On the Mechanism of Action of the Alkylating Agents

I. Interaction of Alkylating Agents with Nucleic Acids*

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SUMMARY

The incorporation of isotopic ethylene-C14-imino-s-triazine (TEM), Myleran, chlorambucil, and sulfur mustard into the DNA fraction of regenerating liver and of various mouse ascites lymphomas was measured. The in vivo incorporation of these alkylating agents was estimated to be of the order of 1 mole of drug per mole of DNA polymer. Results obtained by several different methods of isolation of DNA from in vivo-treated tissues failed to show marked differences in the amounts of isotope coupled to DNA. No correlation was apparent between the susceptibility of the various tumors and the relative amounts of isotope incorporated into tumor DNA fractions. Pretreatment of tumor with nonlabeled triethylene melamine (TEM) failed to alter incorporation patterns for labeled TEM. It was concluded that attack on the DNA itself does not necessarily represent the mechanism of action by which the alkylating agents exert their cytostatic or cytotoxic effects.

Widespread clinical use of the alkylating agents initiated numerous attempts to elucidate the probable mechanism by which they effect cytostasis. Most of the data which led to postulated modes of action have been reviewed recently (11). Investigations revealed that compounds such as the nitrogen mustards, the ethyleneimines and phosphoramides, the alkylepoxides, and some sulfonic acid esters have in common the remarkable capacity to alkylate at physiological pH values, and the term "cytotoxic alkylating agents" has become widely accepted for this group. Since chromosomal aberrations in dividing cells were an outstanding feature of mustard intoxication, most of the mechanism of action hypotheses postulated that the target site in the cell was the genetic material—i.e., the nucleic acids. The following reactions have been suggested for polynucleotide-mustard alkylation reactions with these compounds:

(a) attack on the 3', 5' linkage of DNA or RNA with disruption of the polymer; (b) alkylation of two adjacent phosphoryl hydroxyls within the polymer (intramolecular cross-linking); (c) alkylation of phosphoryl hydroxyls in adjacent chains (intermolecular cross-linking); (d) alkylation of purine or pyrimidine bases with production and incorporation of "base analogs" into the polymer; (e) alkylation of guanine at the 7-position with subsequent opening of the ring, loss of the base, and formation of an a-purinic acid-like structure; (f) formation of cyclic esters on the phosphate by dialkylation. Other possibilities were considered, such as the alkylation of nucleotide-containing coenzymes which would act as metabolic analogs. Lastly, it has been suggested that targets other than nucleic acids are attacked and responsible for the biological effects produced.

In the evaluation of these suggestions, some of the following questions appeared to be pertinent, especially with respect to the more widely accepted cross-linking hypothesis: (a) Was the suggested mechanism compatible with in vivo observations? (b) Was it compatible with the production of specific biochemical lesions induced by these agents? (c) Was it compatible with phenomena of drug susceptibility and the development of drug
resistance? and (d) Was it compatible with the observations that the alkylating agents are mutagenic and carcinogenic? Elmore et al. (9), who initiated the studies on mustard-nucleic acid interactions, suggested that the attack probably occurred on the phosphate moieties of RNA and DNA. Subsequently, Goldacre et al. (10), Butler et al. (5), and Alexander et al. (4) published similar interpretations, and the latter reported that an estimated 5–40 per cent esterification of the phosphoryl hydroxyl group was obtained by treatment of nucleic acids with alkylating agents in vitro (1).

Studies conducted at the Southern Research Institute and in this laboratory with isotopically labeled alkylating agents demonstrated that radioisotope from drugs such as HN2-C\textsubscript{14}, TEM-C\textsubscript{14}, or Myleran-C\textsubscript{14} could be recovered with nucleic acid fractions as isolated from treated animals (13, 17, 18). The data of Wheeler et al. (18) and ours, however, indicated that the incorporation of drug isotope achieved in vitro was of a much lower order of magnitude. Even with the assumption that all isotopic carbon from methyl-C\textsubscript{14}-labeled HN2, isolated by Wheeler et al. with the purines, or all the isotope from ethylene-C\textsubscript{14}-labeled TEM, isolated by Nadkarni et al. with liver sodium nucleates, constituted drug fragments attached to nucleic acid fractions, only one out of 10\textsuperscript{4} to one out of 10\textsuperscript{5} nucleotide units appeared to undergo alkylation in vivo. On the basis of these data it was concluded that the number of possible interactions between nucleic acid fractions and alkylating agents in vitro differed from those observed in vivo by a factor of at least 1000.

The question remained whether or not the incorporation into nucleic acids in vivo, although of a very low order, could account for the mechanism by which the alkylating agents exerted their effects. In an attempt to resolve at least some of these questions the following experiments were carried out.

Alkylating agents labeled in various portions of their molecules were administered to animals with either regenerating liver or with tumors which had varying susceptibilities to the drugs. In the most susceptible tissue, a study was made of the recovery of isotope employing various isolation procedures for nucleic acids.

**MATERIALS AND METHODS**

**Radioactive compounds.**—Dimethanesulfonoxymethane (Myleran) was synthesized in our laboratory, once labeled 1,4-butane-C\textsubscript{14} and once labeled sulfur-S\textsubscript{35}. The carbon-labeled drug had a specific activity of 2.1 X 10\textsuperscript{4} counts/min/mg, and the sulfur-S\textsubscript{35}-labeled compound had an initial specific activity of 2.9 X 10\textsuperscript{6} counts/min/mg. Ethylene-C\textsubscript{14}-imino-s-triazine (TEM) and bis-(beta-chloroethyl-C\textsubscript{14})-p-aminophenyl butyric acid (chlorambucil) were also synthesized in our laboratory and had specific activities of 0.8 X 10\textsuperscript{6} and 1.1 X 10\textsuperscript{6} counts/min/mg, respectively. Radioactivity was measured on samples plated at constant geometry in a proportional flow counter. Corrections for self-absorption and for radioactive decay were made where necessary.

**Tissues.**—Partial hepatectomy was performed on male rats weighing 200–250 gm. under ether anesthesia, and the two larger lobes, constituting about two-thirds of the total liver weight, were excised. Pertinent data on the lymphatic mouse tumors used in this study are summarized in Table 1. The tumors were treated by I.P. injection only during the logarithmic growth phase and were harvested following treatment.

**Sodium nucleates.**—The livers were homoge-
nized immediately after excision, and the ascites was removed by dipping the contents of the peritoneal cavity into ice-cold isotonic saline solution. The homogenate was centrifuged in the cold at 12,000 × g for 10 minutes and the pellet suspended in 10 volumes of 10 per cent NaCl. The homogenate was stirred mechanically for 6 hours at 35°C. and the supernate removed by centrifugation. The residual tissue was extracted for 1 hour with 2 volumes of 10 per cent NaCl, centrifuged, and the supernates were combined. The sodium polynucleates were precipitated by adding 2.5 volumes of 95 per cent ethanol. The nucleates were freed from supernate by centrifugation and redissolved in a small volume of distilled water. After addition of 4 volumes of ethanol, the nucleates were collected by centrifugation and dried by repeated washings with alcohol-ether and with ether. The material was weighed, and an aliquot was redissolved for assay of radioactivity and for phosphate and pentose determination.

Preparation of nucleohistones.—The tissue was homogenized in ice-cold 0.1 M NaCl and 0.05 M citrate at pH 7.0 (6). The material was centrifuged at 9,000 × g for 5 minutes, and the supernate was discarded. The pellet was washed once more with saline-citrate solution and then washed twice with distilled water at pH 7. After removal of the supernate, the residue was extracted with distilled water for 16 hours at 4°C. with the aid of glass beads packed tightly in a vibrating bottle. After extraction, the material was strained, and the viscous supernate collected after centrifugation at 12,000 × g. The residue was washed twice with small amounts of distilled water, and the supernates were combined. The solution was made 0.15 M with respect to sodium chloride, and 2 volumes of ethanol were added. After allowing the mixture to stand for 4 hours in the cold, the precipitate was collected by centrifugation, washed with ethanol, ethanol-ether, and dried with ether.

Preparation of DNA and RNA.—This was carried out essentially by the method of Schmidt and Thannhauser (15). The tissue was homogenized in ice-cold 10 per cent trichloroacetic acid (TCA), centrifuged, and washed 3 times with cold TCA. The TCA extracts were combined and extracted with ether to remove the TCA (acid-soluble fraction). The precipitate was made slightly alkaline (pH 8) with sodium carbonate and extracted once with 95 per cent ethanol at 55°C. and once with ether at 30°C. The extracts containing the lipides were pooled, evaporated to dryness, and redissolved in methanol for assay of radioactivity. The defatted tissue was extracted with 10 volumes of 1 N KOH at 4°C. for 16 hours. After centrifuging, the residual material was washed once with 1-2 ml. of water, and the supernates were combined. Sufficient 1 N perchloric acid was added to bring the solution to pH 2.0. After centrifugation, the supernate containing the RNA was separated, and the residue was dissolved in 5 per cent NH₄OH. The potassium perchlorate was sedimented by centrifugation, and the sediment was washed once with water. The combined supernates were acidified with HCl, and the precipitated DNA was collected by centrifugation.

**Colorimetric estimations of RNA and DNA.**—RNA was determined in each sample as ribose by the orcinol method. DNA was measured both by the reaction with diphenylamine and by determination of total phosphorus with the ammonium molybdate reagent (12). Since it was conceivable that during the isolation procedure the drug or drug fragment was again detached from the nucleic acid moiety, the following three different methods were compared regarding the isolation of the nucleic acids from treated animals: (a) the sodium nucleates were prepared by extraction with hot NaCl solution, (b) the nucleohistones were prepared by extraction with distilled water in the cold, and (c) the DNA was isolated according to the Schmidt-Thannhauser procedure. Table 2 illustrates the recovery of isotope in the P-195 lymphoma with the various procedures. Since the values obtained by these methods were of the same order of magnitude, in most cases, the isolation of DNA by the Schmidt-Thannhauser procedure was considered satisfactory as the standard procedure.

**RESULTS**

To investigate the incorporation of TEMethyleneimino-C¹⁴ and of Myleran-S¹⁵ and Myleran-C¹⁴ into regenerating livers of rats, times of 20 hours and 76 hours after partial hepatectomy...
were chosen, because it was known that, about 20 hours after hepatectomy, nucleic acid synthesis reached its maximum rate (14) whereas at 3 days the growth rate of regenerating liver reached a maximum rate (16). From these livers the sodium nucleates were isolated following doses of 0.7 mg. and 1.0 mg. per animal for TEM and Myleran, respectively. The animals were sacrificed 2 hours after injection of the drug. In Table 3, the specific activities of the sodium nucleates of regenerating livers are compared with those of control animals.

From these data it was concluded that under optimum conditions not more than 1 in 60,000 nucleic acid phosphates had been esterified, even though there appeared to be a significant increase in specific activity when nucleic acid synthesis reached a maximum rate. Since it is known that the regenerative rate of liver is affected only by very large doses of irradiation or 

\[\text{HN2}\], it was considered that the nucleic acid moieties of liver were less susceptible to alkylation than those of a known susceptible tissue.

To gain a wider basis for comparison the incorporation of various isotopically labeled alkylating agents into the lymphatic leukemia L1210 in the ascites form was measured. In Table 4 the results of this experiment are shown. The uptake of adenine-8-C\textsuperscript{14} was included in the table for comparative purposes. The finding that Myleran-C\textsuperscript{14} was incorporated into L1210 DNA at an apparently much higher rate than chlorambucil or sulfur mustard was not surprising, since appreciable amounts of radiocarbon derived from Myleran-1,4-C\textsuperscript{14} were suspected to enter the metabolic pool as one-carbon fragments (17) and thus could be utilized in the \textit{de novo} synthesis of the purine ring. In the case of TEM-ethylene-C\textsuperscript{14} conversion of drug isotope into one-carbon or two-carbon fragments was known to be negligible (13), and little or no rerouting of drug isotope through metabolic pathways could be expected.

However, in order to evaluate the significance of the marked incorporation of radiocarbon from Myleran, a study of the incorporation pattern of Myleran-C\textsuperscript{14} into RNA- and DNA-fractions was made in three different ascites lymphomas which had shown appreciable differences in the therapeutic response to the drug. The incorporation rates of Myleran radiocarbon again were compared with those of adenine-C\textsuperscript{14}. The order of susceptibility of the three tumors increased in the order of L1210, P-381, P-195. The data assembled in Table 5 represent the total radioactivity of the RNA and the DNA fractions in terms of the per cent isotope recovered per gram of tissue. The higher incorporation rates into the L1210 tumor reflect the faster growth of this tumor, and the increase in radioactivity of the DNA fractions in all three tumors is

### Table 3

<table>
<thead>
<tr>
<th>Drug and dosage</th>
<th>Time after hepatectomy (hours)</th>
<th>(\mu)moles of drug per mole phosphorus*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEM-C\textsuperscript{14} (0.7 mg/animal)</td>
<td>0</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>16.9</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>9.8</td>
</tr>
<tr>
<td>Myleran-C\textsuperscript{14} (1.0 mg/animal)</td>
<td>0</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>3.74</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>3.40</td>
</tr>
<tr>
<td>Myleran-S\textsuperscript{35} (1.0 mg/animal)</td>
<td>0–72</td>
<td>None</td>
</tr>
</tbody>
</table>

* In the calculations of these data it was assumed that all isolated radioisotope represented reacted drug or drug fragments and that no recycling of drug isotope had taken place. The values reported are the averages of data obtained from three rats in each category.

### Table 4

<table>
<thead>
<tr>
<th>Drug and dosage</th>
<th>Time after injection of the drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myleran-C\textsuperscript{14} (2.0 mg/kg)</td>
<td>10 min.</td>
</tr>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Chlorambucil-C\textsuperscript{14} (1.0 mg/kg)</td>
<td>2</td>
</tr>
<tr>
<td>TEM-C\textsuperscript{14} (4.0 mg/kg)</td>
<td>4</td>
</tr>
<tr>
<td>Sulfur mustard-S\textsuperscript{35} (0.5 mg/kg)</td>
<td>4</td>
</tr>
<tr>
<td>Adenine-C\textsuperscript{14} (1.2 mg/kg)</td>
<td>2</td>
</tr>
</tbody>
</table>

All values are expressed in \(\mu\)moles of drug per mole of phosphorus. Time of treatment: 120 hr. following inoculation.
approximately proportional to their relative growth rates. In contrast, the incorporation pattern of C\textsuperscript{14} derived from Myleran shows an approximately linear increase with time in most fractions, indicating that the uptake of radiocarbon was dependent upon a supply which was not directly available, but rather furnished by enrichment of metabolic pools with labeled fragments. Apparently, there was no correlation between tumor susceptibility and its capacity to utilize Myleran-radiocarbon. The apparent high incorporation of Myleran-C\textsuperscript{14} into the DNA of the tumors under study, however, was not necessarily representative of alkylation or esterification of the nucleic acid moiety.

In the case of TEM-ethyleneimino-C\textsuperscript{14}, however, the recovery of isotope in the general metabolic pool had been shown to be negligible, so it could be assumed that most, if not all, of the radioactivity recovered in the nucleic acid fractions represented essentially TEM or the alkylating moiety thereof. Because of this, the problem of whether or not the compound had interacted with nucleic acid at specific target sites could be attacked more readily. If susceptible key positions in the phosphate moiety of the DNA polymer were occupied by the drug, even the very small amounts of TEM recovered in the nucleic acid fractions might lead to isolation and identification of the target site. If, on the other hand, the esterification of these nucleic acid moieties was random, no such target site could be postulated, and such phenomena as high susceptibility of one tumor as against another tumor, the development of drug resistance, or the induction by alkylating agents of specific mutations would impose certain restrictions on the interpretation of the mode of action of the alkylating agents.

To elucidate this possibility of drug action at a specific site in the nucleotide sequence of the DNA polymer, the incorporation of TEM-C\textsuperscript{14} (4 mg/kg) into L1210 DNA was measured in control mice which had been given injections intraperitoneally 10 minutes previously with saline, and on mice which had received an identical dose of nonlabeled TEM 10 minutes prior to the injection of the labeled TEM. The premise was that if the “sites” could be saturated with alkylating agent, a divergence in the amount of radioactivity incorporated should occur. The specific activities of the tumor DNA fractions were then estimated at various times after the administration of the isotope.

| TABLE 5 |
| INCORPORATION OF ISOTOPES FROM MYLERAN-C\textsuperscript{14} OR ADENINE-C\textsuperscript{14} INTO RNA AND DNA |
| FRACTIONS FROM LEUKEMIC ASCITES CELLS* |
| | L1210 | P-889 | P-193 |
| | 10 min. | 60 min. | 240 min. | 10 min. | 60 min. | 240 min. | 10 min. | 60 min. | 240 min. |
| Adenine-8-C\textsuperscript{14} |
| RNA | 0.45 | 2.08 | 2.50 | None | None | None | 0.33 | 0.3 |
| DNA | 0.73 | 2.71 | 0.66 | 0.051 | 0.073 | 0.292 | 0.104 | 1.55 |
| Myleran-C\textsuperscript{14} |
| RNA | 0.036 | 0.058 | 0.116 | None | None | None | 0.015 | 0.026 |
| DNA | 0.135 | 0.351 | 0.420 | None | None | None | 0.015 | 0.023 |

* Per cent of injected dose per gram of ascites tumor. Each value is the average of two animals.

The results, which are graphically illustrated in Chart 1, indicated that there was no appreciable difference between the saline-pretreated and the TEM-pretreated tumors. The reason for the differences in the specific activities of the RNA and DNA were not evident and will require further study.

DISCUSSION

In the preliminary experiments of this investigation, three different methods for the isolation of nucleic acid polymers were compared in their efficiency to yield a high drug isotope/nucleic acid ratio. It was found that with each procedure the recovery of isotope was of the same order of magnitude. Individually, the preparation of the sodium nucleates appeared to give the highest ratio. On the other hand, the purity of the fractions in respect to nucleic acid moieties was not identical, and it is suggested that the somewhat better yields.

\[ \text{Equation} \]

\[ \text{Equation} \]
obtained in the sodium nucleates and in the nucleohistones was due to contamination with proteins which often tend to show a higher incorporation rate than the nucleic acids (Nadkarni, unpublished data).

From the observations of Wheeler et al. (18) and Cohn et al. (7) who compared the incorporation rates of methyl-C\(^{14}\)-labeled nitrogen mustard and of \(\beta\)-phenylalanine-C\(^{14}\)-labeled nitrogen mustard with those of their respective bis-\(\beta\)-hydroxyethyl analogs obtained by hydrolysis of the chloroethyl compounds, it is evident that appreciable amounts of isotope from unreactive mustard derivatives could be recovered in various isolated fractions. Therefore, it has to be assumed that rerouting of drug isotope took place to some extent in our experiments, depending upon the nature of the alkylating agent and the position of the isotopic label. In this study, recycling of isotope probably was most abundant with Myleran-C\(^{14}\) and was least likely in the case of the TEM-C\(^{14}\).

Our data were calculated with the assumption that all isotope isolated with the nucleic acid fractions essentially represented unaltered drug, yet these incorporation rates constitute only a fraction of those postulated by Alexander (2) as a result of his observations from \textit{in vitro} studies. In the \textit{in vivo} experiments reported here with various tissues, on the average, only one in 10,000 nucleotide units contained isotopic element derived from an alkylating agent. The nature of the label, whether drug fragment or not, and the specific site of its binding were not ascertained. Whereas there is no objection to the postulate that DNA or closely related materials can be alkylated \textit{in vivo}, there is, however, the question as to the biological significance of this very low order of incorporation or alkylation.

If the nucleotide moiety in DNA is attacked by the alkylating agent at the phosphoryl hydroxyl group, spatial arrangement and the postulated size of coding sequences make the inactivation of a "specific" location, at incorporation rates of one in 10,000 or less, unlikely. It could be assumed that any \textit{in situ} alkylation of the DNA molecule will take place in a completely random fashion, and it can and has been argued that a single alkylation is sufficient to distort the shape of the DNA molecule resulting in impairment of biological function (3). Yet some specificity apparently does exist if one considers such phenomena as recurrence of specific mutagenic effects, variations in susceptibility of tissue from one alkylating agent to the other, or the development of resistance after repeated exposures to the drug.

A comparison with the effects of a therapeutic dose of x-radiation on a tumor, such as the L1210 ascites lymphoma, may be justified in view of the known radiomimetic properties of these drugs. Here with a total dose of 300 roentgens at 275 kv. approximately one ion pair was produced for every 6,000 DNA-phosphate residues in the cell nucleus. The number of ion pairs produced within the range of the genetic material was of the same order of magnitude as the possible alkylations which were produced with some of the compounds studied here. However, the mean path of an ionized particle was so short at these energies that it could not be attracted directly to any specific intramolecular target site. The biological effect of ionizing radiation, if based on an effect on the genetic material, also must take place in a random fashion.

Thus, on the basis of the available evidence, two modes of action for the alkylating agents present themselves: (a) The alkylating agent does impair the function of the genetic apparatus by direct alkylation of the DNA. The ratio of drug molecules to DNA molecules being approximately unity or less, the site of action within the DNA polymer is chosen at random, and the development of specific mutagenic effects, resistance, and susceptibility phenomena, etc., has to be accounted for by the relative abundance of specific sensitive genes or coding sequences. (b) Alkylation of DNA is a noncritical event taking place within the cell along with many other reactions. The mechanism of action of the alkylating agents then involves...
other cellular or molecular sites, probably with various degrees of specificity. The effect produced within the genetic apparatus proper could thus be considered an incidental effect.

REFERENCES

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