 1 hr, followed by removal of drug and continued incubation of the cells in the absence of drug for several hours. Cells were analyzed either before or after posttreatment incubation. CCNU (Chart 8, A and B) and chlorozotocin (Chart 8, E and F) were qualitatively similar to BCNU in that the major feature was DNA cross-linking, which increased greatly upon posttreatment incubation. The result 6 hr after 50 /µM CCNU (Chart 8B, open triangles) is of interest because,
although the curve is nearly horizontal, which is consistent with extensive cross-linking, the entire curve is depressed. This is probably due to a component of dead cells the DNA of which eluted in the 1st fraction. (In other experiments we have noted a correspondence between the fraction of instantaneously eluting DNA and the fraction of dead cells by microscopy.)

FCNU (Chart 8, C and D) produced some strand breakage and cross-linking, but the cross-linking did not progress as it did with BCNU, CCNU, or chlorozotocin.

MNU, by contrast, gave an entirely different pattern, the major feature of which is accelerated elution in the direct assay (Chart 8, G and H, open symbols). This type of elution kinetics cannot be accounted for on the basis of random breaks, such as those caused by X-ray. The difference in the shapes of the elution curves produced by MNU as opposed to X-ray is seen in Chart 8H, where, in addition to the elution of 300-R control cells, the elution of the 150-R reference cells is shown. The accelerated kinetics exhibited by MNU-treated cells suggests that breaks continue to form during the assay, i.e., that most of the breaks did not exist as such in the cells but rather were in the form of alkali-labile sites. These lesions were not lethal since, despite the large breakage effects seen in Chart 8 (G and H), there was a less than 50% loss of colony-forming ability.

It is also clear from Chart 8 (G and H) that MNU produced little or no cross-linking for at least 6 hr of posttreatment incubation, during which time the breakage effect was extensive. After 24 hr, however, evidence of cross-linking was sometimes seen; this may reflect the known ability of methylated DNA to form cross-links after depurination (4, 16).

Protein-dependent and Protein-independent Cross-links. The DNA cross-linking measured by alkaline elution may involve either cross-links between opposite DNA strands or links between DNA and protein (13). The 2 types of cross-links are distinguishable on the basis of whether the cross-linking effect can be reversed by treatment of the cell lysate with proteinase-K (12). We take as working assumptions that the proteinase-sensitive component represents DNA-protein links and that the proteinase-insensitive component represents DNA interstrand cross-links. Both contributed to the cross-linking effect of HN2 (Chart 9) and of BCNU (Chart 10). L1210 cells treated with 0.1 µM HN2 for 0.5 hr or 50 µM BCNU for 1 hr were analyzed by alkaline elution with or without incubation of the cell lysate on the filter with proteinase-K for 1 hr. The proteinase was dissolved in the usual lysis solution in alkaline elution experiments, and the enzyme retained its activity under these conditions, as measured by ability to reduce the retention of labeled protein on the filter or by assay with Azocoll (Calbiochem). Proteinase had little effect on the elution kinetics of control cells (Charts 9A and 10A). In HN2-treated cells, proteinase partially reversed the cross-linking effect (Chart 9), and the extent of reversal was not further increased by repeated proteinase treatments.

In cells assayed immediately after BCNU treatments (Chart 10B), proteinase reversed the cross-linking effect almost completely, suggesting that almost all of the cross-links were of the DNA-protein type. After incubation of the cells for 4.5 or 24 hr, however, the cross-linking effect was only partially reversible (Chart 10, C and D). The cross-

**DISCUSSION**

Alkaline elution experiments appear to distinguish 4 types of DNA strand lesions produced in mammalian cells treated...
with nitrosoureas or HN2: (a) DNA strand breaks (double-strand breaks are not distinguished from single-strand breaks), (b) alkali-labile sites, (c) DNA interstrand cross-links, and (d) DNA-protein links.

Strand breaks and alkali-labile sites both increase DNA elution rates. A prototype for randomly distributed strand breaks is ionizing radiation, which increases DNA elution in a manner characterized by an initial component of 1st order kinetics (25). Alkali-labile sites would continuously generate breaks under the alkaline conditions of the analysis; this would produce accelerated elution, with the rate of elution increasing with time. Kinetics of this type were apparent with nitrosoureas or HN2 (Chart 8), which would be expected to produce alkali-labile sites both in the form of apurinic sites (40) and in the form of alkylated phosphates (37). The alkali-labile lesions produced by MNU were repairable and caused very little loss of colony-forming ability. Increased elution rates were also produced by BCNU, CCNU, chlorozotocin, and FCNU, but these increases were relatively small, so that it was not possible to distinguish between alkali-labile lesions and preexisting strand breaks in the case of these drugs.

Cross-linking was assayed in cells exposed to 300 R of X-ray, which was estimated to produce 2.7 single-strand breaks per 10^7 DNA nucleotides (about 5000 single-strand breaks per L1210 cell) (25). We define cross-links operationally as lesions that inhibit the increase in DNA elution normally produced by a standard X-ray dose (13, 25). This effect was most clearly seen with HN2, which produced cross-linking without detectable strand breakage effects (Chart 3). BCNU, on the other hand, produced both cross-linking and breakage effects (Chart 5).

Both HN2 and BCNU produced 2 types of cross-links, distinguishable on the basis of sensitivity to proteinase-K. The proteinase-insensitive component can reasonably be attributed to DNA interstrand cross-links, since both drugs are known to produce such cross-links in purified DNA (24, 27). The proteinase-sensitive component probably represents DNA cross-linked to protein (13). Evidence for DNA-protein linking has previously been reported for HN2 (18, 23) but not for nitrosoureas.

HN2 and BCNU differed strikingly in regard to changes in cross-linking upon incubation of the cells after removal of drug. Whereas the major feature with HN2 was a slow loss of cross-links, the extent of cross-linking after removal of BCNU increased for at least 6 hr. Such delayed increases in cross-linking were also observed with CCNU and chlorozotocin and, to a lesser degree, with FCNU. In the case of MNU, there was no detectable cross-linking 6 hr after treatment, although some experiments did show cross-linking after 24 hr.

These findings are consistent with known or suspected chemical effects of nitrosoureas on DNA. The cross-linking by MNU may be generated by depurination of methylated purines (4, 16), followed by a reaction of the liberated pentose aldehyde group with a site on the opposite DNA strand. This effect, however, was undetectable 6 hr after MNU treatment, even though large numbers of alkali-labile sites were evident at that time. The chloroethynitrosoureas, on the other hand, produced extensive cross-linking at 6 hr, while strand breakage effects were less prominent. The cross-linking by chloroethynitrosoureas, therefore, probably involves another mechanism. A suggested mechanism involves chloroethylation of a DNA base, followed by a delayed 2nd reaction with a site on the opposite strand (24). In this 2nd reaction, a chloride ion would be liberated and ethylene bridge would be formed between the 2 strands.

The repair of cross-links was most evident in the case of HN2. Cross-links were almost totally repaired 24 hr after treatment of L1210 cells with up to 0.2 /¿M HN2 for 0.5 hr. Since approximately one-half of the cross-linking was sensitive to proteinase, both DNA interstrand cross-links and DNA-protein links are repairable. The ability of L1210 cells to survive this dose of HN2 indicates that neither of the 2 types of cross-links is necessarily lethal; cells appear able to recover proliferative capacity after several hundred cross-links of each type have been inserted per cell. Survival may depend on the relative rates of 2 competing processes: repair of DNA lesions as opposed to lethal expression of these lesions prior to repair. A larger number of DNA lesions per cell would give a larger probability of lethal expression before repair is completed.

In our experiments, DNA repair did not correlate with shoulders on the survival curves. The survival curve with BCNU exhibited a shoulder and was similar to results in other cell types (9, 39). In the case of HN2, however, the loss of survival appeared to be exponential, without a shoulder. Nearly exponential survival has also been reported in HN2-treated H. Ep. No. 2 cells (44). Survival curve shoulders have been related to recovery from X-ray-induced sublethal damage (10) and to potentially lethal damage in cells treated with CCNU (2). The presence or absence of a shoulder, however, does not logically determine whether a cell can repair macromolecular damage. For example, if the repair rate is proportional to the frequency of lesions and the repair system is not saturable, then repair would be equivalent to a dose-reduction factor and would not change the shape of the survival curve.

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