Carcinogenicity of Alkylating Agents

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TO A CHEMIST interested in carcinogenesis, one of its fascinating aspects is the great variety of chemicals that after being administered to a test animal can lead to cancer. Some very simple molecules, that is, carbon tetrachloride or diazomethane, are carcinogenic in certain species of animals while in others only complex structures are cancer producing. Moreover, the production of carcinogens that may be involved in the human environment is not wholly the province of man; a number of quite powerful carcinogens are produced by micro-organisms or plants.

One such mycotoxin that has proved to be a potent carcinogen is aflatoxin (fig. 1), a metabolite elaborated by the fungus *Aspergillus flavus*. The discovery of aflatoxin resulted from an investigation of the cause of a mysterious disease that killed thousands of turkey poults in England in 1960, the fatality largely resulting from liver necrosis. The toxin was traced to moldy peanut meal included in the poultry feed. Further study led to the identification of the organism responsible, to the isolation and determination of the structure of aflatoxin (*1*, *2*), and to the discovery that aflatoxin is a powerful hepatocarcinogen (*3*).

Thus it has been estimated that hepatomas in rats are induced by a daily dose of 9,000 μg. per day of *p*-dimethylaminoazobenzene, a classic liver carcinogen, by 700 μg. per day of dimethylnitrosamine, but by only 10 μg. per day of aflatoxin B_1. On a weight basis, therefore, the toxin is 900 times more effective than the standard azo dye.

Hydrogenation of aflatoxin B_1, the most abundant and most carcinogenic isomer, yields aflatoxin B_2, which is much less toxic and carcinogenic and also a relatively minor mold product.

Usually, contamination of peanuts or oil seeds by *A. flavus* occurs when the peanuts are stored in a moist place after harvesting. Expeditious and proper drying of the peanuts can prevent growth of the fungus and eliminate the production of toxin. Incidentally, the amount of aflatoxin formed varies, and a luxurious growth of *A. flavus* does not necessarily lead to a high yield of aflatoxin or vice versa.

Is aflatoxin likely to be a carcinogen in man? It has been reported that certain African populations enjoy moldy corn or moldy peanuts. Whether contamination of these foods by aflatoxin may be a factor in the high incidence of liver cancer in parts of Africa remains to be determined definitely.

Another naturally occurring compound that is carcinogenic is cycasin (*4*), $H_3C–N(O)=N–CH_2OC_6H_{11}O_5$ (glucose), a glycoside isolated from the seeds of *Cycas circinalis* (cycads), a species of palmlike plant found mostly in tropical or subtropical countries. The cycad nuts are also used in certain food products. The

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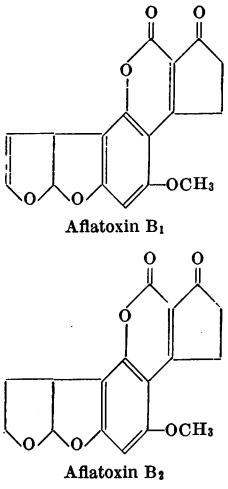

Figure 1. Structure of aflatoxins

crude meal obtained from these nuts was mutagenic to onion seeds and carcinogenic to rats and guinea pigs, causing liver, kidney, intestinal, and lung tumors, and much damage to the liver. Customary processing attempts to eliminate the harmful agent by soaking and washing; complete removal, however, may not be obtained. In germ-free animals in which no organisms are capable of splitting the glycoside to give the aglycone (*5*) methylazoxymethanol, no liver damage occurred over a short term. Feeding the acetate of methylazoxymethanol to germfree rats led to liver damage. Methylazoxymethanol acetate has been synthesized recently by Matsumoto and co-workers (*6*).

In addition to these naturally occurring carcinogens, there are many synthetic organic chemicals, some of very simple structure. Thus diazomethane (CH_2N_2) and some of its precursors are carcinogens. The alkylnitrosamines (R_2–N–NO) are generally potent cancer-producing agents. This property was discovered during an investigation of the causes of the development of symptoms of liver toxicity in pilot-plant workers preparing dimethylnitrosamine (*7*). These compounds are useful as solvents, as intermediates in synthetic processes or production of rocket fuels, as gasoline or rubber additives, and as foaming agents in plastics. Some analogs such as diphenyl- or di-*t*-butylnitrosamines are not carcinogenic (*8*). Recently, Kelly and co-workers (*9*) induced hepatoma in monkeys with diethylnitrosamine in less than 2 years. This is the first time cancer has been produced in primates in such a short time, and it demonstrates the potency of these agents.

What is the mechanism by which all these compounds can be carcinogenic? One common property of these structures is their capability of acting as alkylating agents. Thus the model carcinogenic lactone beta-propiolactone reacts with deoxyribonucleic acid (DNA) to yield the alkylated guanine 7-(2-carboxyethyl)guanine (*10*). Diazomethane (CH_2N_2) reacts with carboxylic acids or phenols to give methyl esters or ethers, reactions frequently used in synthetic organic chemistry. Additionally, with ribonucleic acid (RNA), 7-methylguanine and a small amount of 1,7-dimethylguanine are found upon hydrolysis (*11*). Dimethylnitrosamine is oxidatively demethylated to an intermediate, which then gives rise to either diazomethane (CH_2N_2) or a carbonium ion CH_3^+ that methylates DNA or RNA, affording 7-methylguanine or the terminal histidine of proteins yielding 1- or 3-methylhistidine (*12*). Cycasin acts in similar fashion.

Several of another series of alkylating agents that we have investigated—the nitrogen mustards and related compounds, also the aziridines, the epoxides, and the methanesulfonates—have found clinical use in the treatment of leukemia, Hodgkin's disease, and other neoplastic diseases. In our experiments, A/J mice (strain

Table 1. Carcinogenicity of aromatic nitrogen mustards in A/J mice

Compound	NSC No.[1]	Dose range, millimicromoles per kilogram	Average number of lung tumors per mouse at top dose
5-Aminouracil mustard	34462	0. 06–3. 96	17. 9
Chlorambucil	3088	2. 6–115	8. 9
L-phenylalanine mustard	8806	. 07–4. 68	4. 5
2,3-Dimethoxyaniline mustard	18439	4. 5–72	5. 5
2-Naphthylamine mustard	62209	23–1492	3. 7

[1] Cancer Chemotherapy National Service Center (NSC), National Cancer Institute, Public Health Service.

A mice from the Jackson Laboratories, Bar Harbor, Me.), 4 to 6 weeks old, both male and female, were injected intraperitoneally with these compounds three times a week for 4 weeks. Four or more dose levels were used in each experiment. The top dose was LD_{10} and the other doses were $\frac{1}{4}$, $\frac{1}{16}$, and $\frac{1}{64}$ of that level.

After 39 weeks the mice were killed and their lungs examined for tumor nodules. Because both the percentage of mice with tumors and the number of tumor nodules per mouse can be determined, the statistical evaluation of the experimental data is quite sensitive. Controls averaged 0.5 nodule per mouse. The results obtained indicated that the most active carcinogens were the mustards derived from aromatic amines (table 1).

Most active in causing lung tumor was uracil mustard, followed by chlorambucil and sarcolysin, then a 2,3-dimethoxyaniline and a 2-naphthylamine mustard. The 2-naphthylamine mustard (R48, Erysan, chlornaphazin) has been used in the treatment of Hodgkin's disease or lymphomas. Some patients who survived up to 10 years developed bladder cancer (*13*). The parent 2-naphthylamine is a known bladder carcinogen in man.

The mode of reaction of the aromatic mustards is shown in figure 2. The stability of these compounds is such that a carbonium ion is formed that combines with a nucleophilic center to yield an alkylated product (*14*). Because the second chloroethyl group is still present, a second interaction can occur leading to cross-linking in the molecule, which would prevent opening of the DNA and further duplication of the cell. Brookes and Lawley (*15*) and Abell (*16*) have shown that closely related compounds,

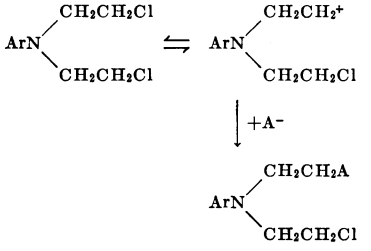

Figure 2. Reaction scheme for aromatic mustards

NOTE: Ar=aryl and A^-=nucleophilic center.

Table 2. Carcinogenicity of aliphatic nitrogen mustards in A/J mice

Compound	NSC No.[1]	Dose range, millimicromoles per kilogram	Average number of lung tumors per mouse at top dose
Nitrogen mustard	762	0. 025–1. 5	2. 3
Benzimidazole methylene mustard	23892	. 35–30	3. 7
Mannitol mustard	9698	. 49–31. 7	2. 3
Cyclophosphoramide (cytoxan)	26271	1. 6–103	1. 8
Hydroquinone mustard (Weatherbee mustard)	18321	. 05–3. 6	1. 4

[1] Cancer Chemotherapy National Service Center (NSC).

the sulfur mustards, alkylate nucleic acids. Furthermore, Byvoet and Busch (*17*) observed that uracil mustard is incorporated into nucleic acids. On the other hand, Cohn (*18*) demonstrated that sarcolysin seemed to be bound more to proteins. The actual intermediates in these reactions have not been established, but the data show that nucleic acids and proteins can be alkylated.

The aliphatic-type mustards we examined were much less effective as carcinogens (table 2). With these compounds, the active intermediate is an imonium ion that attacks a nucleophilic center. The reactions are usually rather fast, within 1 or 2 minutes at 37° for nitrogen mustard (fig. 3).

Strictly speaking, cytoxan or cyclophosphoramide is a derivative of a cyclic phosphodiamidate. However, it hydrolyzes to di-2-chloroethylamine (nor-nitrogen mustard), which then cyclizes to an imine (aziridine), which is apparently the active entity (*14*). See figure 4.

Several aziridines were also tested, and most were found to be only weak carcinogens (table 3). Most active was a benzoquinone aziridine. Two well-known phosphoramides, phosphine sulfide, bis(1–aziridinyl)morpholino–(OPSPA) and phosphine sulfide, tris(1–aziridinyl)– (ThioTEPA), had only weak potency

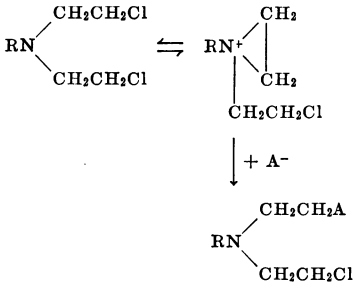

Figure 3. Reaction scheme for aliphatic mustards

NOTE: R=alkyl and A^-=nucleophilic center.

at high dose levels. ThioTEPA was slightly more effective than OPSPA with two aziridine moieties. Since the mechanism by which aliphatic nitrogen mustards react is through cyclization to the imonium ion, similar to the aziridines in structure and reactivity, it is not surprising that the effectiveness of these compounds was of the same order.

In all the most active compounds, the nitrogen mustard is constructed on a nitrogen atom of low basicity, which gives rise to the relatively stable carbonium ion of a measurable half life in aqueous systems. This intermediate therefore may survive until it reaches a nucleophilic target in the lung. The effective level may also be a reflection of secondary detoxification reactions undergone by the molecule. The relatively high dosages tolerated and required for carcinogenic potency of the naphthylamine mustard may be ascribed to detoxification by hydroxylation (probably at the six-position) and conjugation with sulfuric or glucuronic acid, leading to rapid elimination.

Similarly, in the dimethoxyaniline mustard, hydroxylation at the para position or oxidative dealkylation of the methyl groups followed by conjugation may occur. The ring systems of

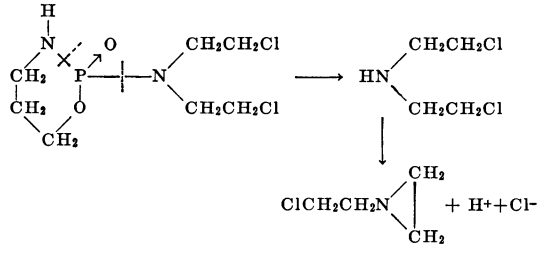

Figure 4. Cyclophosphoramide (cytoxan) hydrolysis

chlorambucil and sarcolysin are less prone to this mode of detoxification, although conjugation of the carboxyl groups with glycine is a possible pathway. With uracil, such reactions are less likely, perhaps explaining why smaller doses were so tumorigenic.

Among the aliphatic mustards, the benzimidazolemethylene mustard, with a structure sterically simulating purines, was most active. Nitrogen mustard was fairly carcinogenic when injected in an aqueous vehicle. In tricaprylin, which allows slow release, it was much less effective. Mannitol mustard, which has a cationic structure (several hydroxyl groups) capable of rapid excretion, required higher doses to yield the same number of tumors as nitrogen mustard. Cytoxan, as we have seen, actually reacts like an aliphatic mustard and was not very potent even at high dosages.

These alkylating agents act to increase the rate and extent of a naturally occurring process, since strain A mice have an inherent tendency to develop lung tumors. Many factors play a role. Agents with a structure simulating a naturally occurring substrate are more active. Those with an aromatic ring system that stabilizes the molecule survive transport to the target organ, especially if the ring is blocked at the usual detoxification positions, and hence are more active. Further aspects are discussed in a detailed manuscript (*19*).

It was of interest to relate the carcinogenicity of the most active compounds to their carcinostatic effect. The data available with respect to

Table 3. Carcinogenicity of aziridines in A/J mice

Compound	NSC No.[1]	Dose range, millimicromoles per kilogram	Average number of lung tumors per mouse at top dose
Benzoquinone aziridine	17262	0. 12–7. 4	2. 9
OPSPA [2]	10429	. 67–43	2. 0
ThioTEPA [3]	6396	. 14–9. 3	1. 7

[1] Cancer Chemotherapy National Service Center (NSC).
[2] Phosphine sulfide, bis(1–aziridinyl)morpholino–.
[3] Phosphine sulfide, tris(1–aziridinyl)–.

lymphocytic leukemia (L1210) indicate that the compounds most active against this disease are not necessarily the most active carcinogens, and vice versa (*19*). Cytoxan, a very weak carcinogen, was the most active agent against L1210. The other agents were intermediate in effectiveness.

In summary, there are several types of natural products and drugs that are alkylating agents and also carcinogenic. They react with important cell constituents such as proteins or nucleic acids. The significance of this property in the actual carcinogenic process is not known, however, and the detailed mechanism is still to be elucidated.

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