The cytotoxic effect of bifunctional alkylating agents has received considerable experimental attention during the last few years, and a variety of mechanisms of action have been postulated to account for the effect. Much of this investigation has centered on the interaction of these agents with deoxyribonucleic acid (DNA) (1, 3, 4, 9, 11, 13, 19—9292) following the initial suggestion by Elmore et al. (8) that sulfur mustard probably attacked the phosphate moieties of the DNA molecule. Rutman et al. (14) have suggested that the biological effectiveness of the mustards may not bear any quantitative relation to the extent of alkylation; rather, the specific type of reaction may be more important than the gross extent of the reaction. Drysdale et al. (6) and Trams et al. (19), however, have discussed the possibility that the attack on the DNA molecule may not represent the specific mechanism of action of these cytotoxic compounds and that other reactions may be of more importance. It must be conceded, therefore, that the specific chemical reaction with the DNA molecule or other cellular constituent that is responsible for the growth-inhibitory properties of nitrogen mustard still remains an enigma at the present time.

Previous studies in this laboratory on the effects of nitrogen mustard on mouse fibroblasts cultured in vitro (9) indicated that at concentrations of 0.1—0.92 μg/micell division ceased but accumulation of cellular protein and RNA continued. DNA replication also occurred, leading to cells containing what was apparently the premitotic amount of DNA, but further DNA synthesis did not occur. It seemed of considerable interest, therefore, to examine the properties of DNA isolated from normal cells, and from cells treated with this growth-inhibitory concentration of nitrogen mustard, in an effort to determine whether the physical or chemical properties of DNA from treated cells differed. In addition, some purified DNA was reacted in vitro with high concentrations of nitrogen mustard and the properties of the resulting material were examined. We shall refer to the three different DNA preparations we studied as “normal DNA,” “treated-cell DNA,” and “HN2-DNA.”

The following properties of DNA were examined: viscosity; hyperchromaticity on thermal denaturation; renaturation on slow cooling; base ratios; and capacity of the DNA preparations to serve as a substrate for pancreatic DNase, as
measured by changes in reduced viscosity and by liberation of cold acid-soluble P\(^{32}\) from P\(^{32}\)-labeled DNA preparations.

**MATERIALS AND METHODS**

Replicate cell inocula were prepared in Blake bottles containing 70 ml. of Eagle’s medium (7) plus 10 per cent bovine serum. The cells were incubated at 37° C. for a 5-day growth period. After 2 days of growth on normal medium HN\(^{2}\) was inoculated into appropriate bottles at a final concentration of 0.2 \(\mu\)g/ml, and the bottles were incubated for the remaining 3 days. Two bottles of untreated cells and two of cells receiving HN\(^{2}\) were utilized for duplicate analyses of cell count, protein, RNA, and DNA according to methods previously described (2).

The remaining bottles, approximately 28 in each group, were used for the isolation of the DNA. The supernatant medium was poured off, and the adherent cells were scraped into 10 ml. of a balanced salt solution. Pooled cells were collected in a refrigerated centrifuge at 1500 r.p.m. The cells were resuspended twice in cold, balanced salt solution and recentrifuged in order to rid the cells of contaminating medium. The final pellet was resuspended in 15 ml. of 2.0 \(\times\) sodium chloride, 0.015 \(\times\) sodium citrate solution.

The cells were lysed by adding 2.5 ml. 5 per cent sodium lauryl sulfate (Dupanol) and stirring occasionally for 3 hours at room temperature. DNA was precipitated on a stirring rod by pouring the cell lysate into twice the volume of cold 95 per cent ethyl alcohol. The DNA was resuspended in 5 ml. of 0.15 \(\times\) sodium chloride, 0.015 \(\times\) sodium citrate solution, and protein was removed by repeated extractions with equal volumes of 10 per cent octanol in chloroform (16) until no residual protein film remained. The DNA was again precipitated in ethanol as previously, resuspended in 5 ml. of the 0.15 \(\times\) sodium chloride-citrate solution, the pH adjusted to 7.0, and incubated with crystalline pancreatic RNase (Worthington, 50 \(\mu\)g/ml) for 2 hours at 37° C. This digest was subsequently treated with trypsin (Worthington 2X recrystallized trypsin, 50 \(\mu\)g/ml) for an additional 2 hours at 37° C. This solution was then dialyzed for 24 hours against either the 0.15 \(\times\) sodium chloride-citrate solution or glass-distilled water.

Normal DNA treated directly with HN\(^{2}\) was prepared by the addition of 50 \(\mu\)g. of HN\(^{2}\) to 100 \(\mu\)g. of DNA suspended in 1 ml. of water. The pH was maintained at 7.5 by titration with 0.01 \(\times\) NaOH. The solution was incubated with mild shaking at 37° C. overnight.

Viscosity determinations were made with a modified Ostwald capillary viscometer (water time = 24.1 sec.). This viscometer was immersed in a 37.00° C. water bath controlled to ± 0.01° C. Before analysis each sample was centrifuged for 10 minutes in the Serval at 15,000 r.p.m. to remove any particulate matter from the solution. Triplicate readings were performed in each sample with 0.1 sec. reproducibility.

Thermal denaturation (hyperchromic effect) of DNA was studied utilizing the temperature-regulated cell holder accessory in the Beckman DK-2 recording spectrophotometer and recording the optical density at 260 m\(\mu\). The solvent used was the 0.15 \(\times\) sodium chloride-citrate solution, and rates of temperature increase of 2° C/min and 5° C/min were investigated. A thermistor was incorporated into the plastic-capped cuvette to obtain exact determinations of the cuvette temperature at the various time intervals. Slow cooling of the DNA was accomplished by immersion of the cuvette into a 6-liter beaker of water heated to 95° C., and allowing the beaker to cool to 35° C. at room temperature. This required about 4 hours, at which time the optical density of the sample was again determined at 260 m\(\mu\).

The base ratios of DNA were determined by hydrolyzing a dried sample of DNA in 0.5 ml. of 95 per cent formic acid in a sealed tube at 175° C. for 2 hours, as described by Chargaff et al. (5). After being dried over solid KOH, the samples were taken up in a small volume of 1 \(\times\) HCl, and the bases were separated by descending paper chromatography in n-butanol, NH\(_4\)OH. The UV-absorbing spots were eluted in 0.01 \(\times\) HCl, and the ultraviolet spectrum was obtained in the recording spectrophotometer.

The kinetic digestion of DNA by pancreatic DNase was measured by changes in reduced viscosity, determined in the capillary viscometer. The reaction mixture (1.4 ml.) contained 1.5 \(\mu\)moles of Tris buffer, pH 7.5; 1.5 \(\mu\)moles MgCl\(_2\); 1.2 \(\mu\)g. DNA; and 0.02 \(\mu\)g. pancreatic DNase. The control in these experiments contained all the components of the reaction mixture except the DNase. The kinetic digestion of DNA was also measured by the liberation of acid-soluble P\(^{32}\) from P\(^{32}\)-labeled DNA. The DNA used in these experiments was obtained from cells allowed to grow for the 5-day period in the presence of 0.1 mc. phosphate-P\(^{32}\) (per bottle). Sixty \(\mu\)g. of the labeled DNA was incubated at 37° C. in a Dubnoff metabolic shaker; the reaction mixture (2.0 ml.) also contained 2 \(\mu\)moles of Tris buffer, pH 7.5; 2 \(\mu\)moles of MgCl\(_2\); and .02 \(\mu\)g. of pancreatic DNase. At various time intervals, 0.1 ml. of the incubation mixture was added to 0.2 ml. ice-cold salmon sperm
DNA (5 mg/ml), serving as a carrier, and the acid-insoluble DNA was precipitated by the addition of 0.5 ml. of ice-cold 5.5 per cent PCA. The solution was centrifuged for 5 minutes at 10,000 r.p.m., and an aliquot of the supernatant solution was counted in a Packard Tri-Carb liquid scintillation counter.

RESULTS

The 23 replicate bottles used in each group yielded approximately 2–3 mg of DNA. RNA and protein contamination of the purified, isolated DNA was less than 1 per cent. The per cent recovery of the initial DNA, as determined by chemical analysis, was in excess of 90 per cent in each experiment. The mustard-treated bottles had less than one-half the number of cells present in the control bottles, and the biological effectiveness of HN\textsubscript{2} on the mouse fibroblast cell was confirmed both by microscopic examination of the very large cells and by chemical determination of DNA, RNA, and protein. On a per cell basis, the mustard-treated cells uniformly had 1.6–1.8 times as much DNA as the untreated cells and 5–6 times as much RNA and protein as has been previously reported (2).

PHYSICAL PROPERTIES OF DNA

Viscosity.—The reduced viscosities for normal DNA, DNA obtained from treated cells, and the in vitro HN\textsubscript{2}-reacted DNA are summarized in Table 1. Each sample represents a separate preparation. DNA from treated cells had a consistent, slightly higher viscosity as compared with normal DNA. The sharply lowered reduced viscosity reported for the in vitro reacted DNA is not the result of the overnight incubation procedure used in treating the sample, since normal DNA subjected to the same procedure but without the addition of HN\textsubscript{2} showed no change in reduced viscosity.

Heat denaturation.—The melting temperature ($T_m$) and hyperchromicity of the different DNA's on heat denaturation are given in Chart 1 and Table 2. A number of experiments from different preparations have shown a range of from 32 to 38 per cent hyperchromicity on heating, with less than a 5 per cent difference between normal DNA and DNA isolated from nitrogen mustard-treated cells. Similar results were obtained in experiments with a 2° C/min or 5° C/min rate of increase in temperature. The in vitro HN\textsubscript{2}-reacted

![Chart 1: Melting curves of DNA.](chart1.png)

**TABLE 1**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\eta$ (Deciliter/gm)</th>
<th>Normal DNA</th>
<th>Treated-cell DNA</th>
<th>HN\textsubscript{2}-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41.8</td>
<td>44.8</td>
<td>28.7</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>45.5</td>
<td>49.1</td>
<td>26.6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>42.1</td>
<td>46.2</td>
<td>26.1</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2**

<table>
<thead>
<tr>
<th>Type of DNA</th>
<th>% Hyperchromicity at 95° C.</th>
<th>% Residual hyperchromicity after slow re-cooling</th>
<th>$T_m$ °C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal DNA</td>
<td>$36.3 \pm 0.2$</td>
<td>$16.1 \pm 0.15$</td>
<td>$85.2 \pm 0.2$</td>
</tr>
<tr>
<td>HN\textsubscript{2}-DNA</td>
<td>$34.7 \pm 0.47$</td>
<td>$16.9 \pm 0.15$</td>
<td>$86.4 \pm 0.3$</td>
</tr>
</tbody>
</table>

Values given are in each case the means and standard errors of five separately isolated samples. The “melting temperature,” $T_m$, was determined from “melting curves” as shown in Chart 1, and represents the temperature at which half of the eventual hyperchromic effect was observed.
DNA showed only 1–4 per cent hyperchromic effect on thermal denaturation (Chart 1). The renaturation of normal DNA and DNA from treated cells in all samples ranged between 15 and 19 per cent, with little or no differences between the two samples. In vitro HN2-reacted DNA showed little or no renaturation (hypochromaticity) on slow cooling (Table 2).

**TABLE 3**
**BASE RATIOS OF DNA FROM NORMAL CELLS, CELLS TREATED WITH HN2, AND DNA REACTED IN VITRO WITH HN2**

<table>
<thead>
<tr>
<th>Ratio</th>
<th>Normal DNA</th>
<th>Treated-cell DNA</th>
<th>HN2-DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/T</td>
<td>1.08</td>
<td>1.08</td>
<td>0.90</td>
</tr>
<tr>
<td>G/C</td>
<td>1.09</td>
<td>1.09</td>
<td>0.94</td>
</tr>
<tr>
<td>A+T/G+C</td>
<td>1.36</td>
<td>1.32</td>
<td>1.25</td>
</tr>
</tbody>
</table>

After hydrolysis of the nucleic acid in formic acid, bases were separated by descending paper chromatography in n-butanol (7 volumes), 1 N NH4OH (1 volume). The DNA reacted in vitro with nitrogen mustard was dialyzed against distilled water for 2 hours before acid hydrolysis in order to remove small molecular weight products of the reaction. Chromatography of the nondialyzable material, after strong acid hydrolysis, revealed the presence of a fifth UV-absorbing compound, moving between adenine and cytosine. Its ultraviolet spectra resembled that of adenine.

**DISCUSSION**

There is no doubt that nitrogen mustard, and other alkylating agents, can and do react with nucleic acids under both in vitro and in vivo conditions (1, 3, 11, 22). Since these reagents, however, also react vigorously with proteins, other macromolecules, and smaller molecules as well, it becomes difficult to ascribe their biological effectiveness in halting cellular division to any particular reaction. Holzer, for example, has presented evidence (15) that the alkylating agents owe their biological effectiveness to a reduction in intracellular diphosphopyridine nucleotide concentration, and Green and Bodansky (10) have recently demonstrated that Ehrlich ascites tumor cells from mice treated with nitrogen mustard have elevated diphosphopyridine nucleotidase activity, as well as...
depressed diphosphopyridine nucleotide concentrations.

If, in fact, nitrogen mustard exerts its effect by alkylation or cross-linking of DNA, it seems reasonable to suppose that some alteration of the physical or chemical properties of DNA isolated from cells treated with a low, but biologically effective, dose of the alkylating agent could be demonstrated. The concentration of HN₂ used here, 0.2 μg/ml (10⁻⁴ M), is in the range of concentration estimated to be present in the intact animal after a therapeutic dose (12). Its biological effectiveness in our system was confirmed by the presence of greatly enlarged, protein and RNA-rich cells. These cells had stopped dividing and contained an elevated (presumably premitotic) amount of DNA. Yet in almost every respect we studied, the DNA obtained from these cells was indistinguishable from DNA obtained from normal cells.

With respect to base ratios; Tₘ; hyperchromaticity on heating; renaturation on slow cooling; and capacity to serve as a substrate for pancreatic DNase, there was no substantial difference between the two samples of DNA (Tables 2, 3; Charts 1–3). The data shown in Table 1, however, do demonstrate a slightly greater viscosity of DNA obtained from mustard-treated cells than from normal cells. The difference is small and overlaps the variations seen in normal DNA samples obtained on different occasions, yet it has been a consistent finding. Its significance, if any, remains obscure. It is possible that DNA obtained from mustard-treated cells tends to be fully double-stranded, preparatory for a division cycle which cannot begin, and thus is in a somewhat different physical form from DNA obtained from a rapidly growing cell culture, the cells of which are in all stages of the mitotic cycle. On the other hand, it could also be interpreted as supporting a hypothesis of intermolecular cross-linking, leading to a gel-like DNA configuration (1).

We conclude from these data that treatment of cells growing in vitro with a biologically effective dose of nitrogen mustard does not produce any measurable change in these physical or chemical properties of DNA.

In marked contrast to the results discussed above, DNA which reacted in vitro with high concentrations of HN₂ shows many alterations in both chemical and physical properties, as many previous investigations have already noted (11). There is a profound reduction in viscosity (Table 1), possibly due to the alkylation of phosphate groups leading to main chain scission (1, 17). Reactions with purines (especially adenine) would also seem to have occurred in the experiments reported here, since after dialysis of the reaction mixture (which would remove small molecular weight products of the reaction) base ratio analysis showed a loss of about 10 per cent of the adenine and 6 per cent of the guanine (Table 3). The one reaction product isolated by the alkaline paper chromatographic separation used in these studies had the typical ultraviolet spectrum of adenine, but it is possible that other reaction products were also present and traveled with the unreacted bases.

In vitro HN₂-reacted DNA still served as a reasonably good substrate for pancreatic DNase (Charts 2, 3), although there are very definite differences in the rate of liberation of acid-soluble P₃₂ from HN₂-DNA as compared with the other two DNA preparations (Chart 3). Tamm et al. (18) have shown that various degraded or depolymerized DNA preparations of different physical properties all served as substrates for DNase; however, the loss of purines resulted in diminished activity as a substrate. In our experiments, depurination was not extensive (data of Table 3 indicate a loss of less than 20 per cent of the purines), and thus our data are harmonious with these studies.

It may of course be argued that HN₂ has reacted to a small, but biologically significant, extent with the DNA of our cultured cells, and our methods of preparation and examination of the DNA are not sufficiently fine to detect biologically important differences in the DNA molecule. In this connection, it would be of considerable interest to study the capacity of the three types of DNA preparations discussed here to serve as priming material for the DNA polymerase system, and it is hoped that such studies will be undertaken shortly. For the present, however, we must conclude that biologically effective doses of nitrogen mustard do not lead to any measurable changes in several physical and chemical properties of purified DNA preparations obtained from mouse cells growing in vitro. The data, therefore, lend no support to any hypothesis suggesting that alkylation or cross-linking of DNA is responsible for the growth-inhibitory properties of nitrogen mustard.

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Effects of Nitrogen Mustard on the Physicochemical Properties of Mouse Fibroblast Deoxyribonucleic Acid

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