DNA Damage in Rat 9L Cells Treated with Nitrogen Mustard and 1,3-Bis(2-chloroethyl)-1-nitrosourea Assayed by Viscoelastometry and S1 Nuclease

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ABSTRACT

The techniques of viscoelastometry and S1 nuclease digestion were applied to the analysis of DNA damage in rat 9L cells treated with nitrogen mustard and 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). Results of the S1 nuclease assay permitted quantitation of the amount of single-strand (or alkali-labile) break formation as well as DNA interstrand cross-link formation. In the presence of 2% detergent, only cells treated with nitrogen mustard showed evidence of DNA cross-link formation as determined by this assay. Viscoelastic analysis of cell lysates under denaturing conditions (pH 12.15) showed that cells treated with nitrogen mustard led to substantial increases in both the viscoelastic retardation time and recoil, consistent with the presence of DNA cross-links, while treatment with BCNU led to decreases in these two properties, consistent with the induction of single-strand breaks. Viscoelastic analysis of cell lysates under nondenaturing conditions (pH 11.15) showed that nitrogen mustard produced an increase in retardation time, consistent with single-strand break induction, along with a fast recoiling component that eventually led to gel-like behavior, suggesting the possibility of drug-induced intermolecular DNA-DNA cross-links. BCNU treatment resulted in a decrease in retardation time. This decrease is consistent with induction of DNA interstrand cross-links by BCNU and shows that the single-strand breaks observed at denaturing conditions were due to the presence of alkali-labile sites rather than true strand breaks. While other methods using denaturing conditions have resulted in evidence for DNA cross-links following BCNU treatment, both viscoelastic and S1 nuclease experiments showed negative results in this regard. Further work is needed to clarify this point.

INTRODUCTION

The problem of detecting and characterizing DNA damage and its repair in mammalian cells is of paramount importance in understanding the mechanism of action of both antitumor drugs and chemical carcinogens. A variety of methods are presently available for the analysis of DNA damage and have been recently reviewed (18). Ideally, an assay for drug-induced DNA damage should be both sensitive and quantitative. In practice, sensitivity requires that the assay itself does not produce significant levels of DNA damage, while quantitation requires a model that relates lesion number to experimental observables. This paper presents our initial results using viscoelastometry as a method for detecting drug-induced DNA damage in mammalian cells. Because this is a new approach to the DNA damage problem, we also present, for the purpose of comparison, results obtained using S1 nuclease digestion. Viscoelastometry is the more sensitive of the 2 techniques but has not yet been sufficiently developed to permit quantitation of damage. The S1 nuclease assay has this capability and permits detection of strand breaks in the presence of cross-links.

Most of the assays presently in use (including S1 nuclease digestion) require alkaline denaturation of the DNA. Exposure to alkali creates a certain level of DNA damage, thereby reducing sensitivity somewhat. However, a great deal of our knowledge concerning the effects of nitrogen mustard [bis(2-chloroethyl)methylamine] and BCNU* on cellular DNA has been derived from such methods.

Alkaline sucrose gradient sedimentation has been used to study, on a qualitative level, the effects of strand breaks and DNA interstrand cross-links induced by nitrogen mustard (12, 26, 32). Quantitation of strand breaks following treatment with BCNU has also been achieved (7, 8). Sensitivity, however, is limited by the need to avoid anomalously sedimenting DNA. Approximately 1000 rads of X-rays are required to obtain well-behaved sedimenting DNA (6, 22). While this technique is capable of quantitating strand breaks, quantitation of cross-links is not yet possible.

Alkaline elution has been shown to be a sensitive assay for the detection of strand breaks and cross-links induced by BCNU and cross-links induced by nitrogen mustard (9–11). While other methods have provided evidence for DNA-protein cross-linking following nitrogen mustard treatment (14), alkaline elution is the only method capable of sensitive measurement and quantitation of DNA-protein cross-links (10, 19). Low levels of X-ray-induced strand breaks, e.g., 30 rads, can be quantitated by alkaline elution. However, in situations where cross-links and strand breaks are produced simultaneously, as in the case of nitrogen mustard, detection of strand breaks is not always possible (10, 11). Also, quantitation of strand-break and cross-link frequencies leads to apparent values rather than true values (13).

Alkaline denaturation-renaturation techniques have been

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* The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ICP, independent-chain polymer; SDS, sodium dodecyl sulfate.

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used extensively to demonstrate cross-links following treatment with nitrogen mustard (4, 12, 13, 20, 27, 32) and cross-links in DNA treated in vitro with BCNU (17). Because of the need for prior isolation of the DNA, the earlier applications of this approach were neither sensitive nor quantitative. Detection and quantitation of both strand breaks and cross-links induced by nitrogen mustard were achieved in 1973 by Jolley and Ormerod (13) by modification of a denaturation-renaturation technique. However, due to rather extensive assay damage (>2000 rads), sensitivity was not high.

**Description of the Viscoelastic Method.** The viscoelasticometer, developed by Zimm et al. (2, 15, 16, 25), is essentially a concentric cylinder viscometer, consisting of an outer glass cylinder, called the cassette, and an inner plastic cylinder, called the rotor. Cells are lysed directly in the cassette, and the rotor is lowered into the lysis and maintained submerged below the surface. During the windup, an electromagnetic torque is applied to make the rotor turn at low shear rates, the angular position of the rotor being monitored optically. After the rotor has undergone a certain angular displacement (usually 70° in our experiments), the torque is removed. The rotor first slows down and then rotates in the opposite direction before coming to a halt. Typical experimental traces can be found in Chart 1.

The effect of the applied torque is to stretch out the large polymers; removal of the torque results in the polymers returning to their unstretched conformation. This leads to the recoil of the rotor in the direction opposite to the motion induced by the torque. The rotor recoil is described by a series of decaying exponentials with time, each characterized by a time constant, or retardation time $\tau$. Experimentally, the largest $\tau$ value is the most reliably determined and is roughly a measure of the time required for the rotor to undergo 63% ($1 - 1/e$) of the total recoil. If the molecules are in an ICP form (3), the retardation time can be related to the molecular weight of the largest species in solution (33). This selectivity is both an advantage and a disadvantage. High sensitivity can be expected since the largest DNA molecules will be the most susceptible to a given dose of damaging agent. However, information cannot be obtained on the whole population of different-sized molecules.

Viscoelastic studies on nonmammalian systems have been reviewed by Roberts et al. (28). Uhlenhop (30) first demonstrated large effects on the retardation time of the DNA from mammalian cells treated with X-rays or nitrogen mustard. Since no independent measure of the solution conformation of the DNA was made, however, interpretation of his preliminary results is difficult. We have made a more extensive study of the effects of lysis time, lysis pH, and exposure to $\gamma$-rays on the retardation time of the DNA in mammalian cell lysates (3). In that same study, the strandedness of the DNA was determined by S$_1$ nuclease digestion.

In lysates of rat 9L cells at pH values less than 11.4, S$_1$ nuclease digestion showed the DNA to be double stranded at least held in register at various points such that rapid renaturation is possible upon transfer to the S$_1$ assay buffer (31). At pH values greater than 11.9, the DNA was single stranded (3). It is interesting to point out that these results are consistent with the observation in the alkaline elution technique that, in the pH range of 11.3 to 11.9, the elution of DNA changes from almost zero to a maximum (18).

**DNA Damage Assayed by Viscoelastometry and $S_1$, Nuclease Digestion.** Viscoelastic measurements made at pH 11.15 (to be referred to as nondenaturing conditions) showed that the retardation time increased following exposure to low doses (50 to 200 rads) of $\gamma$-rays and then decreased with higher doses (200 to 1000 rads). This behavior suggests that a certain degree of supercoiling exists in the DNA under nondenaturing conditions. The initial increase in $\tau$ arises from a loss of supercoiling due to single-strand breaks. This change in conformation results in a more unfolded DNA characterized by a greater hydrodynamic volume and a larger $\tau$. The subsequent decrease in $\tau$ arises from DNA degradation due to further breaks. A more complete discussion of this can be found in Ref. 3.

Viscoelastic measurements on lysates at pH 12.15 (to be referred to as denaturing conditions) showed behavior predicted for ICP single-stranded DNA (3). Measurements on lysates of untreated cells revealed the molecular weight of the largest single-stranded species of DNA to be $2.5 \times 10^{6}$.

Due to the presence of supercoiling under nondenaturing conditions, the DNA is not in an ICP form. Thus, under non-denaturing conditions, the quantitative relationship between $\tau$ and molecular weight, developed for ICP forms of macromolecules (33), is of doubtful validity. However, we assume that the qualitative behavior is correctly predicted, i.e., that an increase in $\tau$ signifies an increase in either molecular weight or hydrodynamic volume (2, 15, 16, 33). Since the retardation time is sensitive to the hydrodynamic volume, it is possible to detect effects of damaging agents on the tertiary structure of the DNA. In addition, experiments can be done at different pH's, thereby permitting distinction between alkali-labile sites and true strand breaks.

In summary, based on the experiments described above, we can expect single-strand breaks to result in an increase in $\tau$ at low levels of damage under nondenaturing conditions. Under denaturing conditions, strand breaks will lead to a decrease in $\tau$. Also, DNA-DNA interstrand cross-links will prevent complete strand separation under denaturing conditions, resulting in an increase in retardation time due to the larger size of the cross-linked molecules.

**S$_1$, Nuclease Digestion.** Under denaturing conditions, a DNA interstrand cross-link facilitates the rapid renaturation of single-strand regions near the cross-link. Increasing the number of cross-links increases the fraction of rapidly renaturable DNA, whereas decreasing the molecular weight decreases the fraction of rapidly renaturable DNA. It is possible to calculate the number of interstrand cross-links and single-strand breaks from the fraction of rapidly renaturable DNA if the number-average molecular weight and molecular weight distribution of the DNA are known (13). In the present study, exposure to increasing doses of $\gamma$-rays following drug treatment is used to create a known distribution of molecular weights.

The use of S$_1$, nuclease digestion to determine the fraction of rapidly renaturing DNA has several attractive features. The theory of Jolley and Ormerod (13) can be unambiguously applied for the quantitation of strand breaks in the presence of cross-links. Also, the technique is rapid, requiring less than 4 hr to perform. However, like all methods that involve alkaline denaturation of DNA, it cannot distinguish alkali-labile breaks from true strand breaks and yields no information on drug-induced changes in the large-scale tertiary structure of the DNA. Quantitation of DNA damage by the S$_1$ nuclease assay
provides data complementary to those of our viscoelastic experiments, which focus on the damage in the largest DNA species.

MATERIALS AND METHODS

Cell Culture. Rat 9L cells from an N-methylnitrosourea-induced brain tumor were seeded at 1 x 10^6/flask; those used in the S1 nuclease studies were labeled with [3H]thymidine (0.3 μCi/ml; 2 Ci/mmol; Amersham/Searle Corp., Arlington Heights, Ill.). The cells were grown for 24 hr (early log phase) in a humidified 5% CO2 atmosphere in medium containing 10% fetal calf serum. Then cells were treated with one of the 2 drugs. Nitrogen mustard (Mustargen; Merck Sharp & Dohme, West Point, Pa.) was diluted in 10^-4 M HCl, and added to the flasks for a period of 1 hr. BCNU (courtesy of Dr. V. A. Levin, Brain Tumor Research Center, University of California, San Francisco) was initially dissolved in ethanol, diluted in Hanks’ balanced salt solution, and then added to the flasks. For a 4-hr treatment, the medium was replaced after 1 hr with drug-free medium. Cells were then trypsinized and resuspended in 1 M NaCl-0.01 M tetrasodium EDTA, pH 9.5.

S1 Nuclease Assay. Cells treated with nitrogen mustard or BCNU were resuspended at a concentration of 4 x 10^6 cells/ml and then exposed on ice to a 137Cs source for various times at a dose rate of 454 rads/min. In order to lyse the cells, 1.5 ml of cells were added to a test tube, and then 1.5 ml of an alkaline solution and 0.3 ml of a detergent solution were added simultaneously, and the test tube was quickly inverted twice. The alkaline solution consisted of 0.25 M Na2HPO4, 0.032 M sodium acetate, and 0.003 M ZnCl2 (pH 4.5). Then 0.25 IU of S1 nuclease (Calbiochem-Behring Corp., La Jolla, Calif.) was added, the mixture was inverted twice, and digestion proceeded at 50° for 1 hr. Undigested double-stranded DNA was then precipitated at 4° with the addition of 0.3 ml of 70% perchloric acid and 75 μg of calf thymus DNA. The precipitate was pelleted 30 min later in an IEC refrigerated centrifuge. The supernatant was carefully decanted into a scintillation vial, and the precipitate was dissolved in 3 ml of 5% (v/v) perchloric acid at 90° for 30 min and added to a scintillation vial. PCS (8 ml; Amersham/Searle Corp.) was added to the vials, and 3H activity was determined. The unnormalized fraction of single-stranded DNA was taken as the ratio of counts in the supernatant to the sum of counts in the supernatant and precipitate, after subtracting background. The normalized fraction of single-stranded DNA was adjusted for the amount of digestion occurring in the untreated DNA in denatured and non-denatured lysates. Cells lysed at the non-denaturing pH of 11.0 yielded 6% single-stranded DNA, while uncross-linked DNA lysed at a pH of 12.15 yielded 84 to 87% single-stranded DNA.

The fraction of non-renaturable DNA FSn determined by the S1 nuclease assay satisfies the following equation, according to the analyses of Jolley and Ormerod (13) and Ewig and Kohn (11):

\[ F_{Sn} = \left( \frac{2b}{2b + c} \right)^2 \]  

where \( b \) and \( c \) are the probabilities or frequencies for single-strand break formation and cross-link formation, respectively. The value of \( b \) includes single-strand breaks from the lysis and assay procedures, as well as from the γ-irradiation and the drug treatment. Strickly speaking, the same considerations hold for the value of \( c \), although the drug treatment is certainly the major source of cross-links. The nuclease digestion results were analyzed by rewriting Equation A as:

\[ F_{Sn} = \left( \frac{2b + 2b_{at}}{2b + 2b_{at} + c} \right)^2 \]  

where \( b \) is the contribution to \( b \) from γ-irradiation and \( b_{at} \) is that part of \( b \) due to the effects of the lysis and assay treatment and the drug treatment. We assume that only the drug treatment contributes to \( c \). Values of \( b_{at} \) and \( c \) were obtained by fitting the experimental results to Equation B using nonlinear regression. Following the method of Ewig and Kohn (11), all the parameters in Equation B are expressed in rads, yielding a value of \( c \) in rad equivalents of cross-links.

Viscoelastometry. Cells were grown and resuspended in the NaCl solution described above without the radioactive label and without exposure to γ-rays. Cells were then filtered through a 20-μm pore-size nylon Nitex filter and resuspended at a concentration of 2 x 10^3/ml for denaturing lysis, as determined by electronic cell counting (Royco Instruments, Inc., Menlo Park, Calif.). The denaturing lysis procedure was identical to that described above, except that lysis was carried out directly in the viscoelastic cassette. For non-denaturing lysis, the alkaline solution was identical to that described above, except that the pH was 11.30 and the final lysis pH was 11.15. The higher concentration of SDS in the lysate than what we used previously (3) led to significantly enhanced stability in determining the viscoelastic behavior of the lysate. Following 2 to 3 hr of lysis, the viscoelastic response of the lysate was determined. The rotor was gently lowered into the lysate and forced to the bottom of the cassette containing the lysate. The height servo was engaged, the rotor was brought to its proper height, and then the windup was performed. The windup was 70° at a shear rate of 1 x 10^-2/sec. Results were reproducible from experiments carried out over long periods of time and from one batch of cells to another. For example, measurements made over a period of 1.5 years yielded an average value of 1205 ± 102 (S.D.) sec for the retardation time of control cells (total of 15 experiments with values ranging from 1000 to 1320 sec).

The recoil curves were digitized into a Digital pdp11/70 computer and analyzed in terms of a 1- or 2-exponential decay process (16) using a nonlinear iterative least-squares-fitting algorithm. Results are presented in terms of retardation times and recoil. The latter gives the angular displacement after the removal of the applied torque and is an approximate measure of the concentration of recoiling species.

RESULTS

Nitrogen Mustard. Viscoelastic analysis, under denaturing
conditions, of lysates of cells treated with nitrogen mustard revealed large changes from untreated cells, as illustrated in Chart 1 and Chart 2, A and B. Both the retardation time and the recoil increased with increasing dose. This increase in $\tau$ is consistent with the presence of DNA interstrand cross-links which prevent complete strand separation. The retardation time reached a plateau value at 1 $\mu$M nitrogen mustard, while the recoil reached a plateau value at 5 $\mu$M. Nondenaturing lysis of cells treated with nitrogen mustard gave the viscoelastic behavior shown in Chart 2, C and D. Here, 2 exponential terms were required to fit the recoil of lysates from treated cells, indicating the presence of a rapidly recoiling species in addition to a slowly recoiling species. Both retardation times increased with increasing concentration of nitrogen mustard. Only the recoil of the faster component increased with increasing drug concentration. At a concentration of 3 $\mu$M, the recoil (~90%) consisted of a single, large component, suggesting the presence of a gel-like structure dominating the viscoelastic behavior.

The fraction of nonrenaturable DNA following treatment with several doses of nitrogen mustard and subsequent irradiation with increasing doses of $\gamma$-rays is plotted in Chart 3, along with the best fit to Equation B. The resulting values of $b_{ss}$ and $c$ for all doses assayed are shown in Table 1. Apparently, drug-induced breaks are not significant until the highest concentration of nitrogen mustard is reached. This is consistent with other studies which reported no strand-breaking activity at lower nitrogen mustard concentrations (11) and significant activity at higher concentrations (13). As Table 1 indicates, the cross-linking activity of nitrogen mustard increases with increasing concentration. A plot of $c$ as a function of concentration (Chart 4) shows this dependence to be approximately quadratic.

We point out here and discuss more fully below the fact that strand breaks did not appear in the $S_1$ assay until a dose of 10 $\mu$M was reached. In contrast to this result, the retardation time in nondenatured lysates showed a significant increase following treatment with only 1 to 2 $\mu$M nitrogen mustard.

**BCNU.** When cells treated with 50 $\mu$M BCNU for varying amounts of time were lysed under denaturing conditions, the

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**DNA Damage Assayed by Viscoelastometry and S$_1$ Nuclease**

Chart 1. Experimental trace of angular position of rotor as a function of time.
Zero time coincides with removal of the applied torque; linear portion prior to zero time, rotor motion during windup under the influence of the applied torque; curved portion, motion of rotor during recoil. Denatured lysates at 6 x 10$^8$ cells/ml; Curve a, untreated cells; Curve b, cells treated with 10 $\mu$M nitrogen mustard for 30 min.

![Chart 1](image1.png)

Chart 2. Viscoelastic analysis of cells treated with nitrogen mustard. A, retardation time $\tau$ of denatured lysates; B, recoil of denatured lysates; C, retardation time $\tau$ of nondenatured lysates; D, recoil of nondenatured lysates; O, slow component; $\Delta$, fast component.

![Chart 2](image2.png)

Chart 3. Results of $S_1$ nuclease digestion assay of cells treated with nitrogen mustard (O, 3 $\mu$M; $\Delta$, 10 $\mu$M) and exposed to various doses of $\gamma$-rays. krads, kilorads.

![Chart 3](image3.png)

Table 1

<table>
<thead>
<tr>
<th>Concentration ($\mu$M)</th>
<th>$b_{ss}$ (rads)</th>
<th>$c$ (rads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>228 $\pm$ 381$^c$</td>
<td>28 $\pm$ 41$^c$</td>
</tr>
<tr>
<td>3.0</td>
<td>319 $\pm$ 21</td>
<td>73 $\pm$ 4</td>
</tr>
<tr>
<td>5.0</td>
<td>216 $\pm$ 60</td>
<td>108 $\pm$ 23</td>
</tr>
<tr>
<td>10.0</td>
<td>1085 $\pm$ 242</td>
<td>841 $\pm$ 129</td>
</tr>
</tbody>
</table>

$^a$ Calculated from nonlinear least-squares fit of $S_1$ nuclease results to Equation B.

$^b$ $b_{ss}$, single-strand break frequency including effects of assay as well as drug treatment; $c$, cross-link frequency.

$^c$ Mean $\pm$ S.E. calculated from nonlinear least-squares fit.
retardation time decreased somewhat, and the amount of viscoelastic recoil showed a large decrease. These decreases indicate a loss of large molecules, suggesting the presence of BCNU-induced single-strand breaks. These results are shown in Chart 5, A and B. When cells treated with BCNU were lysed under non-denaturing conditions, the retardation time decreased somewhat after 4 hr of drug treatment, as shown in Chart 5, C and D. As discussed below, this may be evidence of DNA interstrand cross-links. The recoil remained relatively constant, indicating no significant change in the concentration of recoiling species.

It was not possible to detect a rapidly renaturable fraction of DNA 4 hr after a 50 μM BCNU treatment using the above protocol (data not shown). Treated and untreated cells gave maximum amounts of single-stranded DNA. Since evidence for DNA interstrand cross-links in cells exposed to BCNU has been reported in alkaline elution studies (10, 11), somewhat less harsh lytic conditions were tested that might reveal the presence of such cross-links. When detergent was eliminated from the lysis procedure and the pH was maintained at 12.15, the BCNU-treated cells gave more single-stranded DNA than did untreated cells, as shown on the ordinate of Chart 6. When the lysis pH was lowered to 11.45 and the detergent concentration was lowered to 0.4%, cells treated for 4 hr with BCNU gave somewhat less single-stranded DNA than the untreated cells. However, BCNU-treated cells lysed with 2% detergent at a pH of 11.45 yielded the same amount of single-stranded DNA as did untreated cells.

**DISCUSSION**

**Nitrogen Mustard.** Under denaturing lysis conditions, viscoelastic experiments showed a rapid increase in retardation time at low drug concentrations with a plateau value attained by 1 μM nitrogen mustard (Chart 2). This concentration corresponds to approximately one log cell kill. Further dose increases evidenced little or no effect on the retardation time. The value of τ at 1 μM nitrogen mustard was approximately 4 times that of untreated controls. As discussed earlier, an increase in τ is expected in the presence of DNA interstrand cross-links. Since the viscoelastic recoil is dominated by the largest species in solution, the effect of DNA interstrand cross-links can be discerned when only a small amount of the cross-linked species is formed. The presence of the smaller, strand-separated molecules does not interfere with the detection of the larger, cross-linked species. At doses beyond 1 μM, more cross-linked molecules continued to be formed, as suggested by the fact that the recoil continued to increase. Examination of Table 1 shows that 1 μM nitrogen mustard is equivalent to 28 rads of damage, as quantitated by the S1 nuclease assay. Because of the somewhat large error associated with this number, we can take 45 rads as a reasonable estimate of the damage and calculate that the plateau level in τ is reached at one cross-link approximately every 1 x 10^10 daltons [based on a figure of 44 eV/single-strand break (21)].

The effect of nitrogen mustard treatment on the viscoelastic behavior of lysates under non-denaturing conditions was manifested in 2 major changes. At low doses, the retardation time

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5 D. F. Deen, personal communication.
increased while, at higher doses, a second, fast component appeared in the recoil. The increase in $r$ of the slow component was similar to that observed following exposure to low doses ($\sim 50$ rads) of $\gamma$-rays (3), suggesting the presence of single-strand breaks. Such a small number of breaks could not be detected in the $S_1$ nuclease test because of the assay damage of 200 to 300 rads (see below).

At higher doses of nitrogen mustard, the second component, characterized by a small retardation time, led to large values of the recoil. At the highest drug concentration, the signal was completely dominated by the faster component, with a recoil in excess of 90%. Such high recoils are characteristic of gel solutions. Thus, this result would be expected of an extended network of cross-linked molecules, as might arise from intermolecular cross-links. This type of DNA damage following treatment with nitrogen mustard has been reported before (1).

An analysis of the $S_1$ nuclease results presented in Table 1 indicates that nitrogen mustard induces DNA interstrand cross-links at all concentrations studied and single-strand breaks only at the highest concentration ($10 \mu M$). The relative constancy of the parameter $b_{\text{ns}}$ at all concentrations but the highest suggests that the $S_1$ nuclease assay results in 200 to 300 rads of assay damage. While the viscoelastic studies under nondenaturing conditions showed evidence of single-strand breaks at lower concentrations, this level of assay damage most probably prevented detection of such lesions. Single-strand breaks were observed in an earlier study at doses of $5 \mu M$ nitrogen mustard (13); lower doses could not be studied due to limited sensitivity. No evidence of single-strand breaks following treatment with nitrogen mustard was obtained in several alkaline elution studies, ranging in concentration from 0.1 to 1.4\% (10, 11). On a qualitative level, strand breaks have been observed in alkaline sucrose gradient sedimentation studies (32).

The quadratic dependence of the cross-linking frequency, $c$, on nitrogen mustard concentration seen in Chart 4 is somewhat unexpected. One possible explanation is that the effect of intermolecular DNA-DNA cross-links on the susceptibility to $S_1$ nuclease digestion becomes important only at the higher concentrations. Thus, the linear portion of the curve reflects the effects mainly of DNA interstrand cross-links while, at higher drug levels, both interstrand and intermolecular cross-links are manifested. The high recoil obtained in lysates of cells treated with the highest dose of nitrogen mustard and analyzed under nondenaturing conditions also suggests the possible presence of intermolecular cross-links.

The preceding discussion suggests that treatment of cells with nitrogen mustard results in DNA strand breaks, DNA interstrand cross-links, and possibly DNA-DNA intermolecular cross-links. To the extent that strand breaks were observed at pH 11.15 as well as at pH 12.15, there may be a significant contribution from true breaks as distinguished from alkali-labile breaks. Also, the viscoelastic method appears to be more sensitive than the $S_1$ nuclease assay for detection of single-strand breaks and cross-links.

**BCNU.** The viscoelastic behavior of cell lysates following treatment with BCNU was quite different from that observed following treatment with nitrogen mustard. Under denaturing conditions, both the retardation time and the recoil decreased with increasing exposure to $50 \mu M$ BCNU (Chart 5). This behavior is consistent with the production of single-strand breaks, leading to smaller and fewer single-strand molecules. Unlike nitrogen mustard, there was no evidence of DNA interstrand cross-links. As mentioned above, the viscoelastic recoil is dominated by the largest molecular species. Thus, the lack of evidence for cross-link formation cannot be due to a masking effect of single-strand breaks.

Under nondenaturing conditions, the viscoelastic retardation time decreased with increasing time of treatment, while the recoil remained relatively constant (Chart 5). Since strand breaks would lead to an increase in $r$, the observed behavior rules out this type of lesion. Thus, the decrease in retardation time and recoil obtained at higher pH values must be due to the presence of alkali-labile sites rather than true strand breaks. The decrease in $r$ under nondenaturing conditions is consistent, however, with the presence of DNA interstrand cross-links. A similar decrease in retardation time was obtained for cells treated with the combination of 8-methoxypsoralen and UV. Such cross-links would lead to a more compact form of DNA in lysates at pH 11.15. The lack of evidence for cross-links in viscoelastic experiments at denaturing conditions suggests that these cross-links are not stable at high pH.

The $S_1$ nuclease assay also failed to reveal interstrand cross-links arising from BCNU treatment under normal assay procedures, i.e., lysis conditions identical to those used in the high pH viscoelastic studies. Lysates of both treated and untreated cells yielded maximum amounts of single-stranded DNA (data not shown). When the detergent was eliminated from the lysis solution, the cells treated with BCNU exhibited a greater fraction of single-stranded DNA than controls, consistent with drug-induced strand breaks (see ordinate of Chart 6). However, it was not possible to quantitate the number of breaks since the untreated DNA no longer yielded a maximum level of single-strandedness. Such behavior is required, as can be seen by inspection of Equation B, which predicts unity for the single-stranded fraction of DNA in the absence of cross-links. Examination of Chart 6 reveals a value close to 0.58 in this case.

When the lysis pH was lowered to 11.45 and the detergent was maintained at 2%, lysates of both treated and untreated cells yielded equal fractions of single-stranded DNA. This result implies that the single-strand breaks observed at the $S_1$ nuclease studies at higher pH were generated from alkali-labile sites, consistent with the viscoelastic studies. However, when the detergent concentration was decreased to 0.4\% at pH 11.45, lysates of treated cells gave lower levels of single-stranded DNA than untreated cells. This dependence on detergent concentration is more characteristic of a noncovalent drug-induced association that hinders strand separation than of covalent cross-links.

Thus, under denaturing conditions, neither viscoelastic analysis nor $S_1$ nuclease experiments revealed conclusive evidence of DNA cross-links following BCNU treatment. Several workers have reported such cross-links in cell-free systems using mm concentrations of BCNU or other 1-(2-haloethyl)-1-nitrosoureas (17, 23, 24). Evidence for cross-links following treatment of cells comes from alkaline elution studies (10, 11). We note, however, that lysis was carried out with 0.2\% detergent, and the eluting buffer contained no detergent. Those studies detected cross-links at concentrations as low as 16\mu M while ours used 50\mu M. As the cell survival curve for 9L-cells treated with

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BCNU is similar to that reported for the L1210 cells used in the alkaline elution studies (5, 10), it would appear that the lack of evidence for cross-links in the viscoelastic studies was not due to insufficient drug concentration. Clearly, this point requires further investigation.

From the results on both S1 nuclease digestion and viscoelastic experiments, it is reasonable to conclude that BCNU induces alkali-labile sites in DNA rather than true strand breaks. Also, this drug appears to induce an association between DNA and perhaps some other material, such as protein, which is dispersed by detergent. Further experiments are in progress to confirm the interpretation of the decrease in γ in undenatured lysates as arising from DNA interstrand cross-links.

CONCLUSION

We have demonstrated the applicability of viscoelastometry to the analysis of DNA damage induced by several chemical agents. The viscoelastic behavior of lysates of cells treated with nitrogen mustard was shown to be consistent with and sensitive to the strand-breaking and cross-linking activity of this drug. In the case of BCNU, evidence for the induction of alkali-labile sites was obtained, along with evidence suggestive of DNA cross-links at nondenaturing conditions. Since the viscoelastic behavior of lysates is dominated by the largest DNA species, information pertaining to all of the DNA is not available. Thus, these studies were accompanied by S1 nuclease digestion experiments which permitted quantitation of the drug-induced DNA damage. Results from these 2 techniques were consistent and complementary.

The justification for developing a new method for assaying DNA damage lies in its potential advantages over currently available methods. There are several such advantages of the viscoelastic technique. (a) It is capable of sensitive detection of conformational changes in DNA tertiary structure, e.g., loss of supercoiling, as well as strand degradation and DNA interstrand cross-links. It may be possible also to detect DNA-DNA intermolecular cross-links. Interpretation of results is not subject to the difficulties encountered in sedimentation studies on large molecules. (b) DNA intermolecular cross-links can be discerned in the presence of strand breaks without the necessity of concomitant exposure to ionizing radiation. (c) Experiments can be carried out at pH values as low as 7 to 8 (29), thereby permitting separation of effects due to alkali-labile sites from true strand breaks. Such studies are currently under way in our laboratory. This may also permit the detection of double-strand breaks. On a practical level, untreated cells give well-characterized viscoelastic behavior, no internal standards are necessary, and results are reproducible from experiments performed on different batches of cells. It is not necessary to run a control lysate for each series of treated lysates. Also, no radioactive labels are required, yet experiments can still be done on small amounts of material, e.g., 2000 to 6000 cells or 10 to 30 ng DNA in nondenaturing lysates.

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