The Mechanism of Action of Alkylating Agents

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SUMMARY

The term "alkylating agent" has been defined and a detailed discussion made of the mechanisms (Sn1 and Sn2) by which they interact with nucleophilic centers. The various nucleophiles likely to be encountered in vivo are discussed in terms of their relative reactivities toward alkylating agents.

With regard to Sn2 reactors it was possible to group them into four sections on the basis of their chemical reactivity and the "spread" in their affinity toward different nucleophiles. The groups comprised (a) the epoxides, ethyleneimines, and $\beta$-lactones; (b) the primary alkyl methanesulfonates; (c) primary alkyl halides; and (d) $\alpha$-halo acids and ketones.

An attempt has been made to correlate the reactivity of the Sn2 and Sn1 reactors with their known pharmacological properties and the likelihood of their reaction with the various cellular constituents. In particular, the effects of the alkylating agents on nuclear material and cell division, the blood components, male fertility, and the immune response have been discussed in detail, and the chemistry involved in the interaction of the alkylating agents with compounds of biological importance such as the nucleic acids, proteins, and peptides has been critically examined.

The importance and limitations of the alkylating agents as chemotherapeutic drugs and as tools for examining the basic mechanisms involved in carcinogenesis and mutation have been considered in the light of recent findings.

No attempt will be made to review the whole of the literature relevant to the mode of action of alkylating agents, since this has been done many times in the past, and more recently by Ross (72) and by Wheeler (87). Because of the growing complexity of the field, it might be instructive to examine the chemical reactivities and alkylating potentials of some of the different classes of alkylating agents in relation to what is known about their pharmacological properties. The different types of compound which can function as alkylating agents or electrophilic reagents include alkyl halides, alkyl methanesulfonates, alkyl sulfates, alkyl phosphates, halogenomethyl ethers, 2-chloroethyl sulfides, 2-chloroethylamines, epoxides, $\beta$-lactones, ethyleneimines and ethyleneimides, diazoalkanes, activated ethylenic compounds, halogenomethyl ketones and esters, methylolamines, etc., and, although only a few of these classes are useful as palliatives in clinical therapy, all are capable of reacting with at least some nucleophilic centers with the result that even inactive compounds can help in the elucidation of fundamental mechanisms of action.

The term "alkylating agent" in its widest sense denotes those compounds capable of replacing a hydrogen atom in another molecule by an alkyl radical, and this of course involves electrophilic attack by the alkylating agent so that the definition must be extended to include those reactions involving addition of the radical to a molecule containing an atom in a lower valency state—for example, formation of a sulfonium compound from a sulfide. The alkylating agents exhibit a diversity of pharmacological properties including the capacity to interfere with mitosis, to cause mutations, and to initiate and promote malignant tumors; some of them have a stimulatory action on the nervous system, and others are powerful vesicants or lachrymators. When attempting to find a parallelism between the pharmacological effects elicited by the alkylating agents and their chemical reactions at particular cellular sites, it is important to consider, among other variables, the particular
mechanism of alkylation involved, since this will largely determine the chemical groups which will be attacked inside the cell and their position within the cell. Almost every review which has been written on the alkylation agents has included at least some description of the mechanisms involved in unimolecular and bimolecular reactions, and the justification for indulging in this again in detail is inherent in the problem with which we are concerned. The alkylation agents, unlike many other drugs used in medicine, produce their effects by binding covalently with cell constituents, and their sites and extents of binding, although dependent to some extent on factors such as solubility, will be determined mainly by their electrophilic (or alkylation) potential; and this involves consideration of ease of electron displacement, resonance energies, bond strengths, steric factors, etc.

There are two generally accepted basic mechanisms of alkylation, first-order nucleophilic substitution (S_n1) and second-order nucleophilic substitution (S_n2), although care should be taken in making too rigid a classification. In the former, the rate-controlling stage in the reaction is ionization to form a solvated carbonium ion, followed by a rapid combination with a solvent molecule or new nucleophilic reagent such as an anion or sulfide (Chart 1). Since the driving force of the reaction is a displacement of electrons away from R, then reactions of this type will be facilitated by the presence of electron-repelling substituents (such as methyl) in R. The carbonium ion, being an extremely unstable and hence reactive species, will be to some extent indiscriminate in its combination with nucleophilic centers. The more unstable carbonium ions, such as those derived from i-propyl methanesulfonate, will tend to react rapidly with solvating water molecules, whereas in some cases more discrimination is possible as demonstrated by Ogston (63) in the case of mustard gas. Mustard gas (see later) yields a carbonium ion which is stabilized in the form of a three-membered ring, and this accounts for its greater capacity to discriminate between anions. This stability is even greater in the case of the immonium ion formed from aliphatic 2-chloroethylamines due to the greater basicity of nitrogen than sulfur (Chart 2).

Each nucleophile (carbonium ion, immonium ion, etc.) has a competition factor which was measured by Ogston in the case of mustard gas by observing the extent of reaction in aqueous solution with various anions (Table 1). In the case of compounds such as thiols, amines, and acids, account must be taken of the amount in the reactive form at a particular pH in considering likelihood of reaction with an alkylation agent. If one remembers that alkylation agents pick out centers of high electron density, thiols and acids will be most reactive when ionized, in contrast to amines which become unreactive when protonated. If the competition factor for a particular group is F, c is its concentration, and f is the amount in the reactive form, then in a particular system the relative amount of any group reacting is proportional to

\[
F \cdot c = \frac{1}{k} \cdot \frac{c}{f}
\]

However, Alexander (1) has drawn attention to the fact that apparent competition factors of groups in macromolecules may be increased by adsorption of the agent onto the macromolecule, thereby increasing effective concentration. Ross (74) assessed the reactivity of a number of chloroethylarylamines by measuring their rates of hydrolysis in aqueous acetone and found that electron-releasing substituents in the benzene ring such as p-methyl greatly increased the rate of hydrolysis, whereas it was retarded by p-nitro-, p-phenylazo-, or other electron-attracting substituents.

The other essential features of S_n1 reactions are (a) that the rate of any particular reaction will
be essentially independent of both the concentration and the nature of the other reagent. Thus, an
ionized thiol, a powerful nucleophile because of the electron distribution on the sulfur atom, will
react at the same rate as, e.g., an amine or hydroxide ion, less powerful nucleophiles, and (b) that no reaction will take place until primary ionization has occurred. Both these factors are in
contrast to what is observed in Sn2 reactions and are probably very significant when comparing the pharmacological properties of the two classes of reactant.

Mustard gas (dichlorodiethyl sulfide) and the aromatic nitrogen mustards are the Sn1 reactors with which we will be concerned in this discussion. Mustard gas is an extremely reactive compound (half-life in water about 3 min. at 37° C.) because of the presence and position of the electron-releasing sulfur atom relative to the chlorine atoms which facilitates formation of a carbonium ion through the unstable ring system shown. The carbonium ion (which may exist in the form of a three-membered ring) can discriminate between nucleophiles to some extent, but it is far too reactive and, hence, toxic to be of any use in therapy (Chart 3).

The aromatic nitrogen mustards were developed because they retained the capacity to react by an
Sn1 mechanism; but the electron-withdrawing capacity of the aromatic ring greatly reduced the rate of carbonium ion formation so that compounds could be synthesized which could reach distant sites in the organism before reacting with tissue constituents.

![Chart 3](image_url)

**Chart 3.**—Mechanism of ionization of mustard gas

In second-order nucleophilic substitution a carbonium ion is not formed, but a transition com-
plex is formed involving both reactants, and hence the rate of reaction is dependent upon both concentra-
tions (Chart 4). In this case the reagent helps in the breaking of the R-X bond, and this will occur most readily if the energies of the Y-R and R-X bonds in the transition state are high, if the electron affinity of X is high compared with that of Y, and if the resonance energy of the trans-
sition state is high compared with the initial state. Bond strength is obviously important, since, whereas electron affinity increases in the order I < Br < Cl < F, replacement by an alkylating agent increases in the reverse order, I being most easily expelled.

Compounds such as primary alkyl halides, methanesulfonates, and phosphates are not par-
ticularly powerful reactors, their rate of reaction with water and other nucleophiles being usually relatively low. Thus, whereas mustard gas has a half-life of 8 min. in water at 37° C., ethyl meth-
anesulfonate has a half-life of 56.3 hours and that of methyl bromide is 160 hours (75). The reaction rate increases considerably in the presence of centers of high electron density, especially

![Table 2](image_url)

**Table 2**

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>k/aW</th>
<th>Nucleophile</th>
<th>k/aW</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>1</td>
<td>HS-</td>
<td>1.8X10⁴</td>
</tr>
<tr>
<td>CH₃COO⁻</td>
<td>500</td>
<td>Thiosulfate</td>
<td>4.0X10⁴</td>
</tr>
<tr>
<td>Pyridine</td>
<td>4000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPO₄⁻</td>
<td>6300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OH⁻</td>
<td>16000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Anions. Thus, the ability of Y to replace X in R-X by the Sn2 mechanism can be assessed by comparing reaction rate with Y as compared with water. This has been done by Swain for methyl bromide (84).

The major nucleophilic centers present inside cells are ionized thiol groups, ionized carboxylic acids, ionized phosphates, and unionized amines, and, ignoring accessibility and concentration factors, reaction would proceed most readily in the order thiol > amine > phosphate > carboxyl.

Compounds such as α-halogenocarboxylic acids and ketones are highly reactive Sn2 reagents toward certain nucleophiles by virtue of the electron-attracting capacity of the carbonyl group (Chart 5). However, for reasons to be discussed later, its reactivity toward weak nucleophiles such as the carboxyl ion or the phosphate ion is low. In this context a significant factor in considering Sn2 reactors is the variation in relative reactivity of a particular reagent toward different nucleophilic centers. For example,

\[
\frac{\text{rate constant (thiosulfate)}}{\text{rate constant (acetate)}} = 205
\]

is 205 for ethyl methanesulfonate, 4880 for methyl bromide, and 16,600 for the iodoacetate ion (75,
p. 22). Thus, it is obvious that the “spread” in reactivity toward different nucleophilic centers varies considerably from reagent to reagent and is of fundamental importance in determining the reactions which a particular drug will undergo in vivo and hence its pharmacological properties.

The factors which determine whether an agent will react by an Sn1 or an Sn2 mechanism are obviously complex, and it is dangerous to be too rigid in this type of classification, since even solvent changes can sometimes lead to a modification in mechanism. There occur also those compounds such as the epoxides and ethyleneimines which, by virtue of their strained three-membered ring structure, fall into a different category from most other Sn2 reactors, as will be considered later. In the case of alkyl halides and alkyl methanesulfonates, the rate and mechanism of reaction depend on whether the reactant is primary, secondary, or tertiary. In some cases, such as in the reaction of alkyl bromides with pyridine, the rate of reaction to form an ethylene derivative is in the order tert. > sec. > prim., whereas in the simultaneous reaction to form quaternary salts the order is reversed. The differences in the reaction rates of primary, secondary, and tertiary alkyl compounds are generally attributed to the inductive effect. The replacement of a hydrogen atom by a methyl group is acid-weakening—i.e., it induces a negative charge on the atom on which substitution has occurred. It therefore increases the ease of ionization of a halide ion from the molecule. This ease of separation can be more than compensated for by the smaller attraction of the reacting base. Consequently, the primary halides require a stronger basic reagent than the tert-halides, but this order is reversed when the reagent acts as an ionizing rather than a basic reagent (61).

In general methyl halides react almost exclusively by an Sn2 process, but introduction of another methyl (electron-releasing) substituent (giving ethyl) leads to a reduction in the rate of reaction by the Sn2 mechanism, the factor usually being about 10, as in the reaction with amines. Introduction of another methyl group as in iso-propyl halides leads to a further drop in Sn2 rate, but now the Sn1 mechanism becomes of importance, and the two mechanisms operate simultaneously and at a comparable rate (37). The reason for this is that the extra methyl group facilitates primary ionization of the halide ion (Sn1) but retards the Sn2 process for electronic reasons and because it hinders the approach of the nucleophile and subsequent formation of the transition complex (primary steric effect). The presence of three methyl groups as in tert-butyl halides causes a complete changeover to the Sn1 mechanism, and the rate of hydrolysis becomes faster than the Sn2 hydrolysis of methyl halides under the same conditions (61).

A change in mechanism can also be observed sometimes if an attacking nucleophile is replaced by one of less nucleophilic potential under the same experimental conditions. At first the effect will be to decrease the velocity of the Sn2 process, but with even weaker nucleophilic reagents a point will be reached in which the reagent is no longer able to assist in the breaking of the R-X bond which must, therefore, occur unaided by the reagent, and the mechanism will become Sn1—probably coupled with a decreased reaction rate (39). For example, in the substitution of the trimethylsulphonium ion by various anions it was found that the specific rates were OH(743), PhO-(13.4), CO2-(7.4), Br-(7.8), Cl-(7.3), obviously indicating an Sn2 mechanism for the first two anions and an Sn1 mechanism at a lesser rate for the others. A situation similar to this probably occurs in the case of a-haloketones and acids, indicating their tremendous potential to react with powerful nucleophiles such as ionized thiols by an Sn2 process and their relative unreactivity toward weaker nucleophiles such as ionized acid groups (hence the misnomer “specific thiol reactors”). The earlier data concerning the alkyl halides is probably of fundamental importance in considering the biological properties of the various agents. For example, the greater reactivity of methyl halides (or methanesulfonates), as compared with the corresponding ethyl derivatives, probably explains the greater toxicity of the former in vivo. One surprising finding is the fact that dimethylmyleran (2,5-dimethanesulfonyloxyhexane) is so similar to Myleran (1,4-dimethanesulfonyloxybutane) in its pharmacological properties (see later) (Chart 6). Unlike Myleran, it will tend toward an Sn1 mechanism in its reactions, forming a very unstable carboxonium ion extremely reactive toward its own solvating water in aqueous media and hence unreactive toward other nucleophiles. However, the possibility of its reacting by an Sn2 mechanism may become more important in non-

\[ X^- + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{CH}_3\text{COOH} + I^- \]

**Chart 5.**—Mechanism of reaction of iodoacetic acid

\[ \text{CH}_3\text{SO}_2\text{O} \cdot \text{CH}_3 \cdot \text{CH}_2 \cdot \text{CO}_2\text{H} = \text{CH}_3\text{SO}_2\text{O} \cdot \text{CH}_2\cdot \text{CO}_2\text{H} \]

**Chart 6.**—Dimethylmyleran and Myleran

In general methyl halides react almost exclusively by an Sn2 process, but introduction of another methyl (electron-releasing) substituent (giving ethyl) leads to a reduction in the rate of reaction by the Sn2 mechanism, the factor usually being about 10, as in the reaction with amines. Introduction of another methyl group as in iso-propyl halides leads to a further drop in Sn2 rate, but now the Sn1 mechanism becomes of importance, and the two mechanisms operate simultaneously and at a comparable rate (37). The reason for this is that the extra methyl group facilitates primary ionization of the halide ion (Sn1) but retards the Sn2 process for electronic reasons and because it hinders the approach of the nucleophile and subsequent formation of the transition complex (primary steric effect). The presence of three methyl groups as in tert-butyl halides causes a complete changeover to the Sn1 mechanism, and the rate of hydrolysis becomes faster than the Sn2 hydrolysis of methyl halides under the same conditions (61).
aqueous media, and the implications of this will be considered later.

Striking differences in pharmacological effects have been found on the one hand between alkylating agents which react essentially by an \(Sn_1\) mechanism, and many of those which react by the \(Sn_2\) mechanism, between monofunctional and bifunctional agents of both types, and between individual members of the \(Sn_2\) type. The differences observed between the various \(Sn_2\) reactors are probably explicable on the basis of the foregoing discussion, and from the point of view of chemical reactivity and “spread” in affinity toward different nucleophiles the \(Sn_2\) reactors can be broadly classified into four groups:

- Group (a): Comprises compounds which bear a resemblance in structure to the ethyleneimmonium ions which are derived from aliphatic nitrogen mustards and which react by an \(Sn_2\) mechanism with nucleophiles (see earlier) (Chart 7).
- Group (b): The primary methanesulfonates, differ from the epoxides in their potential of reactivity, because no strained ring is present and the \(Sn_2\) mechanism will mainly operate in which bond-making will be more important than bond-breaking, so that both the concentration of an attacking reagent and its nucleophilicity will be of prime importance, although it should be mentioned that the true \(Sn_2\) tendency will be greater in the methyl esters than the ethyl. Because of their relative unreactivity compared with epoxides, there is found a greater “spread” in their affinity toward nucleophiles, and their reactivity toward thiol groups will be far greater than toward amines or acids. In this connection Brookes and Lawley (21) have found that both ethyl methanesulfonate and Myleran have much less reactivity toward guanine in DNA than the mustards and epoxides, whereas Roberts and Warwick (65, 66) have demonstrated that almost all the urinary reactivity excreted after injection of these compounds could be accounted for by reaction with the powerfully nucleophilic thiol group in vivo. In the case of ethyl methanesulfonate much of the \(C^14\) in the drug was exhaled as \(C^14O_2\) which could have been derived either by hydrolysis of esters formed by reaction with carboxyl groups or phosphate groups. In the case of Myleran, much less of the drug was metabolized in this way. The capabilities of an alkylating agent to react with various nucleophiles is reflected faithfully in the biological and pharmacological effects which it elicits, leading in the case of these two agents to effects much less potent than were observed with the mustards, epoxides, etc. Thus Myleran is much less toxic than mustard gas, possesses none of its vesicant properties, has only moderate activity against transplanted tumors, causes far fewer chromosome

\[ R-CH-CH_2 + X + H \rightarrow \text{RCH-CH}_2X \]

\[ \text{OH} \]

\[ \text{Chart 7.—Mechanism of epoxide reactions} \]
“breaks” (33), and, although it decreases the number of circulating granulocytes, it has far less effect on the peripheral lymphocyte count than the mustards and epoxides (30); and its effects on the immune response and on male fertility in rats again differs fundamentally from the mustards and ethyleneimines (see later).

Group (c), comprising the primary alkyl halides, represents, with the exception of the methyl compounds, agents even more unreactive than the primary alkyl methanesulfonates. Differences in the nucleophilicity of the Br\(^-\) and CH\(_2\)SO\(_2\)O\(^-\) anions coupled with differences in the C-Br and C-OSO\(_2\)CH\(_3\) bond strengths and a weak capacity to undergo Sn\(_2\) reactions makes the halides (e.g., ethyl bromide and di bromobutane) even poorer candidates than the methanesulfonates as far as diversity of reactions in \(v\text{ivo}\) are concerned. Thus a decreased reactivity toward all nucleophiles is observed, so that even fewer biological effects would be anticipated; and this is so, since 80 mg. of dibromobutane can be administered safely to a 300-gm. rat compared with 4 mg. of Myleran, and this compound produces none of the pharmacological effects observed with Myleran. The increased reactivity of methyl compounds has been discussed earlier, and this is reflected in the high toxicity of methyl bromide compared with ethyl bromide.

Roberts and Warwick showed that dibromobutane reacted with cysteine \(v\text{ivo}\) in a manner similar to that of Myleran leading to a sulfonium ion (66). Since dibromobutane lacks the pharmacological properties of Myleran, this evidence could be used to discount the importance of such a reaction in \(v\text{ivo}\) and, indeed, the importance of thiol reaction in general. However, such reasoning is probably not valid, because it does not take account of the different thiol groups in the cell, their different reactivity, or the difference in alkylating potential between Myleran and dibromobutane. The major thiol reaction in \(v\text{ivo}\) will be with glutathione, which will probably have little permanent effect on cellular function; reaction with thiol proteins or enzymes might have greater significance, and there is no evidence yet that dibromobutane will react with these \(v\text{ivo}\). The site of thiol reaction inside the cell is probably very important, since it is now almost certain that every alkylating agent will combine extensively with thiol groups in \(v\text{ivo}\), and yet compounds such as the mustards, methanesulfonates, halides, and \(\alpha\)-haloketones and acids manifest very different effects on cells. There are two conclusions to be drawn from this: (a) that thiol reaction in no case contributes to pharmacological end-effects, or (b) that thiol re-action is a contributor and that the site at which it occurs and its extent differ with those of the various agents leading to different biological effects. There is no proof yet which alternative is correct, and yet certain vital experiments, which remain to be done, may make the situation clearer.

The past literature is full of examples implicating the importance of thiol reactions in all sorts of biological phenomena (10), and yet a really critical assessment of some of these claims can leave little doubt that they were based on naive or oversimplified assumptions leading to a very confused situation. The potential importance of thiol reaction remains (58), and yet more research needs to be done to discard outdated ideas and to bring the whole field into line with modern concepts. Speculative “paper chemistry” leads us nowhere, except possibly in circles, and there is no substitute for doing the vital experiments which can finally clear up these points. More will be said on -SH reaction later.

Group (d) comprises those agents, the \(\alpha\)-haloacids and ketones, which in their reaction mechanism lie in the other extreme from undoubted Sn\(_2\) reactors. With these potent Sn\(_2\) reagents the process of bond-making far outweighs in importance the process of bond-breaking, and so the nature of the attacking nucleophile is vitally important, leading to extreme reactivity toward powerful nucleophiles such as ionized thiol groups and a very weak level of reactivity toward less nucleophilic centers. Thus as mentioned earlier

\[
\frac{k(\text{thiosulfate})}{k(\text{acetate})} = 16,600
\]

is 16,600, as compared with 205 for ethyl methanesulfonate.

 Probably many resonance forms contribute to the transition state (20), but the peculiar reactivity of compounds of this type is a direct result of the presence of the powerful electron-attracting carbonyl group. Because of this, compounds of this type were once regarded as specific thiol reactors, and all their effects from enzyme inhibition to general toxicity in \(v\text{ivo}\) were ascribed on the basis of \(-\text{SH}\) reaction, despite warning by Michaelis and Schubert (59) that amine reaction could also occur. In this connection Anson and Stanley (5) later showed that, although tobacco mosaic virus was not inactivated by \(p\)-chloromercuribenzoate (a powerful \(\text{SH}\) reactor), it was inactivated by iodoacetamide and iodoacetic acid, implicating reaction at another site. More recently, Stark, Stein, and Moore (83) have shown that the inactivation of ribonuclease is due to reaction with the ring nitrogen of histidine. This reaction with tertiary
amines is of interest, since Roberts and Warwick (67) have shown that bromoacetic acid reacts readily with the purine portion of deoxyguanylic acid and that a low level of reaction with the guanine of DNA also occurred. It remains to be seen how significant such reactions are in vivo, however, because of the high competition by other groups such as thiol in the cytoplasm. In this connection, the same authors have shown (67) that, after incubating Krebs ascites cells with C14-bromoacetic acid, there is a steady uptake of label and the ratio

\[
\frac{\text{specific activity (protein)}}{\text{specific activity (DNA)}}
\]

was approximately 22; and it has yet to be established whether any DNA reaction other than that associated with residual DNA protein has occurred. It is significant that in the case of mustard gas and Myleran the ratio was nearer 1 (21).

From the foregoing discussion it is probably true to say that, with some exceptions, the different types of alkylating agent are potentially capable of reacting with all nucleophilic centers and that the pharmacological properties of a particular agent will depend on (a) ease of penetration, (b) some extent mechanism of alkylation, and (c) “spread” in capacity to react with different nucleophiles. The mustards, for example, probably produce such diverse effects because they can easily penetrate cells, their chance of reaching most parts of the cell is high because of their unreactive nature until they are ionized (a relatively slow process), and the carbonium ions once formed are reactive toward most nucleophiles, leading to some reaction at most parts of the cell. The various S_n2 reactors fall into different categories, and compounds such as the epoxides and ethyleneimines produce effects similar to the mustards because of their relatively low reactivity which enables them to reach most parts of the cell unaltered, coupled with a satisfactory gradient in the “spread” of their affinity toward different nucleophiles.

Some of the biological and pharmacological properties of the alkylating agents will now be discussed in the light of their chemical reactivities. The time will surely come when the barriers which divide chemistry from biology must crumble and they will merge into one conceptual framework. If the static concepts of chemistry could be made more dynamic, and the dynamic aspects of biology more static, then the meeting point would seem much nearer.

**The Effect of Alkylating Agents on Nuclear Material and Cell Division**

When it was discovered that in many respects the bifunctional chloroethylamines and chloroethylsulfides produced cytological and pharmacological effects (e.g., cell death, chromosome abnormalities, degenerative changes in the bone marrow and testis, suppression of the immune response) similar to those produced by ionizing radiation, the term “radiomimetic” was applied to them (28). Although certain of the alkylating agents produce end-results which appear superfluously like those produced by radiation, detailed investigation has often revealed significant differences between them, and the term in many respects leads to confusion in the minds of chemists and biologists alike. Loveless and Revell (58) set forth their precise biological criteria upon which chemical substances were to be classified as radiomimetic, and this involved restriction “to those substances which can be shown unequivocally to react upon the resting cell in such a way as to produce an alteration of the genetic material which is revealed by the appearance of chromosome breakage and rearrangement in subsequent divisions.” Certain compounds which caused pyknosis, and which Dustin had referred to as radiomimetic, were not covered by this definition. Others have preferred to retain the original broad definition, but since this time so many alkylating agents have been discovered to mimic superfluously the effects of radiation in, say, one respect only, that the term ceases to have great significance. Ethyl methane sulfonate, for example, shows none of the properties of radiation except that it is a powerful mutagen (53). Myleran, possibly atypical in some respects, nevertheless has a profound destructive effect on granulocyte production (39), causes certain chromosomal abnormalities (45), depresses the immune response (12) (in a manner very similar to radiation) and causes infertility in rats (48) (also in a manner reminiscent of radiation); but, unlike certain mustards, it has only a moderate inhibiting effect on the transplanted Walker rat carcinoma. Thus these compounds would seem to be “radiomimetic,” partially “radiomimetic,” or “nonradiomimetic,” depending on the particular biological phenomenon being considered. Koller (46) has compared the effects of several alkylating agents with those of radiation and concludes “that in view of the dissimilarities, it would be an error to infer a similarity of the mode of action of alkylating agents and x-rays, basing the inference on the similarity of some end products.”

Of the various types of alkylating agent which have been tested for tumor growth-inhibiting
properties, those which have emerged as the most active include the Sn1 reactors such as the aromatic nitrogen mustards and the Sn2 reactors of the HN2 type, epoxides, ethyleneimines. Other Sn2 reactors such as the aliphatic methanesulfonates (Myleran) show a low order of activity against the transplanted Walker tumor, whereas those Sn2 reactors such as the α-halo acids and ketones have been found to be inactive while possessing a high order of general toxicity. The other factor of importance in determining efficacy in this respect is functionality, and it is now well known that bifunctionality leads to a profound increase in capacity to interfere with mitosis, provided the reactive centers are spaced at an optimum distance apart. Thus in the chemical sense greatest activity is associated with undoubted Sn1 reactors or those Sn2 reactors which, as discussed earlier, show some tendency toward the Sn1 mechanism in the bond-breaking and bond-making process and which have a reasonable reactivity toward most nucleophiles. Bieseke et al. (18), in discussing the chemistry of alkylating agents, postulated that the most active compounds in causing chromosomal aberrations were those which possessed or could be transformed into unstable three-membered rings. In a comment on this postulate, Loveless and Ross (57) drew attention to the "radiomimetic" properties of 1,4-dimethanesulfoxyloxybutane (Myleran) in inhibiting the growth of Sarcoma 180 and in its effect on the blood and bone marrow, and the capacity of dimethylsulfate to produce chromosome breakage in plant material. Since both these compounds are incapable of forming three-membered ring systems, the postulate of Bieseke et al. was obviously an oversimplification, and Loveless and Ross (57) argued that capacity to form a carbonium ion was a more general feature of "radiomimetic" chemicals. Actually, it is doubtful whether epoxides, ethyleneimines, and methanesulfonates do, in fact, form true carbonium ions under conditions likely to be met with in the organism, and it is difficult therefore to accept this generalization as it is worded. As indicated earlier the term "radiomimetic" is often applied far too indiscriminately to sometimes ill-defined biological phenomena, and it would seem more profitable and less misleading to define carefully the biological effects manifested by each alkylating agent rather than to refer to some property as "radiomimetic" because it may vaguely correspond to a particular property of radiation. Any apparent similarity between the biological properties of radiation and the alkylating agents undoubtedly results from the capacity of each entity to modify cellular structure and function by direct combination with cellular components such as nuclear and cytoplasmic material. The most "radiomimetic" chemicals will be those capable of penetrating deep into the cell and of reacting with a diversity of cellular sites, whereas less reactive chemicals or those which alkylate by a different basic mechanism may possess far fewer "radiomimetic" properties.

Tumor inhibition with the alkylating agents results from their cytotoxic action, which probably is the result of a complex series of changes varying in fundamental mechanism with different types of alkylating agent, and even from cell type to cell type. Lethal cytotoxic action is usually of two types called interphase death and mitotic death. Interphase death occurs without cells’ going into division or attempting to go into division. Alkylating agents vary in their capacity to kill cells at this stage, and individual cell types vary in their susceptibility to this type of death. Thus fully differentiated lymphocytes tend to be extremely sensitive to the action of mustards (but not to Myleran), whereas most other differentiated cells do not. The mechanism of interphase death is probably complex and results from damage to many cellular sites.

In mitotic death the primary damage is thought to occur in the "resting stage," and chromosome aberrations and other effects become cytologically apparent at the time of division. Many changes occur, such as clumping and bridging of chromosomes, and some of the effects are cumulative during successive cell divisions leading eventually to nonviable cells. The action is not restricted to malignant cells, but extends to proliferating cells of all types (35).

Various differences in detail of action have been reported to exist between the mustards (e.g., HN2) and the methanesulfonates (e.g., dimethylmyleran). Thus in comparing their actions on lymphoma cells in culture, Alexander (3) found that HN2 immediately arrested cell division, whereas dimethylmyleran behaved like x-rays, and at least one division occurred before cell proliferation stopped. Koller (45) has compared in detail the cytological effects produced by various alkylating agents and again found differences in their modes of action and also differences in response to varying doses of alkylating agent. He found that in a series of dimethanesulfonyloxy esters, the greatest diversity of effects on cells of the Walker tumor was shown by Myleran. He also found that it could produce suppression of mitosis, and "radiomimetic" and cytotoxic effects, although these probably differed in detail from those observed with the mustards. Alexander reported
(3) that, after all treatments, there was an increase in volume of cultured lymphoma cells; with HN\(_2\) the cell volume doubled, and then there was degeneration, whereas with x-rays and dimethyl-mylaran a wide spectrum of cell sizes was obtained, including "giants," although nuclear granulation similar to that seen with HN\(_2\) was not observable after 24 hours.

Alexander\(^1\) has also found that iodoacetic acid produces different effects from either the mustards or the methanesulfonates on lymphoma cells. It seems to have little effect on cell division, but when a critical dose level is reached the cells break up. This would be consistent with the reactivity of this compound mentioned earlier, resulting in its major reactions occurring in the cytoplasm (67). The need for bifunctionality and a certain critical level of nuclear reaction (whether this be with nucleic acid, protein, or cofactor) seem essential for "radiomimetic" cell death. "Cytoplasmic death" may be a factor of importance in considering mustard action on fully differentiated lymphocytes and indeed in the inhibition of tumor growth in general. Koller (45) considers that many factors must be considered when assessing the causes of cell death in a dynamic population and that "the ability of the alkylating agents to produce radiomimetic injuries may not be the most important property that determines the effectiveness of these drugs in the therapy of cancer."

EFFECTS ON THE BLOOD

Elson (30) showed that the effects of whole-body x-radiation on the number of circulating platelets, lymphocytes, and neutrophils could be essentially reproduced by the administration of suitable combinations of Chlorambucil (a bifunctional mustard) and Myleran, and he concluded therefore that each of these chemicals was only partially "radiomimetic." Elson, Galton, and Till (32) consider that Myleran appears to act on the resting stage and prolongs the intermitotic interval, whereas mustards appear to act mainly on cells undergoing mitosis and possibly during the latter part of the DNA synthetic period. They also exert a particularly destructive action on mature lymphocytes which Myleran does not. They found that the effects of Chlorambucil appeared rapidly, were transient, and were accompanied by conspicuous mitotic abnormalities. Recovery proceeded rapidly except in the lymphoid tissue, and there was a transient overcompensation in the granulocyte series. Myleran, on the other hand, produced gradual effects, depression of hemo-

poiesis was prolonged, cytological abnormalities were not seen (cf. 45) at the doses used, and mitosis proceeded normally. They explain these different effects on the basis of the capacity of the mustards to attack ruthlessly nuclear material in the resting stage and to lyse fully differentiated lymphocytes, whereas they consider that Myleran prolongs the intermitotic interval in all proliferating cells irrespective of the stage in the mitotic cycle at which they are exposed. Differences in normal intermitotic levels which vary with different cell types could explain the effects. Lajtha (47) has indicated that erythropoietic cells normally divide about twice as often as granulopoietic cells, and delay in each series would explain why depletion of erythropoiesis occurs more rapidly than that of granulopoietic cells. The authors do not consider that Myleran has any selectivity for particular cell types and "that the apparent resistance of lymphoid tissue to Myleran may depend as much on the ratio of mitotic frequency to life-plan as to the lack of damage to mature lymphocytes."

In their effects on the circulating granulocytes and lymphocytes, Elson has found (30) that the epoxides and ethyleneimines produce effects similar to those produced by the mustards—not surprising in view of the similarity in reaction kinetics discussed earlier—whereas he found the effects produced by the carcinogens 4-aminostilbene and the polycyclic hydrocarbons to be more like those of Myleran, a result difficult to explain at present but possibly of great significance. Ethylmethanesulfonate (half-Myleran) was not active.

As in the case of tumor growth-inhibition, bifunctional alkylating agents of all series were much more efficient than the corresponding monofunctional derivatives, and in the Myleran series optimum activity was found when \(n = 4\) or \(5\) (31). Potent Sn\(_2\) reactors such as \(\alpha\)-halo acids and ketones apparently have not been tested as extensively as the foregoing compounds, whereas alkyl halides such as dibromubutane (group [c] Sn\(_2\) reactors) possess no detectable activity (note lower chemical reactivity).

EFFECTS ON MALE FERTILITY

Very interesting results which may contribute to an elucidation of the mode of action of x-rays and the alkylating agents have been found by comparing the effects of these agents on the process of spermatogenesis in rats and mice. The process follows the path spermatogonia \(\rightarrow\) spermatocytes \(\rightarrow\) spermatids \(\rightarrow\) spermatozoa in epididymis, and it has been found that different agents can affect different stages in the process. Once again it tran-

\(^1\) P. Alexander, personal communication.
spires that Myleran most closely follows the effects of radiation.

Intraperitoneal injection into mice of aliphatic mustards produced destructive changes in the testis, and recovery was observed 3–4 weeks later (48), whereas in rats potency returned after 2–4 months (34). It was of interest, however, that two aromatic nitrogen mustards, Melphelan and Chlorambucil, did not interfere with the fertility of male rats (42, 44) at doses which were adequate to cause transient leukopenia in the peripheral white count (29) and inhibit tumor growth (18). Jackson (43) concludes, therefore, that capacity to cause transient leukopenia in the peripheral white count and inhibit tumor growth or to interfere with the bone marrow does not imply capacity to damage germinal epithelium. This, of course, may have been due to poor penetration rather than to a different mode of action.

2,4,6-Triethyleneimino-1,3,5-triazine (TEM), which was placed with the epoxides as far as chemical reactivity is concerned, produced effects indicative of selective interference with certain stages of spermatogenesis (19), mainly, probably, with spermatocytes (11, 42), since the infertility was found at 4 weeks and recovery was rapid after a single dose of 0.2 mg/kg. Five daily doses of 0.2 mg/kg, however, caused sterility for several weeks, and early and late stages of spermatogenesis were affected, and probably mature sperm in the epididymis were also attacked (as with the mustards).

The relative insusceptibility of spermatogonia is in contrast in that they are the germinal tissue most sensitive to radiation (62, 79) and to Myleran.

After one dose of Myleran (11 mg/kg) the testis histology remained normal until the 8th week, when sterility rapidly developed (26, 42), indicating an effect on an early stage of spermatogenesis. The other germinal cells present at the time of treatment appeared to develop normally for about 45 days, resulting in a systematic depletion of spermatogonia, spermatocytes, spermatids, and spermatozoa, in that sequence (42). Increased doses still did not affect other spermatogenic cells which continued to proliferate.

Dimethylmyleran produced similar effects at a lower dose level (44). This apparent specificity of action of Myleran is probably in some way related to its chemical reactivity. The fact that the effects which it produces are similar to those produced by radiation and unlike those produced by the mustards and TEM is probably highly significant.

EFFECTS ON THE IMMUNE RESPONSE (12)

Different effects were found on the immune response, depending on the agent tested. Thus, it was found that irradiation and Myleran suppressed antibody production maximally if given about 2–4 days before the antigen, whereas nitrogen mustard and TEM did so when given about 2 days after the antigen, and antibody production could be suppressed for some weeks in each case, following a single injection given at the appropriate time interval. Once antibody production is under way, however, these agents have no further inhibiting effect. Berenbaum tentatively explains these relative effects by assuming that immunologically competent cells are produced from primitive stem cells which give rise to more differentiated cells which can produce antibody. He envisages early cell types to be sensitive to the action of x-rays and Myleran, but not to TEM or the mustards, whereas the later, more differentiated forms are sensitive to the latter agents, but not to the former. Other alkylating agents have unfortunately not yet been tested, but the foregoing results will be discussed later in the light of known reactions of these agents in vivo.

CHEMICAL INTERACTIONS WITH CELL CONSTITUENTS

General considerations.—In discussing the basic chemistry of the alkylating agents it was found logical to separate these compounds into various types or groups depending on whether they were essentially Sn1 reactors or Sn2 reactors, and further subdivision was possible when a more detailed assessment was made. In considering some of the pharmacological properties of the alkylating agents, it was found that, where comparative assessments have been made, in a broad sense these faithfully reflected the type of alkylating agent involved. Thus, one can distinguish between the pharmacological properties manifested by the mustards, the epoxides and ethyleneimines on the one hand, and the alkyl methanesulfonates on the other, in considering their pharmacological effects. Although fewer biological studies have been carried out on the alkyl halides and the α-haloketones and acids, it is evident that again these differ from one another and from the above compounds.

Since these complex processes involving cells and alteration of cellular structure and function almost certainly involve direct chemical combination with cellular constituents, and since the level of any particular combination will depend on many variables, the most important of which is probably chemical reactivity, then an examination of cellular components after treatment, or of altered biochemical integrity after treatment, should help to pin-point the lesions involved. This type of reasoning, valid or misleading, has
guided most workers studying the mechanism of action of alkylating agents for many years. The general opinion seems to be that, in the case of compounds such as the carcinogenic hydrocarbons, the carcinogenic azo dyes, etc., we must keep an open mind whether these agents cause their effects by actually binding to cellular constituents or whether they are doing something much more subtle—such as finding their way between macromolecular strands and exerting their effects by essentially physical means; however, in the case of the alkylating agents, cellular binding is of prime importance.

To digress a little, there is good reason for believing that the carcinogenic hydrocarbons and azo dyes do, in fact, need optimum structural requirements and particular electron distributions (7) which may enable them to alter cellular function by some kind of physical means; but there is growing evidence that compounds of this type can in fact bind firmly to cellular constituents, both to proteins and to nucleic acids. To quote but two examples, Heidelberger (86) has shown dimethylbenzanthracene to combine both with protein and DNA in vivo, whereas the Millers (60) and others have shown dye binding to liver proteins following administration of carcinogenic azo dyes, and, more recently, Roberts and Warwick (68) have shown the combination of a metabolite of dimethylaminoazobenzene with nucleic acids in vitro. These findings tend if anything to cloud the complicated situation even further; they may be misleading, but if direct covalent combination with cellular constituents is proved to be a potent force in producing all the pharmacological properties of the alkylating agents, then these findings might be of the utmost importance in elucidating at least some of the phases of the complex carcinogenic process with these and other agents.

In the case of the alkylating agents where one compound can be cytotoxic, carcinogenic, and mutagenic, the correlation between chemical reactions at the cellular level with any of these manifestations becomes exceedingly difficult. However, attempts have been made to study the types of chemical reactions which some of these reagents can undergo inside cells, and in the main the results obtained have indicated a close parallelism between the types of reactions observed in vitro using purified chemicals and those observed in vivo, and it has also been found that levels of binding to various constituents were as one might have anticipated on the basis of the reactivity of the drug concerned. These studies are probably important if only to show that definite covalent binding can occur between alkylating drugs and cellular constituents, the nature of this binding in some cases, and that in vitro studies can often help to elucidate tantalizing in vivo problems. The progress made so far, much as it seems when one assesses it against the almost complete void which existed 10 years ago, is still extremely limited; and, although we are in a position to make some intelligent guesses as to the mechanism by which the alkylating agents exert some of their effects (e.g., their cytotoxic action), it would be unwise to make any dogmatic assessments at this stage, no matter how justifiable they may seem. However, the surface has been scratched, and doubtless the task of a reviewer at the end of another 10 years will be a comparatively reassuring one.

Metabolism of ethyl methanesulfonate and Myleran.—The first clear-cut demonstration that a pharmacologically active alkylating agent did in fact alkylate nucleophilic centers in vivo was obtained when Roberts and Warwick (65) demonstrated the presence of N-acetyl-S-ethylcysteine and related compounds in the urine of rats which had received C14-ethyl methanesulfonate by intraperitoneal injection. The bulk of the radioactivity which appeared during the first few hours was probably derived from reaction with glutathione known to be present in high concentration in mammalian cells and with which ethyl methanesulfonate reacted readily in vitro. That which appeared later probably came from ethylated protein. Because of the related structure of 1,4-dimethanesulfoxoxybutane (Myleran) and its enhanced pharmacological properties, it was considered of interest to examine the urinary products excreted after its injection. Because of the bifunctional nature of the drug it was considered that some type of cross-linked product might be formed in vivo. However, the major urinary metabolite was found to be 3-hydroxytetrahydrothiophene and its 1,1-dioxide (66), which was probably derived from a sulphonium compound formed by the interaction of both alkylating arms with one thiol group. This was followed by the removal of tetraphrothiophene and its further metabolism to the hydroxysulfone. The reaction is of interest, because it involves not only modification of an existing thiol group but its later removal from the amino acid moiety in the form of tetraphrothiophene. In the case of protein reaction, this could lead to the production of a new sequence of amino acids by the replacement of an existing molecule of bound cysteine by a molecule of bound lantihione or bound aminoaacrylic acid (89). Bound lantihione would result if the sulphonium compound was attacked by another molecule of ionized glutathione, whereas bound aminoaacrylic acid would result by
hydrolytic decomposition (Chart 8). The dehydropeptide or dehydroprotein might undergo addition of a molecule of glutathione or other thiol forming bound lanthionine.

It is extremely difficult to assess the importance of these reactions when considering the mechanism of action of Myleran. It has been stated (21) that the low level of reaction of Myleran with nucleic acids in vitro and in vivo at therapeutically effective doses makes these centers seem unlikely vital points of attack in this case. It is possibly relevant that, in the series of dimethanesulfonyloxy esters, those having four or five methylene groups are the most active in depressing granulocyte count and are also those which could undergo the ring closure of the optimum spacing of the reactive centers, since if reactivity toward anions is low and for steric reasons ring closure and dethiolation are not possible in this case, then the relevance of these changes to the mode of action of Myleran would be questionable. Work on this aspect of the problem is in progress, with highly tritiated dimethylmyleran.

In connection with the mode of action of Myleran, Timmis (85), on the basis of its similarity in action to thioguanine, has suggested that it might combine with nucleic acids in vivo or with nucleotides to form a type of antimetabolite which could act by lengthening intermitotic intervals rather than directly killing cells. He suggested that Myleran might first interact with homocysteine to form a compound capable of alkylating purine bases in a similar manner to adenosyl-methionine.

Metabolism of aromatic nitrogen mustards and mustard gas.—It was considered of interest in view of the ring closure reaction of Myleran to determine whether similar reactions occurred in vitro and in vivo in the case of the bifunctional mustards. Reaction with thiol groups in vivo seemed probable, and in the case of the bifunctional compounds ring closure also seemed probable in view of the optimum spacing of the reactive centers, since six-membered rings are normally particularly stable.

Under in vitro conditions with the use of bis-chloroethylaniline, bis-chloroethyl-p-anisidine, or mustard gas, and either cysteine or glutathione, evidence was obtained that intermediate sulfonium compounds were in fact formed (70), but their instability was such that isolation proved impossible. In the presence of excess cysteine or glutathione the only products isolated were the cross-linked cysteinyl or cross-linked bound cysteinyl compounds, in contrast to the situation found with Myleran. Thus, in this case dethiolation did not occur. Assuming the formation of an intermediate sulfonium ion, the reaction sequence followed in the case of glutathione and aniline mustard would be as shown in Chart 9. The secondary reaction of the sulfonium ion with glutathione was obviously so fast that hydrolytic cleavage to 4-phenylthiazan was not possible. The interesting feature of these reactions is, of course, the point of attack on the sulfonium compound by the
glutathione molecule, leading to ring opening, to form cross-linked products instead of dethiolation and ring release as in the case of Myleran.

On the basis of these observations, cross-linking of protein strands in vivo following treatment with reactive bifunctional mustards seems likely (71), and because of the chemical properties of the mustards discussed earlier, restriction as to the cellular sites involved would seem less likely than with powerful Sn2 reactors such as iodoacetate. Thus, the mustards because of their unreactive nature prior to ionization would be more capable of deeper penetration into cells and, hence, reaction with a greater range of thiols than the Sn2 reactors of the iodoacetate type. The arguments used in the past to dismiss the importance of thiol reaction in determining any of the important pharmacological properties of the alkylating agents are for these reasons not valid (74, 82). The range of so-called specific -SH reactors tested for their capacity to inhibit tumor growth, etc., has been limited; the compounds have often been monofunctional, when it is known that bifunctionality is a requisite for high antitumor activity; and most of the bifunctional agents tested have not had their reactive centers spaced an optimum distance apart (58). In addition, account was obviously not taken of the probability that, because of the inherent chemical reactivity of such compounds, they would probably never reach cellular sites accessible to agents such as the mustards, epoxides, ethyleneimines, and, to a lesser extent, the aliphatic methanesulfonates. Now that it is known that both the mustards and the aliphatic methanesulfonates are capable of extensive thiol reaction with -SH groups in vivo, coupled with the knowledge that the mustards can cross-link, while Myleran can bring about a unique type of dethiolation and at cellular sites probably inaccessible to -SH reactors of the iodoacetate type, it will be necessary to reconsider the possible relevance of particular types of -SH reaction to the mode of action of the alkylating agents. Greater knowledge of the site and extent of reaction is required and, in particular, whether thiol reaction in the nucleus plays any role in inhibition of cell division or in those processes leading to malignancy. If in the future alkylaion of proteins or peptides and, in particular, alkylaion of those bearing reactive thiol groups is shown to have no part to play in determining any of the important pharmacological properties of the alkylating agents, the experiments leading to such conclusions will be mainly those which now remain to be done, since those which have been done can lead us to no definite conclusion. The writer is not a supporter of any theory of mode of action whose foundations rest on particular reactions at particular cellular sites, which excludes other possibilities, but he wishes to draw attention to certain oversimplifications in reasoning which have led to premature conclusions regarding -SH reaction, where in fact a great deal of work remains to be done. It may transpire that much of the reasoning used in the past to explain the mode of action of alkylating agents has its foundation in oversimplification, and although theories to explain certain pharmacological events are often useful in leading to further experimentation, they can be a drag on rapid progress if their exponents and others regard them as an end in themselves.

Reaction of alkylating agents with nucleic acids.— Since the importance of the nucleic acids in cellular metabolism was established, many workers have been attracted to the idea that all the important biological properties of the alkylating agents can be explained on the basis of reaction with nucleic acids, particularly DNA. Since the alkylating agents cause gross mitotic abnormalities and can effect gene mutations, this hypothesis has always had many followers. Nobody would doubt that the alkylating agents lead to an alteration in the structure and function of nuclear DNA and consequently in cellular integrity, but there is evidence that some of these alterations might be mediated by indirect effects at many sites, such as those indicated earlier when thiol reaction was being considered.

The effects of the alkylating agents on DNA, RNA, and protein synthesis have been recently well reviewed by Wheeler (87). It transpires that the mustards are capable of inhibiting DNA synthesis to a greater extent than RNA synthesis, whereas the methanesulfonates such as Myleran have little effect on either—results which may help to clarify the apparent differences in pharmacological properties of the two classes of compound.

Considering the popularity of the concept of direct reaction on DNA it seems incredible that the actual loci of the reactions should be overlooked for so long. Despite the work of Young and Campbell (90), who first drew attention to the reactivity of the guanine moiety, and of Wheeler, Morrow, and Skipper (88, 89), who pointed out that quaternization of a ring nitrogen atom was likely in the reaction of certain purines with alkylating agents, attention continued to be directed toward reaction with ionized phosphate groups as the most probable points of reaction in the nucleotides (9, 74). Although esterification of phosphate groups seems probable in some cases it is a difficult reaction to demonstrate because of the lability of the
products formed and because differences in the liberation of acid, interpreted as being due to esterification, also occur following quaternization of the ring nitrogen atoms. It remained for Lawley and Wallick (50), possibly stimulated by the remarks of Skipper (80), to demonstrate that dimethylsulfate reacted with guanylic acid to form 7-methylguanine, and it was further shown by Lawley (49) that the product formed from deoxyguanylic acid and dimethylsulfate was much more labile and that liberation of 7-methylguanine occurred appreciably at pH 7.2 (37° C.). Thus, a mechanism was established by which alkylation of guanine moieties of DNA could effect changes at pH 7, and in this case breakdown probably occurs with the formation of an aldehydropyrimidine derivative by ring opening (Chart 11).

In the case of reaction with DNA, the apurinic acid formed after removal of the alkylated guanine will be relatively unstable and could lead to main chain breakdown.

These reactions may be of fundamental importance in explaining some of the pharmacological effects of the alkylating in mechanistic chemical terms. Thus it was postulated (55) that the greater efficiency of certain bifunctional alkylating agents in killing cells and interfering with mitosis might be due to their capacity to cross-link parts of DNA, or DNA to protein, or protein to protein. We have seen how proteins might be cross-linked (71), some evidence is available for linking of nucleic acid to protein (77), and Alexander, Cousins, and Stacey (4) obtained evidence of cross-linking of DNA in nucleoprotein with nitrogen mustards. This they interpreted as being solely due to reaction with phosphate group, but in the light of the findings of Brookes and Lawley (21) that bis-guanine products are formed after reaction of various alkylating agents with nucleic acids, they have had to reconsider their interpretation, and it is the belief of Lett, Parkins, and Alexander (51) that in some cases the alkyl groups of phosphate esters can transalkylate to the 7-position of guanine accounting for the apparently higher reactivity of guanine when in DNA than when it appears in nucleosides (75, p. 79). The main evidence which they present for this comes from spectral data, the U.V. changes which accompany base alkylation being delayed in some cases as if two reactions were occurring; and they also interpret changes in “coiling” as being due to esterification. Whatever the true interpretation, the fact remains that parts of DNA can be cross-linked in vivo, and DNA will be more or less inactivated and degraded depending on the extent of reaction. Some of the cytotoxic effects of, say, the mustards will be explicable on this basis, but whether the whole complex process of lysis of fully differentiated lymphocytes, for example, can be explained entirely by such mechanisms remains to be tested.

Brookes and Lawley (21, 22) have studied the extent of combination of various alkylating agents with DNA and have found a simple rule to apply—the more reactive the compound and the greater its tendency to react with all nucleophiles, the greater the extent of reaction with the guanine moiety. Thus the mustards reacted extensively, whereas the methanesulfonates, halides, and sul-

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**Chart 10.** Reaction of deoxyguanylic acid or guanylic acid with dimethyl sulfate.

**Chart 11.** Breakdown of an alkylated guanylic acid to form an aldehydropyrimidine derivative.
fates reacted much less extensively and more slowly. These findings again reflect the differences in pharmacological properties displayed by the various compounds. Thus the mustards, highly reactive toward most nucleophiles, able to penetrate deep into cells, able to cross-link nucleic acids and probably proteins, are able to create havoc among cells, causing effects from inhibition of cell division to complete cellular disruption and lysis in contrast to the less reactive reagents such as Myleran which would be expected to react very little with DNA at therapeutic doses and unable to cross-link proteins through their thiol groups (see earlier). The greater efficiency of mustards in causing cell death probably results from their heightened capacity to react with all nucleophiles within the cell, not merely to their greater reactivity toward DNA.

The low level of reaction of Myleran with nucleic acids in vivo leads one to seek for other explanations for its pharmacological effects. There is no scope in this discussion for considering other implications of DNA reaction such as mutagenesis and the difficulties which have been found in interpreting some of the biological phenomena encountered—for example, the "uniqueness of ethylation" (23, 51, 53); but let it suffice to say that, although difficulties have been encountered in interpreting mutation in the light of phosphate esterification (2), a working hypothesis has been put forward by Lawley and Brookes (24) which seems to fit most of the facts and which can probably be tested.

**Mutation and Cancer**

Although bifunctionality in alkylating agents is a normal requisite for high killing action against cells, this is not the case with regard to mutagenesis and carcinogenesis, since in these respects monofunctional agents are often as active or more active than their bifunctional counterparts, and this is in line with the concept that cross-linking is lethal. Some evidence exists that the physiological state of the cell influences the occurrence of 'spontaneous' or induced chromosomal abnormalities (15–17) in some cases; Loveless (54) has strongly supported the hypothesis that mutation in the case of the alkylating agents involves direct attack on nucleic acid. The fact that monofunctional alkylating agents can be both mutagenic and carcinogenic could be used as evidence to support the mutational theory of cancer. On the other hand, one should not ignore appealing alternatives which involve much more extensive alteration of cellular integrity and alteration in delicately balanced cellular homeostasis. There is much evidence to support an alteration in the integrity of mitochondria and the endoplasmic reticulum during administration of carcinogens (8, 9), a consequence of the alteration of lipo-protein membranes which constitute the structural support of the organized multi-enzyme complexes. Thus 3'-DAB (dimethylaminonozobenzene) has a profound effect on microsomal swelling when fed for 4 weeks (6), in contrast to the inactive 2-methyl DAB, and the former compound also produces a significant decrease in the mitochondrial population in the cell (64, 78). During the early stages of carcinogenesis with any agent the cell population is being continuously modified, and many cells die as a result of excessive interference with their cytoplasmic and nuclear elements. Those extensively modified cells which survive will be those which have been able to adapt themselves to the unfavorable conditions; but with many of their complex feedback controls and fine structure damaged they will be unable to respond to the normal stimuli of intercellular dependence and will cease to obey the stringent rules of organismal organization (81). The great complexity of the intracellular effects produced by the alkylating carcinogens, ranging from slight alterations in cell structure to cell death, makes the problem of sorting out the chain of events leading to malignancy one of the utmost difficulty. The relatively long time lapse between treatment with the carcinogen and the emergence of malignant tumors is probably related to the multitude of changes occurring in the cell population during the 'adaptive' period. The oncogenic viruses are probably more specific in their effect and faster acting because of their information content and may eventually prove to be better tools for elucidating the chain of events leading to malignancy than the chemical carcinogens which in many ways have not lived up to earlier expectations.

**Conclusion**

No attempt has been made to discuss the actual biochemical lesions such as effects on cellular synthetic mechanisms which result from the action of the alkylating agents. Effects on glycolysis, enzymes, and so on have been well reviewed by Wheeler (87). Rather, an attempt has been made to correlate the basic alkylating capacities of the various compounds with gross biological end products, and some attempt has been made to classify the various agents into groups or classes as far as possible.

Some conclusions can be reached regarding
these comparisons, the main one being that the most reactive compounds of the Sn1 or pseudo-Sn1 type with a satisfactory "spread" in their reactivity toward all nucleophiles are those which manifest the most diverse pharmacological effects. It must also be relatively obvious from the foregoing discussion that none of the studies carried out on the mechanism of action of alkylating agents has given any information as to how better drugs with a greater selectivity of action might be designed, although suggestions have been made as to why certain drugs are more efficacious than others. This statement is depressing, and yet the obvious must be faced—and this is that the alkylating agents so far designed are far too crude in their killing properties to be of any permanent use in cancer therapy. Of course one must always leave room for the unexpected breakthrough which may take the form of a drug with a special "carrier" or a drug possessing "latent" activity, and yet attempts which have been made so far to make this kind of breakthrough have proved somewhat disappointing. Thus, for example, Ross and Warwick (76, 86) made a series of mustard derivatives of azobenzene which would remain chemically unreactive until reduction of the azo linkage occurred in vivo, and it was hoped that some selectivity of action might be achieved. However, the compounds are relatively toxic, and none (40) has undergone clinical trial with disappointing results. Yet more of this type of research is needed.

Compounds possessing all the various types of chemical reactivity have been tried, and no obvious solution to the problem of what to try next has been forthcoming. Thus, compounds such as Myleran, Chlorambucil, etc., which are now used as palliatives in the treatment of chronic leukemia and Hodgkin's disease, have certainly played a part in relieving distressing symptoms and have enabled certain patients to remain active when otherwise they may have been bedridden; and in this sense the efforts which have gone into planning them and synthesizing them has probably been justifiable. However, whether many more of these compounds should be made is a debatable point.

The alkylating agents have stimulated much research on fundamental problems concerning cell metabolism, mutagenesis, carcinogenesis, and so on, and it is probably in these fundamental ways that they have proved most useful. However, their very number, the diversity of pharmacological effects which they manifest, and the number of cellular sites with which they interact preclude facile explanations for their mode of action. We now have some idea of the chemical sites with which a few of them interact intracellularly, but from a very mechanical organic chemical viewpoint. Much more work now needs to be directed to the study of their effects in relation to cell population dynamics and their effects in relation to intercellular dependence and to dedifferentiation, since it is in these dynamic aspects that the explanation of the carcinogenic process lies.

Wheeler (87) indicates at the conclusion to his review that there is a need "to pinpoint the critical site of alkylation and the real cause of anticancer activity." Assuming that there is a "critical site of alkylation," which is debatable, one wonders whether knowing it would help in designing better chemotherapeutic agents. We now have drugs which will attack almost every cellular site, and it is difficult to envisage a new type of alkylating agent which has not yet been tried.

More research on the effects of combinations of drugs on tumors might be worth while—there would seem to be endless possibilities to exploit—and the recent work of Dean and Alexander (27) demonstrating the potentiating effect of iodoacetamide on the destructive effect of radiation on cultured lymphoma cells and bacteria is encouraging. In this connection, Roberts and Warwick observed the effect of pretreatment of rats with a large dose of ethyl methanesulfonate prior to injection with Myleran on the peripheral blood count. It was hoped that ethyl methanesulfonate, while inactive itself against granulocytes, might potentiate the effects of Myleran, since it is itself a thiol reactivator. Preliminary results have proved disappointing, but in some respects rats are not ideal test subjects from the point of view of following minor variations in peripheral blood count due to large background fluctuations, and the experiment will probably be repeated in some other way. Bearing in mind the chemical, as well as the biological, properties of the various agents, some interesting combinations which have not yet been tried might come to light.

At this Institute further attempts are being made to exploit differences between normal and tumor tissue in several ways which have been described at length by Ross (72), and the attempts being made by Connors and Elson (25) to lower general toxicity while maintaining antitumor activity by prior treatment with certain compounds such as thiols, etc., is worthy of comment. Some of these studies assume different pH levels between tumor tissue and normal tissue, and results are

* Unpublished data.
awaited with interest. Studies on the mechanisms of resistance are urgently required, but this subject is to be covered by Dr. Wheeler.

Some fresh approaches are needed to lift cancer chemotherapy from the somewhat unsatisfactory situation in which it finds itself, but most of all is needed a really critical assessment from all points of view of the situation as it stands at the moment.

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