Numerous investigations have yielded results that indicate that cellular deoxyribonucleic acid (DNA) might be the primary and perhaps the critical site of alkylation by cytotoxic alkylating agents (21). The biologic systems used in most of these investigations, however, have not permitted a direct correlation between alkylation of DNA and the observed biologic effects; that is, there is a lack of evidence whether alkylation of DNA is essential to the biologic effect. The availability of an alkylating agent sensitive tumor and a subline of the tumor that is resistant to the respective agent offers the opportunity for attempting to correlate biochemical and biologic events. Previous studies in this laboratory (23, 24) have already demonstrated the utility of the biologic system consisting of cyclophosphamide-sensitive (5) and cyclophosphamide-resistant (18) plasmacytomas growing bilaterally in hamsters. It has been found that following the administration of alkylating agents labeled with C14 to hamsters bearing bilaterally-growing sensitive and resistant tumors, approximately equal quantities of C14 were present in the corresponding subcellular fractions and the DNA's of the 2 tumors (23). Equal extents of fixation of C14 in the DNA fractions were observed even when enough radioactive agent was administered to the animal to cause regression of the sensitive tumor while the resistant tumor continued to grow. The question remained unanswered, however, whether the C14 was fixed in the same way in the 2 tumors—one might visualize quantitatively similar but qualitatively different fixation processes. The availability of the biologic system consisting of cyclophosphamide-sensitive and cyclophosphamide-resistant plasmacytomas growing bilaterally in hamsters offers the opportunity for attempting to correlate biochemical and biologic events. Previous studies in this laboratory (23, 24) have already demonstrated the utility of the biologic system consisting of cyclophosphamide-sensitive (5) and cyclophosphamide-resistant (18) plasmacytomas growing bilaterally in hamsters. It has been found that following the administration of alkylating agents labeled with C14 to hamsters bearing bilaterally-growing sensitive and resistant tumors, approximately equal quantities of C14 were present in the corresponding subcellular fractions and the DNA's of the 2 tumors (23). Equal extents of fixation of C14 in the DNA fractions were observed even when enough radioactive agent was administered to the animal to cause regression of the sensitive tumor while the resistant tumor continued to grow. The question remained unanswered, however, whether the C14 was fixed in the same way in the 2 tumors—one might visualize quantitatively similar but qualitatively different fixation processes.
tively different fixations of alkylating agents in the 2
tumors with consequent differences in biologic effects, as
suggested by Rutman et al. (17). The present study was
undertaken because of the possibility that qualitative
differences in fixation or alkylation might be detected by
determining the thermal properties (15) of DNA's of
sensitive tumors and resistant tumors after exposure in
vivo or in vitro to alkylating agents. Incidental to com-
paring the effects of the agents upon the thermal properties
of DNA's of the tumors, it was also possible to compare the
thermal properties of the DNA's of the tumors from un-
treated animals, and the results of such a comparison have
a direct bearing upon certain possible mechanisms of resis-
tance to alkylating agents.

MATERIALS AND METHODS

Procedures used in obtaining tumors.—Cyclophospha-
mine-sensitive (5) and cyclophosphamide-resistant plas-
macytomas (18) were implanted bilaterally into the axil-
lary regions of golden Syrian hamsters. Animals that
were treated in vivo received daily I.P. injections of cyclo-
phosphamide at a dose level of 10 mg/kg on days 13
through 18 after implantation of the tumors (by day 13 all
of the tumors were of multigram size), and the animals
were killed by asphyxiation with carbon dioxide on day 19.
The control animals of the above group received I.P.
injections of saline instead of the cyclophosphamide.
During the course of treatment, the sizes of the tumors
were determined by daily measurements with calipers.
Animals that received no in vivo treatment were killed on
day 18 or 19. The tumors were quickly removed from the
animals and placed in ice-cold beakers, where they re-
mained until used for isolation of DNA.

Isolation of DNA.—The tumors were minced finely
free-hand with knives, and the minced tissue was forced
through a tissue press. The extruded tissue was ground in
a motor-driven homogenizer having a glass tube and a
Teflon pestle, with a measured volume of 0.25 M sucrose
solution buffered with 0.02 M Tris to pH 8.0, and containing
0.018 M calcium chloride. After grinding, more sucrose
solution was added to the homogenate to give an extruded
tissue:sucrose solution ratio (w/v) of 1:9. The resulting
sample was centrifuged in a refrigerated centrifuge for
10 min. at 544 X g, and the supernatant was discarded.
The sedimented material was either taken up directly or
washed once with the sucrose solution with subsequent
sedimentation at 544 X g and then taken up in 6% sodium
p-amino-salicylate solution and used for the isolation of
fibrous DNA by an adaptation of the procedure of Kirby
(11) including digestion with ribonuclease. In some ex-
periments the sedimented material was used for the isola-
tion of DNA by the procedure of Marmur (13) with minor
modifications to adapt the procedure for mammalian
cells.

Incubation of DNA with nitrogen mustard (HN2).—The
DNA, either commercially available salmon sperm DNA
or DNA isolated from sensitive and resistant plasmacyto-
mas grown bilaterally in untreated hamsters, was dis-
solved in a solution of 0.01 M sodium chloride and 0.001 M
sodium citrate (pH 7.0 ± 0.2) to yield a solution containing
20 μg of DNA per ml. Nitrogen mustard hydrochloride
[methyl bis(2-chloroethyl)amine hydrochloride] (HN2) was
dissolved in demineralized distilled water, and appropriate
volumes of the resulting solution were added immediately
to portions of the DNA solution to yield the desired final
concentration of HN2—in all instances the volume of the
HN2 solution that was added was small enough that the
concentration of the DNA solution was reduced only very
slightly, and equal volumes of demineralized distilled water
were added to the control portions of DNA solution.
After the control solutions and the solutions containing
HN2 were incubated overnight at 37°C, they were used for
the determination of thermal properties as described below.

In 1 experiment, which was performed to determine
whether alkylated DNA having altered thermal properties
would be precipitable in the Kirby procedure (11) for
isolating DNA, commercial calf thymus DNA was dis-
solved in water at a concentration of 4 mg/ml, and 100 mg
of sodium acetate trihydrate were added per milliliter of
solution. To 6-ml portions of this solution (pH 7.0) was
added a solution of nitrogen mustard hydrochloride and/or
water to give a total volume per sample of 7 ml. In this
way samples containing HN2 at concentrations of 0, 10⁻⁴,
and 10⁻⁵ M were prepared. After these solutions had incu-
bated for 16 hr. at 37°C, they were dialyzed against cold
sodium acetate solution (100 mg of the trihydrate per ml)
overnight with 1 change of solution. Addition of 1 volume
of ethyl Cellosolve caused precipitation of the DNA from the
0 and 10⁻⁴ M HN2 samples in a form that permitted wind-
ing the DNA on a glass stirring rod, but the precipitate
that formed in the 10⁻⁵ M sample was finely particulate and
clung to the wall of the tube upon centrifugation. The
precipitates on the rods or upon the wall of the tube were
washed twice with 75% ethanol and once with absolute
ethanol, then dried over calcium chloride in a vacuum
desiccator. These samples of DNA were later used for
determination of thermal properties.

Apparatus and procedure for determining thermal prop-
erties of the DNA.—Cuvettes (1 cm X 1 cm) containing
samples of the incubated solutions described above or solu-
tions of DNA's isolated from plasmacytomas, or precipi-
tated after in vitro treatment, were placed in the cell com-
partment of a Beckman DU spectrophotometer equipped
with thermostaters with antifreeze solution (ethylene
glycol base) flowing through the inner pair of spacers and
with tap water flowing through the outer pair of spacers.
The temperature of the circulating antifreeze solution was
controlled by the activation of a heating coil, submerged in
an external reservoir, by means of a Bronwill thermoster-
gulator suspended in the solution in the reservoir. A small
hole was drilled in the cover of the cell compartment to
admit the thermoster probe (YSI, No. 401) for a YSI
model 43 Tele-thermometer, and the probe was immersed
in an aqueous solution contained in a cuvette in the No. 4
position of the sample holder. The cell compartment, the
external reservoir, and the tubing for transmitting the
antifreeze solution were insulated to minimize heat loss.

The thermoster was set approximately at the de-
sired temperature, and after the bath had reached this
temperature a period of 10–15 minutes was allowed for
equilibration between the temperature of the thermoster
and the samples. The actual temperature of a solution in

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the cell compartment was read on the Tele-thermometer, and the temperature and absorbance at 260 m\(\mu\) of the respective samples were recorded. Temperatures were estimated to tenths of a degree, and the mean values were rounded off to the nearest degree.

For the determination of the degree of reversibility of thermal denaturation of a sample of DNA, the procedure was similar to that described above except that, following the determination of the absorbance at a specific temperature, the sample was removed from the cell compartment, immersed in ice water for a period of about 10 min. and returned to the cell compartment where it was equilibrated by means of the thermospacers to a temperature of 20—25°C., whereupon the absorbance was determined at this lower temperature. The temperature of the sample was then raised to another specific temperature, slightly higher than previously, and the procedure of reading absorbance, quenching in ice water, equilibrating to 25°C., and redetermining absorbance was repeated. By repetition of this cycle of manipulations, it was possible to obtain data demonstrating the degrees of denaturation at the various temperatures and the extents of reversibility of this denaturation upon rapid cooling.

RESULTS

Effects of exposure to HN2 in vitro.—Table 1 contains the data obtained in the experiments where commercial DNA and DNA's from plasmacytomas grown in untreated hamsters were treated in vitro with HN2. The first property listed in the table is the \(T_m,\alpha\), which is the midpoint of the absorbance-temperature transition profile (15). It is known that the temperature at which the transition occurs is dependent upon the ionic strength of the solution (2, 3, 15), and solutions of several different ionic strengths were examined in the initial experiments of this investigation to select a suitable concentration. Solutions in which the transition midpoint was above 80°C. were not considered to be satisfactory, because of heat losses from the apparatus with consequent long periods of equilibration at the higher temperatures, loss of solvent from the samples by evaporation during equilibration, and creeping of the solution out of the cuvettes during equilibration. A solution that was 0.01 M with respect to sodium chloride and 0.001 M with respect to sodium citrate was selected because in its transition of the untreated DNA's occurred in the temperature range of 60—80°C., temperatures in this range being easily attained with the apparatus while still being sufficiently above room temperature to permit significant measurement of decreases of transition temperatures resulting from treatment of the DNA. In some experiments in which the degree of reversibility of denaturation was determined, the solvent consisted of 0.001 M sodium chloride, 0.0001 M sodium citrate, and 7.2 M sodium perchlorate (6). The values for \(T_m,\alpha\) for commercial salmon sperm DNA (DNA\(_c\)), DNA isolated from sensitive plasmacytoma (DNA\(_s\)), and DNA isolated from resistant plasmacytoma (DNA\(_r\)) were approximately the same. All 3 of the DNA's were affected similarly by treatment with HN2 in vitro; treatment with \(10^{-4}\) M HN2 or \(10^{-5}\) M HN2 caused significant decrease in \(T_m,\alpha\), but \(10^{-6}\) M HN2 did not lower \(T_m,\alpha\).

<table>
<thead>
<tr>
<th>Propertya</th>
<th>Material</th>
<th>Experiment No.</th>
<th>Concentration of HN2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T_m,\alpha), °C.</td>
<td>DNA(_c)</td>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>DNA(_s)</td>
<td>4</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>DNA(_r)</td>
<td>6</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>DNA(_c)</td>
<td>4</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>DNA(_s)</td>
<td>6</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>DNA(_r)</td>
<td>9</td>
<td>66</td>
</tr>
<tr>
<td>(\delta), °C.</td>
<td>DNA(_c)</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DNA(_s)</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>DNA(_r)</td>
<td>9</td>
<td>4</td>
</tr>
<tr>
<td>(\Delta A_{\text{max}}), %</td>
<td>DNA(_c)</td>
<td>5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>DNA(_s)</td>
<td>6</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>DNA(_r)</td>
<td>9</td>
<td>32</td>
</tr>
<tr>
<td>(T_m,\beta), °C.</td>
<td>DNA(_s)</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>DNA(_r)</td>
<td>9</td>
<td>34</td>
</tr>
<tr>
<td>(\Delta T), °C.</td>
<td>DNA(_s)</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>DNA(_r)</td>
<td>9</td>
<td>71</td>
</tr>
</tbody>
</table>

* See the text for definitions and explanations of the various properties. The solvent consisted of 0.01 M sodium chloride—0.001 M sodium citrate.

† DNA\(_c\), commercially available salmon sperm DNA; DNA\(_s\), DNA isolated from cyclophosphamide-sensitive plasmacytoma; DNA\(_r\), DNA isolated from cyclophosphamide-resistant plasmacytoma.

The magnitude of the temperature range over which the transition occurs is indicative of the degree of heterogeneity of the molecular population of the sample, and for purposes of comparing samples of DNA the transition half-width, \(\delta\) (7), is useful. The transition half-width is obtained from the same data that is used in determining \(T_m,\alpha\), and is the temperature range over which the middle 50% of the transition occurs, that is, the range between the temperature at which 25% of the transition has occurred and the temperature at which 75% of the transition has occurred. The data of Table 1 show that treatment of all 3 DNA's with \(10^{-4}\) M HN2 consistently caused small increases in the values of \(\delta\), while the effects of \(10^{-5}\) M HN2 were inconsistent, and treatment with \(10^{-6}\) M HN2 did not alter \(\delta\).

Hyperchromicity is another useful property for comparing the extents of alteration of structure upon thermal denaturation of DNA's. The extent of hyperchromicity is indicated by the value of \(\Delta A_{\text{max}}\), which is the change in absorbance upon complete denaturation, and this change is conveniently expressed as a percentage of the initial absorbance of the sample. The data of Table 1 show that treatment with \(10^{-4}\) M HN2 resulted in a decrease of the percentage of hyperchromicity, and that \(10^{-6}\) M HN2...
caused decreases in some experiments, while 10⁻⁴ M HN2 had no effect. Similar results were obtained for the 3 types of DNA. The decrease is chiefly due to a higher initial absorbance of the sample rather than to a lower maximum absorbance corresponding to maximum denaturation. This higher initial absorbance would mathematically magnify the decrease in percentage of hyperchromicity, because it would not only decrease the value of ΔAₘₐₓ but also raise the base for the calculation of the percentage.

The quantity Tₘ₂₅ is the midpoint of the ultraviolet absorbance-temperature profile measured at 25°C, following quenching as described above (6, 7). Treatment with 10⁻⁴ M HN2 and 10⁻⁵ M HN2 caused decreases in the Tₘ₂₅ values of the DNA's from both the sensitive and resistant tumors, and the amounts of the decreases were similar for the 2 tumors.

The difference between Tₘ₂₅ and Tₘₐₓ is designated as ΔT, and the magnitude of this quantity indicates the degree of reversibility of the denaturation—the greater the value of ΔT, the greater the reversibility. The data of Table 1 indicate that treatment with 10⁻⁴ M HN2 caused a slight increase in reversibility but treatment with 10⁻⁵ M HN2 did not, and that the DNA's from sensitive and from resistant tumors were affected similarly.

In the experiments of Table 1 the thermal properties were determined on the complete incubation mixture without intermediate isolation of the treated DNA. This left open the question whether the treated DNA that had the altered thermal properties would be isolable by the usual methods of isolating DNA from tissues. To determine if the altered DNA was precipitable by the addition of an equal volume of ethyl Cellosolve, as in the Kirby procedure (11), the experiment described in the 2d paragraph of the above section “Incubation of DNA with Nitrogen Mustard” was performed; the thermal properties of the isolated DNA's are given in Table 2. The DNA that was treated with 10⁻⁴ M HN2, and which would not wind on a rod when precipitated, was completely denatured at room temperature, and there was no evidence of transition at elevated temperatures. The DNA that was treated with 10⁻⁵ M HN2, and which wound around a rod, did have altered thermal properties.

**Effects of in vivo treatment with cyclophosphamide.**—Chart 1 shows the effects of daily treatments with cyclophosphamide upon the size of plasmacytomas. S-C, sensitive tumors from control animals; S-Tr, sensitive tumors from treated animals; R-C, resistant tumors from control animals; R-Tr, resistant tumors from treated animals; Days, days following initiation of daily cyclophosphamide treatment (10 mg/kg) of animals bearing established (13-day-old)tumors.

The Tₘ₂₅ (NaClO₄) values for the sensitive control in Experiment 10 and for the resistant control in Experiment 11. Since the samples used for Experiments 8, 10, and 11 were taken from similar lots of DNA isolated from the same pooled tumors, the preponderance of evidence indicating similarity of values for Tₘ₂₅ points to the likelihood that the 2 values referred to above are indeed anomalous. These anomalous values of Tₘ₂₅ are reflected in the values of ΔT(NaClO₄). With the exception of these 2 values, the similarity of the values for the various samples is impressive, although this similarity is axiomatic since the ΔT's were derived from similar quantities.
The in vitro treatments of DNAc, DNAs, and DNAR with different concentrations of HN2 were undertaken to determine if treatment with this alkylating agent could alter the thermal properties of the DNA's and to ascertain approximately what concentration of the agent would be required to cause a detectable alteration. The data of Table 1 and Table 2 show that treatment with $10^{-4}$ M HN2 consistently caused alterations of the thermal properties of each of the DNA's, while the results with $10^{-5}$ M HN2 were somewhat less consistent and no alteration was caused by $10^{-6}$ M HN2. It has been estimated that the approximate concentration in an experimental animal following the injection of a median lethal dose of HN2 is $10^{-4}$ M (16); therefore, the corresponding concentration in vitro altered some of the thermal properties of the DNA but not others. The decreased values of $T_{m,a}$ for the DNA's treated with $10^{-4}$ M and $10^{-5}$ M HN2 indicate that alkylation caused a decreased stability of the helix, since this property is a measure of the disruption of the helical structure without necessarily involving the separation of the polynucleotide strands (15). The slight increases in transition half-widths (7) also suggest a small increase in the heterogeneity of the molecular population as a result of treatment. Although the decreased hyperchromicity of the treated solutions might indicate that there is less separation of the strands in the DNA's exposed to HN2, this interpretation is not necessarily correct, because, as was pointed out above, the decreased percentage of hyperchromicity of these samples is largely due to the higher initial absorbance and not to a decrease in the maximum absorbance of the denatured sample. On the other hand, this higher initial absorbance would indicate that treatment with HN2 has caused some disorganization of the structure that is detectable even at room temperature. A similar effect was observed by Brewer and Aronow (1) upon treating $100 \mu g$ of DNA with $50 \mu g$ of HN2 in 1 ml of water (this solution is approximately $2.6 \times 10^{-4}$ M with respect to HN2).

Although disruption of the residual helical structure of the treated DNA occurred at a lower temperature than for the untreated DNA (as evidenced by the values for $T_{m,a}$), there was a greater degree of recovery of helical structure upon quenching by the DNA treated with $10^{-4}$ M HN2 than by the untreated DNA (as evidenced by the values of $\Delta T$). This increased reversibility might be due to cross-linking of the strands of DNA (15). Geiduschek (6, 7) found that treatment of DNA with approximately $3 \times 10^{-4}$ M HN2 caused a large increase in reversibility of denaturation of the DNA, and he ascribed this effect to cross-linking. Jones et al. (10) found that alkylation of DNA by HN2 resulted in increased reversibility of thermal denaturation, and the extent of reversibility decreased as the extent of alkylation of the DNA decreased. Kohn et al. (12) also found that treatment of DNA with HN2 caused a great increase in reversibility of denaturation of the DNA, and the extent of reversibility decreased as the extent of alkylation of the DNA decreased.

The experimental results therefore indicate that treatment of DNA with $10^{-4}$ M HN2 and $10^{-5}$ M HN2 can alter the thermal properties of the DNA to detectable extents and that the alterations of the properties of DNAc and DNAs are quantitatively similar.

In contrast to the detected effects of in vitro treatment of DNA with HN2, the properties of the DNA's isolated from sensitive and resistant plasmacytomas growing in hamsters that received sufficient cyclophosphamide to cause regression of the sensitive tumor while the resistant tumor continued to grow were similar to the properties of DNA's isolated from sensitive and resistant plasmacytomas growing in untreated hamsters (Table 3). Cyclophosphamide could not be used for the in vitro experiments, resulting in an increase of thermal denaturation, and the extent of reversibility decreased as the extent of alkylation of the DNA decreased.

### Table 3

<table>
<thead>
<tr>
<th>Propertya</th>
<th>Exponent of Method of Isolation</th>
<th>Sensitive</th>
<th>Resistant</th>
</tr>
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<tbody>
<tr>
<td>$T_{m,a}(NaCl)$, °C.</td>
<td>0.6</td>
<td>66</td>
<td>67</td>
</tr>
<tr>
<td>$T_{m,a}(NaClO_4)$, °C.</td>
<td>0.6</td>
<td>45</td>
<td>44</td>
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<tr>
<td>$\delta(NaCl)$, °C.</td>
<td>0.6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>$\delta(NaClO_4)$, °C.</td>
<td>0.6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>$\Delta T(NaCl)$, °C.</td>
<td>0.6</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>$\Delta T(NaClO_4)$, °C.</td>
<td>0.6</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>

a See text for definitions and explanations of the various properties.

b The animals received 5 daily I.P. injections of cyclophosphamide at a dosage level of 10 mg/kg beginning on the 13th day after implantation of the tumor.

(4) (NaCl) indicates that the DNA was dissolved in 0.01 M sodium chloride-0.001 M sodium citrate; (NaClO_4) indicates that the DNA was dissolved in 0.001 M sodium chloride-0.0001 M sodium citrate-7.2 M sodium perchlorate.

K, Kirby procedure used for isolation of DNA; M, Marmur procedure used for isolation of DNA.

### DISCUSSION

The in vitro treatments of DNAc, DNAs, and DNAR with different concentrations of HN2 were undertaken to determine if treatment with this alkylating agent could alter the thermal properties of the DNA's and to ascertain approximately what concentration of the agent would be required to cause a detectable alteration. The data of Table 1 and Table 2 show that treatment with $10^{-4}$ M HN2 consistently caused alterations of the thermal properties of each of the DNA's, while the results with $10^{-5}$ M HN2 were somewhat less consistent and no alteration was caused by $10^{-6}$ M HN2. It has been estimated that the approximate concentration in an experimental animal following the injection of a median lethal dose of HN2 is $10^{-4}$ M (16); therefore, the corresponding concentration in vitro altered some of the thermal properties of the DNA but not others. The decreased values of $T_{m,a}$ for the DNA's treated with $10^{-4}$ M and $10^{-5}$ M HN2 indicate that alkylation caused a decreased stability of the helix, since this property is a measure of the disruption of the helical structure without necessarily involving the separation of the polynucleotide strands (15). The slight increases in transition half-widths (7) also suggest a small increase in the heterogeneity of the molecular population as a result of treatment. Although the decreased hyperchromicity of the treated solutions might indicate that there is less separation of the strands in the DNA's exposed to HN2, this interpretation is not necessarily correct, because, as was pointed out above, the decreased percentage of hyperchromicity of these samples is largely due to the higher initial absorbance and not to a decrease in the maximum absorbance of the denatured sample. On the other hand, this higher initial absorbance would indicate that treatment with HN2 has caused some disorganization of the structure that is detectable even at room temperature. A similar effect was observed by Brewer and Aronow (1) upon treating $100 \mu g$ of DNA with $50 \mu g$ of HN2 in 1 ml of water (this solution is approximately $2.6 \times 10^{-4}$ M with respect to HN2).

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<tr>
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<td>7</td>
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</tr>
<tr>
<td>$\Delta T(NaCl)$, °C.</td>
<td>0.6</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>$\Delta T(NaClO_4)$, °C.</td>
<td>0.6</td>
<td>30</td>
<td>30</td>
</tr>
</tbody>
</table>
because it is not activated in such systems (4). However, the similarity of the observed in vivo effects of HN2 and of cyclophosphamide upon the growth (18, 24) and metabolism (24) of the tumors makes the present comparison of in vitro data for HN2 and in vivo data for cyclophosphamide appear plausible. The experimental data reported here do not indicate that any alkylation or cross-linking of DNA occurred in vivo in either the sensitive or resistant tumor. In addition, the similarity of the values of Tm, a for the DNA's from control and treated animals indicate that the treatment did not alter the base ratios of the DNA's (14). These conclusions are similar to those of Brewer and Aronow (1) who found that treatment of mouse fibroblasts growing in vitro with concentrations of HN2 sufficient to inhibit multiplication of cells (10⁻⁴ M) did not alter the base ratios, the hyperchromicity upon thermal denaturation, the renaturation upon slow cooling, or the capacity to serve as substrate for pancreatic deoxyribonuclease. They are also consistent with those of Golder et al. (8), who observed that the melting behavior, optical characteristics, viscosity, and sedimentation characteristics of DNA isolated from Lettré-Ehrlich ascites cells following in vivo treatment of the host with HN2 were not significantly different from those of DNA isolated from ascites cells of untreated animals. The latter investigators (8) concluded that their data eliminated intermolecular cross-linking or chain scission, exhaustive degradylation, or changes in base composition as major chemical results of alkylation in vivo.

The possibility that fractionation of the DNA's occurred during isolation with consequent loss of alkylated material is recognized, but since similar results were obtained in experiments in which 2 methods of isolation were used (Table 3) and since it was demonstrated that DNA treated in vitro with 10⁻⁴ M HN2 could be precipitated with retention of altered thermal properties (Table 2), this possibility is somewhat countered.

It is recognized that in the regressing tumors some necrosis had occurred and that the discarded necrotic tissue no doubt represented cells that were adversely affected by the cyclophosphamide. Therefore, one might pose the question whether the dead cells thus discarded, and those previously lysed during the regression, contained alkylated DNA that was not isolated in these experiments, while the remaining cells that were used for the isolation of the DNA were not affected by the cyclophosphamide. This would be equivalent to a fractionation of the cells with elimination of those cells in which alkylation of the DNA occurred; hence no alkylated DNA would be isolated. Although such a fractionation would account for the results described, it is felt that such an explanation is not applicable here for several reasons. The appearance of the tissue that was used in the isolation of DNA from the sensitive tumors of the treated animals was intermediate between that of necrotic tissue and tissue of tumors from untreated animals; this tissue, though not dead, was evidently affected by the treatment. Upon the basis of the results of a number of other experiments it would be predicted that, if the animals had not been killed, the sensitive tumors of the treated animals would have continued to regress, that is, the S-Tr line of Chart 1 would continue with a negative slope. This regression would no doubt result from the sequential death and lysis of the cells present on day 4. It has also been observed that regression of sensitive plasmacytomas can be effected by single doses of cyclophosphamide; however, the regression is not instantaneous but is progressive over a period of several days. It seems reasonable to assume that even the last cells to die were affected by the drug and that death resulted from these effects. By analogy, therefore, it also seems reasonable to assume that the tumor tissue used for the isolation of DNA in the present experiments was also progressively deteriorating as a result of the treatment and could be expected to contain alkylated materials.

It is possible that enough alkylation of DNA could occur to cause a biologic or biochemical effect without there being enough alteration of the thermal properties of the DNA to permit detection. It has been shown, however, that DNA isolated from hamster plasmacytomas regressing after treatment with cyclophosphamide served as well as or better than DNA isolated from plasmacytomas of untreated animals as primer for the in vitro DNA polymerase system (A. J. Tomisek, M. A. Bruce, and P. W. Allan, manuscript in preparation). On the other hand, Goldstein and Rutman (9) observed that DNA alkylated in vitro served as primer as well as or better than untreated DNA but that DNA isolated from in vivo treated Ehrlich ascites cells had less primer activity than DNA isolated from untreated cells. They suggested that this reduced activity might be the result of some type of association of in vivo alkylated DNA with protein. This group of investigators had previously shown that following the administration of an LD₅₀ dose of HN2 to mice bearing Ehrlich ascites cells the isolated DNA had a significant quantity of protein associated with it, but that this associated protein did not significantly alter the observed thermal and physical properties of the DNA (8). No determination of protein associated with the isolated DNA was made in the present investigation. The disparity between the results obtained by Tomisek and Allan (manuscript in preparation) and by Goldstein and Rutman (9) with regard to the primer activity of DNA's isolated from tumor cells of treated animals might be due to difference in dosage of agents and to difference in the loci of the tumor cells in the animals.

The results reported here do not provide any support to the possibility that the observed biologic effects of cyclophosphamide are due to alkylation of DNA. This is of interest, since other investigators (19, 20) have obtained other types of data that led them to suggest that direct alkylation of DNA might not be the cause of the observed biologic effects. However, as was suggested in the preceding paragraph, it is possible that a minute extent of alkylation of DNA could cause profound biochemical and biologic effects and that this minute extent of alkylation could not be detected by physical and chemical methods that have been used to date.

All of the data obtained in this study indicate that the DNA's isolated from the sensitive and from the resistant plasmacytomas are structurally and compositionally similar. These facts stand in opposition to certain possible mechanisms of resistance to cyclophosphamide and other
alkylating agents (22)—specifically, those mechanisms dependent upon decreased availability of critical targets, such as decreased concentration of target groups on DNA, chemical masking of target sites on DNA, and physical masking of target sites on DNA.

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REFERENCES

Studies with Mustards: VII. Effects of Alkylating Agents in Vitro and in Vivo upon the Thermal Properties of Deoxyribonucleic Acids from Sensitive and Resistant Plasmacytomases

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