Studies Related to Mechanisms of Resistance to Biological Alkylating Agents

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SUMMARY

Several possible mechanisms of resistance to alkylating agents have been posed and discussed on the basis of presently available experimental data. The two mechanisms that appear to be most plausible are altered cell permeability toward the agents and deactivation of the agents by combination with nonprotein sulfhydryl groups. These two mechanisms might function simultaneously and might contribute to both “natural” and “acquired” resistance. However, definitive evidence supporting any specific mechanism is scant.

In the report of the first chemotherapeutic use of nitrogen mustard for combating neoplasia, Dr. C. P. Rhoads stated, “The tumor regressions induced by these compounds (even with maximum dosages) are temporary, with maximal persistence rarely extending beyond several months,” and, in reference to the treatment of Hodgkin’s disease, “... improvement ... continues only from two weeks to a few months and is followed by fairly rapid relapse. The relapses may respond to further therapy, but the remissions induced are progressively shorter in duration” (76). Thus the problem of development of resistance to alkylating agents was recognized in the earliest studies with these agents, and the development of resistance toward a variety of agents by a variety of human cancers has been reported (45). It is also well known that many types of human cancers (38, 76) and cancers in lower animals (19, 83, 88, 116) do not respond to treatment with alkylating agents and are said to be “naturally resistant.” Knowledge of these facts leads one to pose some interesting questions:

1. Is it possible that “acquired resistance” and “natural resistance” are really the results of similar mechanisms?
2. Is the slight specificity of the effects of alkylating agents for neoplastic tissue compared to host tissues actually due to quantitative differences in the degrees of functioning of the mechanisms of resistance?
3. Is there a common mode of resistance for the various types of alkylating agents?
4. Does cross-resistance to several agents necessarily indicate a common mechanism of action of the agents?
Although the problem of resistance to alkylating agents has been recognized for as long a time as the agents have been used, formal studies of the mechanisms of this resistance have been undertaken only recently. It is believed that information concerning the basic mechanism(s) of resistance to alkylating agents might be of value in (a) preventing the development of resistance in patients who initially have favorable responses to treatment, (b) developing means of obtaining favorable responses in patients who have neoplasms that are "naturally resistant" to alkylating agents, and (c) defining the mode(s) of action of alkylating agents.

Since it is possible that mechanisms of action and mechanisms of resistance might be closely related, it is logical that studies of the two types of mechanisms be carried out simultaneously. Such a unified program would of course be facilitated by the availability of a drug-sensitive line of a biological system and a subline of the same biological system that had acquired resistance to the agent. Recognition of this fact has led investigators to attempt to isolate drug-resistant sublines of a variety of systems including bacteria, animal tumors, and cultured cells, and the success of these attempts is reflected by the list of some of the resistant sublines that are now available for experimental use (Table 1). Another type of dual system that is useful for such studies consists of tumors that are derived from the same tissue of origin but have different degrees of natural resistance toward certain agents—for example, the ascites hepatomas that have various sensitivities to nitrogen mustard N-oxide (110).

It appears that the degree of resistance of cells is under genetic control, since resistance to alanine mustard (52, 53), phenylalanine mustard (75), and mitomycin C (54) was reported to be conferred upon sensitive cells by incubating these sensitive cells in vitro with DNA or nucleoprotein isolated from resistant cells. This resistance was also reported to be stable through several transplant generations in animals (54, 52, 53). Some degree of resistance to phenylalanine mustard was conferred upon Sarcoma 45 by injecting nucleoprotein isolated from phenylalanine mustard-resistant Sarcoma 45 into animals immediately following the implantation of phenylalanine mustard-sensitive Sarcoma 45 (75).

It has often been observed that tissues or cells that possess acquired resistance to one alkylating agent are also resistant to other types of alkylating agents (Table 2). Also, natural resistance toward a variety of alkylating agents by a single animal neoplasm is not uncommon (88, 101, 115). Cross-resistance to ultraviolet radiation has been observed in bacteria, but it appears that animal tumors that have acquired resistance to alkylating agents are not resistant to x-radiation. Also, in a group of rat ascites hepatomas possessing various degrees of resistance to alkylating agents, there was no correlation between sensitivity to x-radiation and sensitivity to alkylating agents (39, 40). Cross-resistance to mitomycin C has been observed, and mitomycin C is included in this report, since it has a number of biological effects similar to those of the common alkylating agents and since it contains an ethylenimino group (104). Cross-resistance to various alkylating agents by human tumors is less common than by rodent tumors, and favorable clinical responses have been reported for a second agent after resistance had developed to the first agent used (14, 25, 54, 85, 99, 110). Although such clinical studies are of great importance, it must be admitted that they are difficult to quantitate.

Although the existence of cross-resistance has frequently been used as evidence to support the idea that the respective drugs have a common mode of action, such a conclusion is not necessarily warranted in the case of the alkylating agents. For example, two alkylating agents might have different specific sites of action that cause their particular biological effects, but they might be prevented from reaching those sites by a common mechanism such as reduced membrane permeability or detoxification. Such possibilities should be kept in mind when one is considering the various possible mechanisms of resistance to alkylating agents and the relationship of the mechanism of resistance and the mechanism of action.

In a number of studies the effects of alkylating agents upon the biological and biochemical properties of sensitive and resistant tumors have been compared for the purpose of pointing out effects that might be critical to the therapeutic effect of the agent and of eliminating effects that are common to both the sensitive and resistant tumors and are therefore of no therapeutic significance. Such investigations are of value in determining the mechanism of action of the agent, but they might yield little or no information related to the mechanism of resistance toward the agent. Table 3 lists experiments of this type and shows the qualitative results obtained.

**POSSIBLE MECHANISMS OF RESISTANCE**

Several authors have listed possible mechanisms of resistance to miscellaneous drugs (9, 35, 59, 77, 113). Although a number of these mechanisms...
might be applicable to alkylating agents, some that might be logical possibilities for agents that act as antimetabolites, such as altered enzyme affinity and increased competition by a normal metabolite for an enzyme, would probably not be possibilities for alkylating agents. On the other hand, other possible mechanisms, such as combination with noncritical cellular components, might be more likely to be applicable to alkylating agents than to some other types of drugs. The remainder of this report will consist of a listing of possible mechanisms of resistance that might be applicable to alkylating agents and a presentation and consideration of presently available experimental results that are related to these possibilities.

Altered Transport of the Agent to the Cells

This type of alteration might result from differences in the degree of vascularization of the tumors

### TABLE 1

**BIOLOGICAL SYSTEMS WITH ACQUIRED RESISTANCE TO ALKYLATING AGENTS**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Biological system</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen mustard</td>
<td><em>Escherichia coli</em></td>
<td>5, 23, 24, 45, 84, 112</td>
</tr>
<tr>
<td></td>
<td>Yoshida ascites sarcoma</td>
<td>42, 53</td>
</tr>
<tr>
<td></td>
<td>Dunning leukemia</td>
<td>46</td>
</tr>
<tr>
<td>Nitrogen mustard N-oxide</td>
<td><em>Escherichia coli</em></td>
<td>23, 112</td>
</tr>
<tr>
<td></td>
<td>Yoshida sarcoma</td>
<td>1, 87, 88, 98, 116, 117</td>
</tr>
<tr>
<td></td>
<td>Hiroaki sarcoma</td>
<td>73, 74</td>
</tr>
<tr>
<td></td>
<td>Rat ascites hepatoma AH-130</td>
<td>102, 103</td>
</tr>
<tr>
<td></td>
<td>Rat ascites hepatoma AH-13</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Rat ascites hepatoma AH-7974</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>Ehrlich ascites carcinoma</td>
<td>98</td>
</tr>
<tr>
<td>Phenylalanine mustard</td>
<td>Sarcoma 45</td>
<td>57, 84, 118</td>
</tr>
<tr>
<td></td>
<td>Dunning leukemia</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>Yoshida ascites hepatoma</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>Walker carcinosarcoma 256, pulmonary form</td>
<td>82</td>
</tr>
<tr>
<td>Chlorambucil</td>
<td>Sarcoma 45</td>
<td>50</td>
</tr>
<tr>
<td>Alanine mustard</td>
<td>Yoshida sarcoma</td>
<td>52, 53</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>Leukemia L1210</td>
<td>55, 56</td>
</tr>
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<td></td>
<td>Hamster plasmacytoma</td>
<td>87</td>
</tr>
<tr>
<td>N-Acetyl-N',N'-bis(2-chloroethyl)-p-phenylenediamine</td>
<td>Walker carcinosarcoma 256</td>
<td>8</td>
</tr>
<tr>
<td>Degranol</td>
<td>Ehrlich ascites carcinoma</td>
<td>18</td>
</tr>
<tr>
<td>Triethylene melamine</td>
<td>Walker carcinosarcoma 256</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>Ehrlich ascites carcinoma</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Sarcoma 180 (ascitic form)</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Hamster plasmacytoma</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>Sarcoma 45</td>
<td>84</td>
</tr>
<tr>
<td>ThioTEPA</td>
<td>Hiroaki sarcoma</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Cultured ovarian cancer cells (9-99)</td>
<td>70</td>
</tr>
<tr>
<td>1-Methyl-3-nitro-1-nitrosoguanidine</td>
<td><em>Escherichia coli</em></td>
<td>23, 60</td>
</tr>
<tr>
<td>1-(b-Chloroethyl)-3-nitro-1-nitrosoguanidine</td>
<td><em>Escherichia coli</em></td>
<td>23</td>
</tr>
<tr>
<td>1-(b-Chloropropyl)-3-nitro-1-nitrosoguanidine</td>
<td><em>Escherichia coli</em></td>
<td>23</td>
</tr>
<tr>
<td>Dopan</td>
<td>Sarcoma 45</td>
<td>118</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td><em>Escherichia coli</em></td>
<td>23, 24, 48</td>
</tr>
<tr>
<td></td>
<td>Hiroaki sarcoma</td>
<td>73, 74</td>
</tr>
<tr>
<td></td>
<td>Yoshida ascites sarcoma</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Jensen rat sarcoma</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>Rat ascites hepatoma AH-13</td>
<td>31</td>
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<table>
<thead>
<tr>
<th>SELECTED FOR RESISTANCE TO:</th>
<th>BIOLOGICAL SYSTEM</th>
<th>Also resistant to:</th>
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<tbody>
<tr>
<td></td>
<td>HN2</td>
<td>Tha</td>
</tr>
<tr>
<td>HN2</td>
<td>Escherichia coli</td>
<td>Y</td>
</tr>
<tr>
<td>Yoshida ascites sarcoma</td>
<td>Jansen sarcoma</td>
<td>Y</td>
</tr>
<tr>
<td>HN2-O</td>
<td>Yoshida ascites sarcoma</td>
<td>Y</td>
</tr>
<tr>
<td>Hirokami sarcoma</td>
<td>Escherichia coli</td>
<td>Y</td>
</tr>
<tr>
<td>Phenylalanine mustard</td>
<td>Dunning leukemia</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Walker 256, pul-</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>monary form</td>
<td>Yoshida ascites</td>
</tr>
<tr>
<td></td>
<td>Hepatoma</td>
<td>Y</td>
</tr>
<tr>
<td>1357</td>
<td>Alamine mustard</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Yoshida sarcoma</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Hamster pla-</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>smycytona</td>
<td>Y</td>
</tr>
<tr>
<td>TEM</td>
<td>Walker 256</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Hamster pla-</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>smycytona</td>
<td>Y</td>
</tr>
<tr>
<td>ThioTEPA</td>
<td>Hirokami sarcoma</td>
<td>Y</td>
</tr>
<tr>
<td>NG</td>
<td>Escherichia coli</td>
<td>Y</td>
</tr>
<tr>
<td>CP</td>
<td>Escherichia coli</td>
<td>Y</td>
</tr>
<tr>
<td>CE</td>
<td>Escherichia coli</td>
<td>Y</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Yoshida ascites</td>
<td>Y</td>
</tr>
<tr>
<td>sarcoma</td>
<td>Jensen rat sarcoma</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Hirokami sarcoma</td>
<td>Y</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td>Y</td>
</tr>
</tbody>
</table>

* Y (yes) indicates that the biological system was also resistant to the agent listed at the top of the column. N (no) indicates no resistance to the agent at top of column. — Indicates that no test was run. See footnote in text for meanings of abbreviations.

† Resistant to low doses but not large doses.
### TABLE 3

**EFFECTS OF ALKYLATING AGENTS UPON SENSITIVE AND RESISTANT CELLS**

<table>
<thead>
<tr>
<th>Property Examined</th>
<th>Test System</th>
<th>Agent</th>
<th>Effect on:</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>Suppressed</td>
<td>20, 22</td>
</tr>
<tr>
<td>Respiration</td>
<td>Sarcoma 45</td>
<td>Dopan</td>
<td>Stimulated</td>
<td></td>
</tr>
<tr>
<td>Respiration</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard</td>
<td>Suppressed</td>
<td>100</td>
</tr>
<tr>
<td>Respiration</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard N-oxide</td>
<td>No change</td>
<td>100</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard</td>
<td>Suppressed</td>
<td>100</td>
</tr>
<tr>
<td>Glycolysis</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard N-oxide</td>
<td>No change</td>
<td>100</td>
</tr>
<tr>
<td>Concentration of nicotinamide adenine</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>96% decrease</td>
<td>21</td>
</tr>
<tr>
<td>diadenine diucleotide</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>74% decrease</td>
<td></td>
</tr>
<tr>
<td>Glycine-1-C\textsuperscript{14} into</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>Suppressed</td>
<td>10</td>
</tr>
<tr>
<td>nucleoproteins</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>No change</td>
<td>10</td>
</tr>
<tr>
<td>Glycine-1-C\textsuperscript{14} into</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>Suppressed</td>
<td>10</td>
</tr>
<tr>
<td>RNA</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>No change</td>
<td>10</td>
</tr>
<tr>
<td>$^3$P into RNA</td>
<td>Sarcoma 45</td>
<td>Phenylalanine mustard</td>
<td>Suppressed</td>
<td>71</td>
</tr>
<tr>
<td>Glycine-1-C\textsuperscript{14} into</td>
<td>Rat ascites hepatomas AH-130 and AH-7974</td>
<td>Nitrogen mustard</td>
<td>Stimulated</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
<td>Rat ascites hepatomas AH-130 and AH-7974</td>
<td>ThioTEPA</td>
<td>Suppressed</td>
<td>69</td>
</tr>
<tr>
<td>Formate-C\textsuperscript{14} into</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard, cyclophosphamide, triethyl-</td>
<td>Suppressed</td>
<td>107</td>
</tr>
<tr>
<td>RNA-adenine</td>
<td>Hamster plasmacytoma in vivo</td>
<td>emelamine, or thioTEPA</td>
<td>No change</td>
<td></td>
</tr>
<tr>
<td>Formate-C\textsuperscript{14} into</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>Suppressed</td>
<td>107</td>
</tr>
<tr>
<td>DNA-adenine</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>No change</td>
<td>107</td>
</tr>
<tr>
<td>Adenine-8-C\textsuperscript{14} into</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>Suppression</td>
<td>107</td>
</tr>
<tr>
<td>RNA-adenine</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>No change</td>
<td>107</td>
</tr>
<tr>
<td>Adenine-8-C\textsuperscript{14} into</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>Suppression</td>
<td>107</td>
</tr>
<tr>
<td>DNA-adenine</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>No change</td>
<td>107</td>
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</tbody>
</table>

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<table>
<thead>
<tr>
<th>Property Examined</th>
<th>Test System</th>
<th>Agent</th>
<th>Effect on:</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate-C&lt;sup&gt;14&lt;/sup&gt; into soluble compounds</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>Suppression</td>
<td>107</td>
</tr>
<tr>
<td>Adenine-8-C&lt;sup&gt;14&lt;/sup&gt; into soluble compounds</td>
<td>Hamster plasmacytoma in vivo</td>
<td>Nitrogen mustard</td>
<td>No change</td>
<td>107</td>
</tr>
<tr>
<td>Orotic acid-2-C&lt;sup&gt;14&lt;/sup&gt; into RNA</td>
<td>Rat ascites hepatomas AH-180 and AH-7974</td>
<td>Nitrogen mustard</td>
<td>No change</td>
<td>63</td>
</tr>
<tr>
<td>Orotic acid-2-C&lt;sup&gt;14&lt;/sup&gt; into DNA</td>
<td>Rat ascites hepatomas AH-130 and AH-7974</td>
<td>Nitrogen mustard</td>
<td>Suppressed</td>
<td>66</td>
</tr>
<tr>
<td>Orotic acid-2-C&lt;sup&gt;14&lt;/sup&gt; into RNA</td>
<td>Homogenates of hepatomas AH-130 and AH-7974</td>
<td>Nitrogen mustard</td>
<td>Suppressed</td>
<td>66</td>
</tr>
<tr>
<td>Content of DNA</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard N-oxide</td>
<td>Decreased</td>
<td>100</td>
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<tr>
<td>Ratio of purines to pyrimidines in RNA</td>
<td>Rat ascites hepatomas AH-180 and AH-7974</td>
<td>Decreased</td>
<td>Decreased less</td>
<td>63, 69</td>
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<tr>
<td>Ratio of purines to pyrimidines in RNA</td>
<td>Rat ascites hepatomas AH-130 and AH-7974</td>
<td>Decreased</td>
<td>No change</td>
<td>63, 69</td>
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<tr>
<td>Glycine-1-C&lt;sup&gt;14&lt;/sup&gt; into protein</td>
<td>Rat ascites hepatomas AH-180 and AH-7974</td>
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<td>No change</td>
<td>63, 69</td>
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<td>Uniformly labeled amino acids into protein</td>
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<td>No change</td>
<td>63, 69</td>
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<td>Methionine-S&lt;sup&gt;35&lt;/sup&gt; into proteins</td>
<td>Leukemia L1210</td>
<td>Nitrogen mustard</td>
<td>Suppressed</td>
<td>63</td>
</tr>
<tr>
<td>Alkaline phosphatase activity</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard N-oxide</td>
<td>Suppressed</td>
<td>63</td>
</tr>
<tr>
<td>Acid phosphatase activity</td>
<td>Yoshida sarcoma</td>
<td>Nitrogen mustard N-oxide</td>
<td>No change</td>
<td>89</td>
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<tr>
<td>Structural viscosity of DNA</td>
<td>Sarcoma 45</td>
<td>Nitrogen mustard N-oxide</td>
<td>Increased</td>
<td>100</td>
</tr>
<tr>
<td>Swelling and fragmentation of mitochondria</td>
<td>Rat ascites hepatomas AH-180 and AH-7974</td>
<td>Nitrogen mustard N-oxide</td>
<td>Increased</td>
<td>100</td>
</tr>
<tr>
<td>Combining of S-RNA with C&lt;sup&gt;14&lt;/sup&gt;-labeled amino acids</td>
<td>Rat ascites hepatomas AH-180 and AH-7974</td>
<td>Nitrogen mustard</td>
<td>Decreased</td>
<td>51</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Occurred extensively</td>
<td>86</td>
</tr>
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<td></td>
<td></td>
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<td>Slight</td>
<td>86</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Suppressed</td>
<td>63</td>
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so that less agent would reach the tumor in a given time; this might be important for agents that have a short half-life in the animal. Contrary to this possibility, however, carbon-14 from phenylalanine mustard-\(^{14}\)C was found to be present in phenylalanine mustard-resistant Sarcoma 45 to a greater extent than in the sensitive Sarcoma 45 following the administration of the radioactive agent to animals bearing the respective tumors (72). This was true even if the two tumors were grown bilaterally in the same animal. Also, cyclophosphamide-sensitive and cyclophosphamide-resistant plasmacytomas grown bilaterally in hamsters contained approximately equal quantities of \(^{14}\)C following the intraperitoneal injection of cyclophosphamide-\(^{14}\)C, nitrogen mustard-\(^{14}\)H, or uniformly labeled thioTEPA-\(^{14}\)C. Thus, for these two types of tumors it appears that the drug, or a metabolic product derived from the drug, reaches the resistant tumor as extensively as it reaches the sensitive tumor.

Differences in the effects of alkylating agents upon the metabolism of sensitive and resistant tumors have also been detected in in vitro systems in which the degree of vascularization of the tumors would not be a factor.

Although it would be expected that the degrees of vascularization of a sensitive tumor and its resistant subline would be similar, it is possible that there are differences in vascularization of tumors of various types and that these differences might partially determine the degree of natural resistance. The possible role of such differences in vascularization has not been investigated.

**Altered Permeability of the Cells**

It is possible that the radioactive compound that reached the tissues referred to in the preceding section was fixed on the outer cell membranes and did not actually enter the cells. However, in the experiments with the hamster tumors, samples of the tissues were homogenized, and crude nuclear, mitochondrial, microsomal, and supernatant fractions were separated by means of centrifugation and assayed for radioactivity. The specific activities and the distribution of radioactivity among the fractions obtained from the resistant plasmacytoma were similar to those for the sensitive tumor. The data would indicate that the radioactive compound not only reached both tumors but also penetrated the cells of both tumors.

The permeability of ascites hepatoma cells (AH-13) and cells of a nitrogen mustard N-oxide-resistant subline of this tumor to nitrogen mustard and nitrogen mustard N-oxide was studied by determining the sulfhydryl content of the intact cells and of cells with membranes destroyed by homogenization, before and after incubation with the alkylating agents (32). It was concluded that there was no significant difference in the permeability of the cells of these tumors to these agents. No significant difference in the cell walls of the cells of the original cell line and the resistant cell line was detected by electronmicroscopy (33). Likewise, no difference in the cell membrane and the microvilli of Yoshida ascites sarcoma cells and of cells of a subline of the Yoshida ascites sarcoma resistant to nitrogen mustard N-oxide was detected (33).

Contrary to the results obtained with the tumors having "acquired resistance," results obtained by other workers who were studying the effect of nitrogen mustard upon the in vitro incorporation of \(^{32}\)P into the nucleotides of sensitive ascites hepatoma cells (AH-130) and naturally resistant ascites hepatoma cells (AH-7974) led to the suggestion that the AH-7974 cells might be less permeable to the agent than AH-130 cells (69). This suggestion was substantiated by the observation that nitrogen mustard inhibited the in vitro incorporation of orotic acid-\(^{14}\)C into the nucleic acids of whole AH-130 cells more than that into whole AH-7974 cells, but the difference in effects upon the two cell lines disappeared when cell homogenates rather than whole cells were used (66). Other studies showed that treatment of sensitive ascites hepatoma cells with Tween 80 increased the sensitivity of the cells to nitrogen mustard N-oxide, but no such effect was observed with sublines of Yoshida sarcoma and rat ascites hepatoma having acquired resistance to the agent (114). It was concluded that impairment of transportation of nitrogen mustard N-oxide through the cellular membrane participates to some degree in determining the degree of natural resistance of ascites hepatoma cells to this agent.

It is of interest that, upon incubation of homogenates or cell suspensions of phenylalanine mustard- and dopan-sensitive and -resistant forms of Sarcoma 45, the sensitive tumors bound more of these agents than did the resistant tumors (4).

The few experimental results that are available at present do not establish the importance of the role, if any, that permeability plays in determining the degree of resistance of cells to these agents. However, these results do suggest, but do not establish, that permeability might play roles of different importance in natural resistance and acquired resistance.

\(^{1}\)G. P. Wheeler, unpublished results.
DEACTIVATION OF THE AGENT

Chemical breakdown or catabolism.—Detoxification of the agent by enzymatic catabolic breakdown in the resistant cell is a possible mechanism of resistance. Although this possible mechanism has not been investigated extensively, there is some evidence against it. When minces of cyclophosphamide-sensitive hamster plasmacytoma and the cyclophosphamide-resistant subline tumor (which was cross-resistant to nitrogen mustard, triethylene melamine, thioTEPA, and Myleran) were incubated with nitrogen mustard-C$^{14}$H$_3$ and alcoholic extracts of these minces were used for two-dimensional paper chromatography and radioautography, no difference in the patterns of radioactive spots was detected. Likewise, no difference in patterns was detected when minces of the tumors were incubated with uniformly labeled thioTEPA-C$^{14}$. The existence of the phenomenon of cross-resistance to structurally diverse compounds such as nitrogen mustard, triethylene melamine, thioTEPA, and Myleran makes it seem unlikely that enzymatic catabolic breakdown is the mechanism, because it would not be expected that the same enzyme would be active in the catabolism of these compounds.

It has been suggested that certain cells can detoxify nitrogen mustard or remove it more rapidly from the blood stream than others and that the types of cells within the capillary bed just beyond the injection site might determine the availability of the agent for transport to the tumor (47). This would be a detoxification by the host tissue rather than by the tumor, and one might even speculate that induction of the formation of specific catabolic enzymes in the host tissues might follow exposure to the agent. Such detoxification might make it appear that the tumor had become resistant to the agent. Such a mechanism would be possible for the initially observed resistant tumor, but this mechanism is not feasible for transplanted resistant tumors in new hosts, because sensitive tumors transplanted into similar hosts respond to the agent. Also, the fact that treatment of an animal bearing bilateral implants of sensitive and resistant tumors results in the regression of sensitive tumor whereas the resistant tumor continues to grow (87) rules out detoxification by the host. The concept that the host does not detoxify the agent is consistent with the fact that a tumor may become resistant to an agent without any alteration in the general toxicity of the agent for the host (8, 79 [pp. 155–57]).

Combination with nonessential nucleophilic centers.—It is well known that the various biological alkylating agents combine readily with nucleophilic centers such as organic and inorganic anions, amino groups, and sulfhydryl groups (78). If a tumor contained a relatively large number of such groups that might be expended without excessive damage to the cells, then the tumor cells might be effectively protected from the effects of the agent and the tumor would be observed to be resistant to the agent. Such a mechanism would be analogous to that of certain agents that protect animals and lower organisms from the effects of radiation by combination with the free radicals produced by the radiation (11) or to that of mercaptans or progenitors of mercaptans and of thiosulfate in protecting organisms from the toxic effects of alkylating agents (91, 92). This mechanism of resistance would require that the resistant tumor contain a larger concentration of available nonessential nucleophilic centers than the sensitive tumor.

Determination of the sulfhydryl contents of Yoshida ascites sarcoma cells and of cells of the nitrogen mustard N-oxide-resistant subline of this tumor showed the two tumors to contain approximately the same total number of sulfhydryl groups, but the resistant tumor contained a larger number of nonprotein sulfhydryl groups (30–32). It was suggested that acquired resistance is chiefly due to increased nonprotein sulfhydryl content (30–32). On the other hand, there was no correlation between sulfhydryl content and the degree of natural resistance (30, 31).

Other investigators who have determined the sulfhydryl content of sensitive and naturally resistant animal tumors found no correlation between tumor sensitivity and the quantities of either protein-bound or acid-soluble sulfhydryl groups but did find a good correlation between tumor sensitivity and the ratio of protein-bound sulfhydryl to acid-soluble sulfhydryl—the more resistant the tumor, the lower the ratio.

In view of the fact that rat hepatoma is more sensitive to alkylating agents than host liver, it is of interest that the hepatoma contains less nonprotein and less protein sulfhydryl groups than does liver (90).

Deactivation resulting from altered pH.—It has been demonstrated that the toxicity of nitrogen mustard for mice could be greatly reduced by lowering the pH of the solution that is injected into the mouse (108) and that the rate of reaction of nitrogen mustard and nitrogen mustard N-oxide with the sulfhydryl groups of rat ascites hepatoma

1 G. Calcutt, T. A. Connors, and W. C. J. Ross, personal communication.
AH-49 was reduced by lowering the pH (36). One might speculate then that the decreased effect of nitrogen mustard upon a resistant tumor in comparison with that upon a sensitive tumor might be due to differences in the hydrogen ion concentrations of the tumors. However, no comparison of the pH of sensitive and resistant tumors has been reported, and such a mechanism might be difficult to correlate with the observation of cross-resistance toward two agents, such as nitrogen mustard and triethylenemelamine, whose reactivities are affected oppositely by changes in pH (79).

Immunological deactivation.—Development of specific antibodies for Myleran by rabbits and by humans has been reported, and the appearance of the antibodies coincided with the development of resistance (19). No report of studies of immunological deactivation of other alkylating agents has been found.

Decreased Availability of Critical Targets

Decreased concentration of target molecules or groups.—Investigation of this possible cause is hampered by the lack of knowledge of the identity of the critical target. A considerable bit of evidence points to DNA as the critical, but not the only, target (105). At present, few data are available comparing the nucleic acid contents of sensitive and resistant tumors. However, Ehrlich ascites cells that were resistant to nitrogen mustard N-oxide contained more RNA than did sensitive cells (95, 97, 98), and mitomycin-C-resistant E. coli cells contained more DNA than did sensitive cells (48). However, it would also be possible to have approximately equal total contents of DNA in the two tumors but have different numbers of target sites in the DNA's; for example, the DNA of the resistant tumor might contain less guanine, which is perhaps the chief target moiety of the DNA (105), than the DNA of the sensitive tumor. Little information on the composition of the DNA's of sensitive and resistant tumors is available, but the similarities of the transition temperatures of DNA's isolated from cyclophosphamide-sensitive and cyclophosphamide-resistant hamster plasmacytomas suggest that the two types of DNA have similar base compositions (12). It was also found that the base ratios of DNA of a sensitive rat ascites hepatoma (AH-130) were similar to those of a naturally resistant hepatoma (AH-7974) (69).

Similar considerations might be given to the proteins, which are perhaps secondary targets of alkylation. Ehrlich ascites tumor cells resistant to nitrogen mustard N-oxide contained more protein than did sensitive cells (95, 97, 98). Although the total sulfhydryl contents of rat ascites hepatoma sensitive to nitrogen mustard N-oxide and of the resistant subline tumors were similar, the sulfhydryl content of the protein of the resistant tumor was lower than that of the sensitive tumor (30–32). The significance of this difference as a cause of resistance to the agent is questionable, however, since, as stated above, the resistance could be due to deactivation of the alkylating agent by combination with the nonprotein sulfhydryl groups rather than due to a decreased number of target protein sulfhydryl groups.

Chemical masking.—One can visualize a chemical masking of a potential critical site of alkylation without alteration of the normal functioning of the molecule. For example, the proteins of resistant tumors might contain sulfide or disulfide groups rather than sulfhydryl groups and therefore be less susceptible to alkylation. This would be consistent with the observation of lowered content of sulfhydryl groups in the proteins of tumors with acquired resistance to alkylating agents (30–32). This mechanism would be similar to the suggested mechanism of protection from irradiation and alkylating agents by sulfhydryl compounds by combination of the protective agent with protein-sulfhydryl to form disulfide bonds (91, 92). Masking of carboxylate and phosphate groups by the formation of esters and amides might be other possibilities. No direct evidence for these possibilities is available.

Physical masking.—One can also visualize a masking of the critical site of alkylation by physical means. In proteins, nucleic acids, and nucleoproteins this might be due to altered secondary or tertiary structure resulting from altered secondary bonding between molecules. Coiling, folding, or knitting of the molecules might make the critical sites sterically unavailable for alkylation. Again, little experimental evidence relevant to this possible mechanism is available. The similarity of the transition temperatures of DNA's isolated from cyclophosphamide-sensitive and cyclophosphamide-resistant plasmacytomas suggests that the structures of the DNA's are similar, but it must be recognized that the physical state of the isolated DNA is probably different from that of the DNA in the cell, particularly since the proteins are no longer associated with the DNA.

Decreased nucleophilic character.—It is possible that changes in nucleophilic character might result from changes in the sequence of amino acids in protein or of nucleotides in nucleic acids, and the reduced nucleophilic character would contribute to the resistant nature of the tumor. At present, how-
ever, too little is known about the influence of such sequences upon chemical properties to permit discussion.

The reactivity of the target group might also be altered by alteration of pH. For example, a sulfhydryl group might be ionized less at lower pH's (79), and the nucleophilic character of the bases of nucleic acids also would probably be less at lower pH's. Thus, resistance could be due to a lower pH in the resistant tumor than in the sensitive tumor, but no comparison of the pH values of sensitive and resistant tumors has been reported.

**Utilization of Alternative Metabolic Pathways by the Resistant Cells**

Utilization of an alternative metabolic pathway that by-passes an inhibited metabolic step would be a mechanism of resistance. Some indirect evidence has been obtained that indicates that cyclophosphamide-sensitive and cyclophosphamide-resistant hamster plasmacytomas utilize similar pathways in the biosynthesis of the purines and nucleic acids. Formate-C^14 was injected intraperitoneally into hamsters bearing bilateral implants of the sensitive and resistant tumors, and alcoholic extracts of the tumors were prepared and utilized for two-dimensional paper chromatography and radioautography. By the technics used (106) it is possible to identify certain radioactive carboxylic acids, amino acids, purine, purine ribonucleosides, and purine ribonucleotides and to perform radioassays upon these various compounds. The data indicate that the two types of tumors fixed approximately the same quantities of C^14 into the soluble compounds, and the distributions of the radioactivity among the various compounds were essentially the same for the two tumors. These results suggest that, in the areas of metabolism illuminated by these experiments, the two tumors utilize the same metabolic pathways at essentially equal rates. When adenine-8-C^14 rather than formate-C^14 was used in experiments similar to those just described, again similar results were obtained for the two tumors. Also, when minced tumors were incubated with formate-C^14 or adenine-8-C^14 and alcoholic extracts were prepared and used as described above for the in *vivo* experiments, similar results were obtained for the sensitive and resistant tumors. In both the in *vivo* experiments and the experiments with minces the sensitive and resistant tumors were equally efficient in utilizing formate-C^14 or adenine-8-C^14 for the synthesis of DNA and RNA. It thus appears that these two tumors utilize the same metabolic pathways for *de novo* synthesis of purines to essentially the same extent and that the two tumors are equivalent in their capacity to utilize preformed purines for the synthesis of nucleotides and nucleic acids.

Other areas of intermediary metabolism have not been compared for the two tumors.

**Increased Production of Essential Material**

*(Enzyme or Compound) To Overcome the Deficiency Caused by the Alkylation*

This mechanism would be dependent upon an increased capacity for the synthesis of a compound or enzyme that would be in short supply as a result of the action of the alkylating agent.

Contrary to this theory, however, it was found that about 2 times as much protein synthesis, measured by the incorporation of methionine-S^35 in *vivo*, occurred in leukemia L1210 tumor as in the cyclophosphamide-resistant subline of this tumor (89). One might assume that this difference would be reflected in the rates of synthesis of enzymes as well as of other proteins.

It has been observed that Ehrlich ascites tumor cells with acquired resistance to nitrogen mustard N-oxide carried out more endogenous oxidative respiration than did the sensitive cells (38, 95–98). The resistant cells also were characterized by increased oxidation of fatty acids, moderately increased oxidation of pyruvate and α-ketoglutarate, slightly increased anaerobic glycolysis, cytochrome oxidase activity, oxidation of fumarate and maleate, and decreased lactate accumulation with glucose as substrate (97). The naturally resistant rat ascites hepatoma AH-7974 consumed more oxygen and inorganic phosphate and exhibited a higher P:O ratio than did the sensitive rat ascites hepatoma AH-130 when the substrate was succinate or α-ketoglutarate (87). No difference in the extent of aerobic or anaerobic glycolysis by homogenates of these resistant and sensitive hepatoma cells was observed, but the rate of glycolysis by the nuclear fraction of the resistant hepatoma was lower than that of the sensitive hepatoma and was about the same as that of normal and regenerating liver (88). These data thus indicate that resistant tumors might have higher rates of respiration and lower rates of glycolysis than do sensitive tumors. On the other hand, there was less endogenous respiration and glycolysis in resistant Yoshida sarcoma cells than in the sensitive cells (100).

Assays of several enzymes were performed on homogenates of rat ascites hepatomas AH-130 (sensitive) and AH-7974 (resistant), and it was found that the two tumors contained about equal amounts of histidase, urocanicase, arginase, aldolase, catalase, choline esterase, cathepsin, and leucine amino peptidase, but the resistant tumor had
lower acid and alkaline phosphatase activities than
did the sensitive tumor; the difference was greater
for the alkaline phosphatase (64). The distribution
of the alkaline phosphatase among the subcellular
fractions was also different for the two tumors—
the resistant tumor had a lower percentage of its
total activity in the nuclear and microsomal frac-
tions and a larger percentage in the supernatant
fraction than did the sensitive tumor. The lower
phosphatase activity might result in higher concen-
trations of nucleotides in the resistant tumor, and
this might be equivalent to greater synthesis of
nucleotides with respect to the availability of com-
ounds essential for the survival of treated cells.

Experiments with cyclophosphamide-sensitive
and cyclophosphamide-resistant hamster plasma-
cytomas in which alcoholic extracts of the tumors
were examined chromatographically and radio-
autographically following injection of formate-C14
or adenine-8-C14 into the host animals yielded data
that indicated that the same compounds were la-
beled to approximately the same extent in the two
tumors. Similar results were obtained in experi-
ments with minces of these tumors. Other studies
showed that treatment of the host animals with
alkylating agents interferes with the de novo syn-
thesis of purine ribonucleotides by the sensitive
tumor but not by the resistant tumor (107), but
the data just referred to would indicate that the
resistance is not due simply to a greater capacity
for de novo synthesis of nucleotides.

Several investigators have used the incorpora-
tion of radioactive precursors into the nucleic
acids of sensitive and resistant tumors as a measure
of the rate of synthesis of nucleic acids. Ehrlich
ascites tumor cells that were resistant to nitrogen
mustard N-oxide incorporated more P32 into the
RNA than did the sensitive cells, but there was no
difference in the DNA turnover for the two cell
lines (97). Phenylalanine mustard-resistant Sarco-
ma 45 incorporated less P32 into the nucleic acid
than did the sensitive Sarcoma 45 on the 16th or
17th day after implantation, but at later times
there was little difference (71). There was no sig-
nificant difference in the extents of in vivo incor-
poration of P32 into the RNA of rat ascites hepatoma
AH-130 (sensitive to nitrogen mustard
N-oxide) and AH-7974 (resistant to nitrogen mus-

tard N-oxide) (69); the two tumors utilized about
the same quantities of orotic acid-C14 for the syn-
thesis of RNA, but the resistant tumor used more
orotic acid-C14 for the synthesis of DNA than did
the sensitive tumor (86). Cyclophosphamide-sen-
sitive and cyclophosphamide-resistant hamster
plasmacytomas were about equally efficient in
utilizing formate-C14 or adenine-8-C14 for the syn-
thesis of the purines of RNA and of DNA in vivo
and in vitro as minces. Thus, there was no consist-
ent difference in the capacities of sensitive and
resistant tumors for synthesizing nucleic acids.

Although there are perhaps differences in the
enzyme levels or activities of sensitive and resist-
tant tumors, particularly in the area of enzymes of
respiration, the possible significance of the higher
respiratory activity for supplying greater quanti-
ties of compounds or enzymes that might be de-
pleted by treatment of the sensitive cells by alkyl-
ating agents cannot be assessed at present because
of the scant knowledge of the metabolic site of
action of these agents.

**Failure To Activate the Agent**

This possible mechanism would obviously be
applicable only to those agents that require activa-
tion before they can act as alkylating agents. This
activation would by definition consist of some
chemical or enzymatic transformation that would
not be expected to occur upon simply placing the
agent in an aqueous medium at physiological pH
and temperature. For example, the formation of
the ethylenimonium ion from nitrogen mustard
when it is placed in buffer at physiological condi-
tions is not considered to be activation, but the
hydrolytic conversion of N-acetyl-N',N'-bis(2-
chloroethyl)-p-phenylenediamine, which does not
readily ionize to N,N-bis(2-chloroethyl)-p-phenyl-
enediamine, which more readily ionizes, would be
considered to be activation (8). The activation of
other compounds might be dependent upon the
presence of esterases, hydrolases, reductases, ox-
idases, or other enzymes (8, 79 [pp. 152–76]) in
tumors, and thus a tumor that lacked the required
enzyme would be resistant to the agent.

It is believed that the acetamido compound
mentioned in the preceding paragraph is actually
converted to the corresponding amino compound
by the Walker rat sarcoma and the resulting amino
compound is the active cytotoxic alkylating agent.
Repeated treatment of the tumor-bearing animals
with the acetamido compound resulted in the de-
velopment of resistance by the tumor, which was
accompanied by decreased peptidase activity of the
tumor (8).

There is some evidence that nitrogen mustard
N-oxide is activated upon contact with cultured
Yoshida sarcoma cells, perhaps by conversion to
nitrogen mustard (37, 41), but resistant sublines
of this tumor and the naturally resistant rat
ascites hepatoma AH-7974 were as active as the
sensitive tumor in reducing the N-oxide, and it
was concluded that the resistance of the tumors to
the anticancer activity of nitrogen mustard
N-oxide is independent of their reducing activity against the agent (37).

Cyclophosphamide requires activation before it serves as an alkylating agent. This activation might consist of hydrolysis of the agent to yield bis(2-chloroethyl)amine, which may be the active cytotoxic agent (2, 3, 79 [p. 163]). This activation is probably enzymatic, because incubation of cyclophosphamide with homogenates of certain tissues results in activation, whereas incubation with homogenates of other tissues does not (16). It has been shown that several types of neoplasms that respond to this agent in vitro do not respond to it in vivo because of lack of activation (6, 15–17). The in vitro utilization of formate-C\(^4\) or adenine-8-C\(^4\) for the synthesis of nucleic acids by minces of either cyclophosphamide-sensitive or cyclophosphamide-resistant hamster plasmacytomas was not inhibited by cyclophosphamide that was added to the incubation mixture, but this utilization was inhibited by nitrogen mustard under similar conditions.\(^2\) Thus, activation of this agent in vivo evidently occurs at some locus other than the tumors, and one would assume that the activated form is transported to both the sensitive and resistant plasmacytomas when they are growing bilaterally in the same host. Therefore, resistance to this agent by this tumor is not due to lack of activation.

The evidence presented above points to three possibilities concerning the activation of agents by sensitive and resistant tumors: (a) the sensitive tumor activates, but the resistant tumor does not; (b) both the sensitive and the resistant tumor activate; and (c) neither the sensitive nor the resistant tumor activates. Although failure to activate the agent may be the mechanism of resistance for certain tumors with respect to certain agents, this is certainly not a general mechanism. Also, cross-resistance of tumors to agents that require no activation and to agents that are activated in different ways is indicative that this is not a general mechanism of resistance.

CONCLUSIONS

Descriptions of many of the investigations to which reference has been made in this review have been available only in the form of abstracts. Therefore, it has not been possible to independently assess the methods and the data, and it must be assumed that the conclusions stated in the abstracts are justifiable.

Experimental evidence indicates that in experimental neoplasms the site of resistance is within the neoplastic cell itself rather than in other tissues of the host (8, 79 [pp. 155–37], 116), and it is probable that this is also true for many primary human neoplasms. As yet no mechanism of resistance to alkylating agents has been definitely established. It is possible that two or more mechanisms are simultaneously contributing to the resistance of a single tumor. It has been suggested that resistance to alkylating agents probably involves a capacity to prevent sufficient alkylating potential from reaching the cell nucleus, which is perhaps the locus of the critical alkylation (79, pp. 155–57). This possibility is consistent with altered permeability of the cell membrane or increased deactivation of the agent, and there is some evidence supporting these mechanisms. At present there is little evidence that indicates that resistant cells have fewer sensitive sites in the target molecules or that the target molecules are less nucleophilic, that the resistant cells utilize alternative metabolic pathways that by-pass an inhibited metabolic step, or that resistant cells have increased capacities for synthesizing compounds or enzymes that might ordinarily be in short supply as a result of the effect of the alkylating agent. Although failure of the resistant cell to activate an agent might be the mechanism of resistance to specific agents by specific tumors, this mechanism is certainly not generally operative.

The experimental results reviewed above make it possible to give partial answers to the questions posed at the beginning of this paper. It appears possible that "acquired resistance" and "natural resistance" might result from similar mechanisms or combinations of mechanisms, but, where two mechanisms operate simultaneously, the relative contributions of the two mechanisms might differ for natural and acquired resistance. For example, low permeability of the cell membrane to the agent and detoxification of the agent by combination with nonprotein sulfhydryl group might contribute to both natural and acquired resistance, but low permeability might be the dominant factor in determining the degree of natural resistance (114) whereas the availability of nonprotein sulfhydryl groups might be the dominant factor in determining the degree of acquired resistance (30–32). One might speculate that the mechanisms of natural resistance operate to a limited extent in host tissues and thus protect them from low concentrations of the agents that are toxic to sensitive tumors, but little experimental evidence related to this possibility has been obtained.

The observations of cross-resistance to different types of alkylating agents in experimental tumors with acquired resistance lead one to assume that there is a common mode of resistance to these various agents. Since the chief chemical property that
these agents have in common is the capacity to form electrophilic centers, then combination of these agents with nonessential nucleophilic centers would be a plausible mechanism of resistance that would be common to different agents. The presence of relatively elevated quantities of nonprotein sulphydryl groups in resistant tumors would be consistent with such a mechanism. A common mechanism of resistance, however, does not necessarily indicate similar mechanisms of action or critical sites of alkylation for the agents.

Although the experimental evidence that is now available does not definitely establish the functioning of any specific mechanism of resistance to these agents, ingenuity in the designing of experiments with presently available experimental systems and techniques should make possible insight into the phenomenon of this resistance in the not-too-distant future.

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Studies Related to Mechanisms of Resistance to Biological Alkylating Agents

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