RECENT CONTRIBUTIONS TO THE PHARMACOLOGY OF BIS(2-HALOETHYL) AMINES AND SULFIDES

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I. INTRODUCTION

At the beginning of World War II, *bis*(2-chloroethyl)sulfide (mustard gas or sulfur mustard) was regarded as an effective combat agent and as a protoplasmic poison causing indiscriminate damage to exposed tissues. This concept seemed well founded in experiences gained during World War I, when the arresting features of mustard intoxication appeared to include vesication of skin, ocular lesions, and severe necrosis of the mucosal lining of the respiratory tract (256, 257). It had been recognized that intoxication involved cells and tissues other than those directly exposed to liquid or vaporous *bis*(2-chloroethyl)sulfide, but

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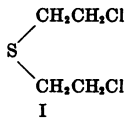
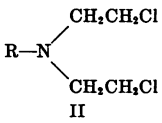
the fundamental significance of such systemic actions had escaped notice (97, 189, 260). During the recent reappraisal of mustard gas fostered by the exigencies of a second world war, greater attention was directed toward systemic aspects of intoxication and these proved of unexpected importance. Intensive study of systemic actions derived impetus from inclusion among the potential war gases (222) of tertiary *bis*(2-chloroethyl)amines (nitrogen or amine mustards) which had been discovered several years prior to World War II. In addition to damaging the exposed integument of the body in a manner closely resembling the local effects of mustard gas (I), the amine analogs (II) appeared to be more readily absorbed percutaneously and, therefore, more hazardous as systemic intoxicants.



I



II

The orientation of investigations toward systemic actions led to the realization that mustards were not merely indiscriminate poisons of protoplasm but actually were highly selective in their cellular actions. Although morbid changes could be elicited with suitably chosen doses in almost any type of cell or tissue, proliferating tissues of the adult mammal were found most susceptible to both I and II. Effects of these agents analogous to those of roentgen radiation were appreciated and on this basis various amine mustards were evaluated in the treatment of neoplastic disease (111, 125, 155, 221). Allied studies characterized mustards as unique in their capacity to disrupt cell reproduction and to cause derangements in chromosomal mechanisms which had previously been produced in similar measure only by use of ionizing radiations (11). Other studies of amine mustards suggested that, as a group, the tertiary 2-haloalkylamines might well become useful as experimental tools and possibly in therapy. This predication was confirmed by the independent discovery that certain 2-haloalkylamines elicit specific adrenergic and/or histaminic blockade (181, 199). Parallel investigations of the reactivity of 2-chloroethyl amines and sulfides with components of biological systems provided important information concerning their biochemical mechanism of action (47, 87, 207).

After the termination of hostilities reviews of previously confidential and unpublished work became available. These served to orient those unacquainted with the new understanding of the biological actions of mustards (40, 47, 55, 87, 111, 114, 207, 212). Even the initial publications made it evident that these agents were no longer to be considered merely brutal accessories of warfare (222). Indeed, they are likely to endure as profitable chemical instruments in biological research. Now that sufficient time has elapsed to permit publication of war-time investigations and the appearance of pertinent post-war contributions, it is appropriate to reconsider the important applications of *bis*(2-haloalkyl) amines and sulfides.

II. HISTORICAL

A. Systemic intoxication. The vesicant and local, necrotizing actions of pure samples of I were first described by Meyer in 1887 (191) and proved to be the predominant features of mustard intoxication in World War I. Evidence for injury by direct contamination of superficial tissues was so abundant that it was reasonable to conclude that I was an escharotic and non-specific protoplasmic poison (254, 255, 256, 257, 258). Lethal intoxication was generally ascribed to overwhelming necrosis of tissues and to infections secondary to local injury, such as bronchopneumonia. Nevertheless, it was evident to some investigators that systemic intoxication by I was a significant factor in fatal poisoning (183, 189, 260). Thus, the pulmonary pathology in dogs which inhaled lethal doses of I appeared insufficient to account for death and failed to approach in severity the lesions obtained in animals exposed to primary pulmonary irritants, such as phosgene or chlorine (183, 260). Moreover, severe mustard intoxication in human casualties was frequently associated with characteristic manifestations suggesting systemic poisoning. These included headache, malaise, vomiting, epigastric distress, anorexia, diarrhea and cachexia (124, 189). Damage to hematopoietic tissues was observed at autopsy and progressive leucopenia developed in severely gassed individuals (176, 177). A possible relation between fatal poisoning and hematopoietic damage was suggested by the coincidence of maximal leucopenia and mortality rate 6 to 9 days after exposure.

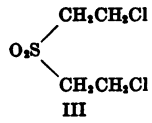
Proof that primary systemic actions could follow exposure to I was obtained experimentally. Dogs inhaling high concentrations of vapor exhibited all the signs of intoxication which ordinarily appear after parenteral administration of I, *i.e.*, salivation, emesis, diarrhea, bradycardia, hyperpnea and clonic-tonic convulsions (183). Furthermore, animals given parenteral doses developed severe, persistent diarrhea and progressive cachexia and succumbed after 3 to 6 days (205, 256). Autopsies revealed an extensive, desquamating, necrotic enteritis confined to the intestinal tract. Finally, leucopenia and necrosis and atrophy of hematopoietic elements in bone marrow and lymphoid organs were found in rabbits given lethal intravenous doses of I (205). Needless to say, manifestations of intoxication in animals given I parenterally occurred in the absence of the cutaneous and pulmonary lesions incurred by battle casualties.

A most dramatic and tragic demonstration of the systemic effects of I was provided by an incident in World War II which is now known historically as the "Bari disaster" (2, 222). As the result of a chain of unfortunate events initiated by a minor aerial bombardment, a number of cargo vessels in the Harbor of Bari, laden with high explosives and mustard-gas, were demolished. The fact that sulfur mustard had become admixed with the oil-slick that coated the surface of the harbor was overlooked during the immediate confusion and not fully appreciated until several days later. Many casualties who had floundered in oil-coated water for a variable number of hours before rescue were kept wrapped in blankets until the overtaxed medical personnel of the port-area could administer individual treatment. This condition proved particularly efficacious for the percutaneous absorption of I. Early casualties exhibited an unusual shock-like syndrome with low arterial pressure and hemoconcentration which resisted

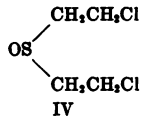
parenteral fluid therapy, transfusion and epinephrine. The vasodepression persisted for 18 hours to 3 days and was followed by death or slow recovery. During the shock-like phase, casualties were unusually depressed and apathetic. A second peak of mortality took place at 8 to 9 days and was associated with extreme leucopenia and failure of granulocyte response in cases with pneumonitis and pneumonia. A survey of the casualties left little doubt that the unique clinical features of the disaster were to be attributed to systemic intoxication by I.

B. Mechanism of action. Proper insight into the mechanism of action of I was seriously hampered during World War I and for many years thereafter by lack of pertinent studies of its interaction with biochemical entities. The rapid hydrolysis of I in aqueous media to nontoxic thiodiglycol with release of Cl^- and H^+ was appreciated (18, 183, 211). In the absence of evidence for a more direct reaction with cellular constituents, the hydrolysis of I became almost by default, the basis for one pharmacodynamic hypothesis (180, 183, 189). It was suggested that cellular permeability to I was related to its high lipid solubility. Subsequent to penetration of the cell, I was believed to hydrolyze and release strong acid in amounts sufficient to damage sensitive intracellular structures. The hypothesis explained satisfactorily the futility of decontamination procedures conducted within a few minutes after tissue contact with I, even though signs of injury were characteristically delayed for several hours. Serious objections to the "intracellular acid" theory were subsequently raised. In a series of analogs of I, rate of acid liberation could not be correlated with degree of vesicant activity (211). Furthermore, it was difficult to believe that the buffering systems of cells could be overwhelmed by the acid produced from the small amounts of I necessary for intoxication (18). Finally, many substances known to release HCl by hydrolysis were not vesicant (18).

An alternative mechanism, proposed at the end of World War I, was that I might be oxidized intracellularly to toxic intermediates, such as the sulfone (III) which is known to be chemically reactive (97).



III

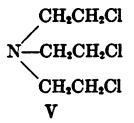


IV

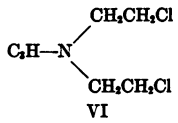
Compound III was found to be toxic and vesicant; it has continued to be of interest, even in more recent studies (18). It was not, however, until some years later that evidence accumulated which indicated the possibility of direct interaction of I and III with tissue constituents. Thus, I, III and also the sulfoxide (IV) were shown to condense with amino groups of amino acids and various secondary and tertiary amines (68, 150, 178). Later, rapid inhibition of glycolysis and respiration by I was demonstrated in mixed tumor tissue *in vitro* (31, 227). Moreover, III proved more effective than iodoacetate in inactivation of the pyruvate oxidase system of pigeon brain (206). It was suggested that inactivation

resulted from the interaction of III with essential —SH groups of the enzyme. Nevertheless, pyruvate oxidase was relatively insensitive to I. Finally, the interaction of I and III with native proteins was convincingly demonstrated (33). Mild treatment of horse serum at pH 8 and at temperatures not greater than 30°C produced specific alterations of proteins which could be detected by serological methods. The absence of serological cross-reactions between I- and III-treated horse serum provided the first satisfactory evidence that *bis*(2-chloroethyl)sulfide might directly affect complex cellular constituents. Subsequent studies during World War II fully exploited this fertile lead.

C. Nitrogen mustards. Prior to World War II, information concerning the biological actions of nitrogen mustards, *bis*(2-chloroethyl)amines, was sparse and limited to brief announcements of the synthesis of a new vesicant, *tris*(2-chloroethyl)amine (V) (184, 190, 253). A synthesis of methyl-*bis*(2-chloroethyl)amine (VI) was also recorded (216). This compound is now commonly known cryptically as HN2 or MBA, while V is referred to as HN3 or TBA.



V

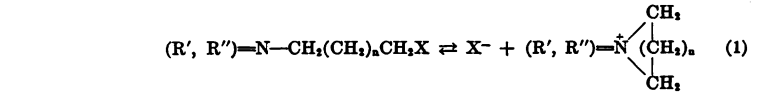


VI

III. CHEMICAL REACTIONS

Compounds having the structure of I and II are relatively stable when undissolved or in non-polar solvents. When activated by water, they transform rapidly into unstable intermediates which readily react with numerous inorganic and organic substances of biochemical interest. Evidence to be presented below clearly establishes the importance of these reactive intermediates in the pharmacological actions of the mustards.

A. Transformation in aqueous media. The initial reaction of nitrogen mustards in water is one which is characteristic of aliphatic amines halogenated in the beta to omega position. Such compounds undergo a distinctive intramolecular transformation (equation 1), with release of halide ion, to form cyclic amines which may be quaternary if derived from tertiary bases (24, 25, 26, 74, 80, 135, 136).



(1)

Although the rate of the reaction is dependent on temperature and influenced by concentration of water and ionic strength, it is not otherwise affected by the solutes present (24, 25, 26, 74). However, in the case of tertiary amines, cyclization cannot occur when the N atom is coordinated with a proton (74). Thus the rate of cyclization of nitrogen mustards, which are more or less weak bases,

is retarded in solutions of low pH. This is of practical importance in their administration as water-soluble hydrochlorides or hydrobromides which exhibit sufficient dissociation to render them more stable in water than the free bases.

The cyclic ethylenimonium derivatives formed by primary intramolecular

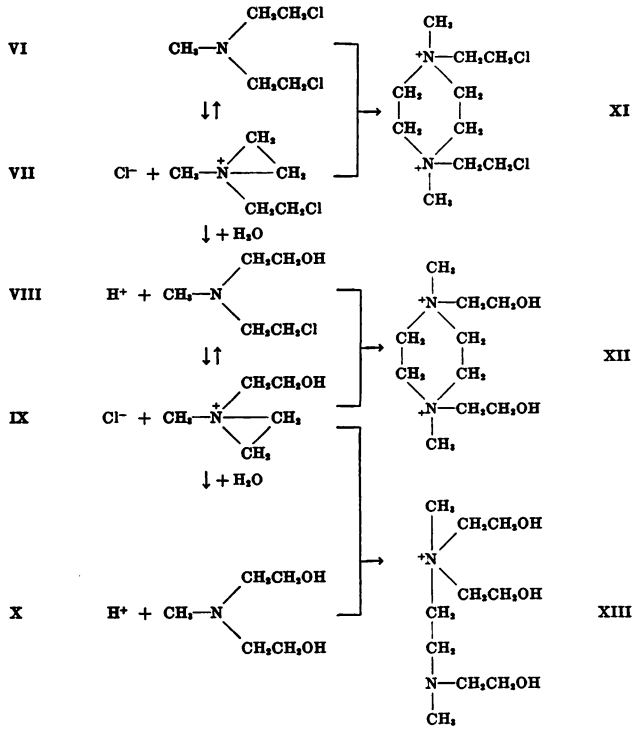


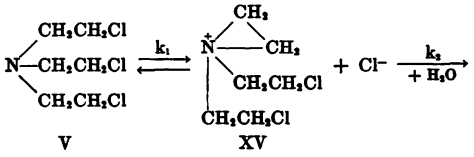
FIG. 1. TRANSFORMATION OF VI IN AQUEOUS MEDIUM FREE OF REACTIVE SOLUTES (122)

reaction are the intermediates responsible for chemical reactivity of nitrogen mustards (25, 74). In aqueous solutions free of other solutes capable of condensing with ethylenimine moieties, back-reactions occur with halide ions which diminish to some extent the over-all rate of formation of cyclic ethylenimonium

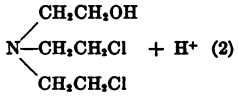
ions. Of more importance is the hydrolysis of the imines which results in formation of tertiary chlorohydrins. These in turn undergo successive cyclisation and hydrolysis.

The series of consecutive reactions outlined above is shown in Fig. 1 for methyl-*bis*(2-chloroethyl)amine (VI). The half-lives of the tertiary amines VI and VIII are of the order of several minutes at 37°C and physiological pH, whereas the imines VII and IX are considerably more stable. It may be seen that the reactions are complicated by attack of the chlorimine (VII) and the hydroxyimine (IX) on tertiary bases present, to form cyclic (XI and XII) and linear (XIII) dimers. As expected, dimer formation is favored by high initial concentrations of VI. The scheme of Fig. 1 has been proven from the kinetics of appearance of Cl^-, H^+ and reactive groups, and also by the isolation of each of the reaction products depicted (25, 74, 121, 122, 135).

Identical series of consecutive reactions have been established for the transformations of ethyl-*bis*(2-chloroethyl)amine (XIV) into ethyldiethanolamine and of *tris*(2-chloroethyl)amine (V) into triethanolamine. The extent of dimer formation in aqueous reactions of XIV and V is less than in the case of VI (24, 74, 103, 135). It appears from kinetic studies of transformations in an extensive series of analogs (74) that the scheme depicted in Fig. 1 would apply generally to nitrogen mustards having aliphatic R-groups (see formula, II). Nevertheless, the reactions of V represent a special case of some importance. Evidence for the formation of the chlorimine (XV) has been obtained from kinetic studies (26, 74), but the rate-constant for its hydrolysis (k_2, equation 2) is too rapid to permit its accumulation and isolation from solution (26, 123).



V XV

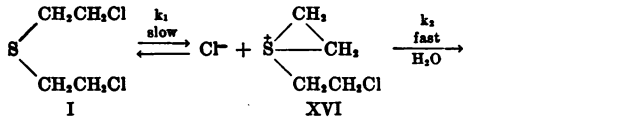


(2)

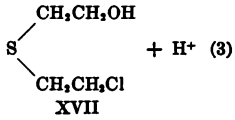
This special circumstance has been associated with the base-strength of V which is weaker than that of VI or XIV, and the observation that reactivity of respective cyclic imines is inversely related to the base-strength of their tertiary parent amines. Conversely, rates of formation of cyclic intermediates (k_1, equation 2) vary directly as base-strength and accordingly V transforms more slowly than VI or XIV (26, 74).

By virtue of its special properties, V has been considered as a transitional substance linking the chemical properties of sulfur and nitrogen mustards (26, 27).

Sulfur mustard (I) is known to undergo two consecutive hydrolytic reactions in water, concerned with the respective formation of a chlorohydrin and thiodiglycol (85, 127, 129, 202, 203, 211, 218, 240, 243). Equimolar amounts of Cl^- and H^+ appear simultaneously during the course of the transformation, and consequently the formation of cyclic ethylensulfonium intermediates is not apparent and has never been proven by their isolation (243). Nevertheless, evidence indicating the possibility of their existence has been obtained in studies of replacement reactions of 2-mercaptoalkyl radicals (106). They also figure in mechanisms which explain satisfactorily the kinetics of I-transformations (27, 218). By analogy with properties of various nitrogen mustards, the weakly basic character of the S atom of I would promote a rate of hydrolysis (k_2) more rapid than the rate of cyclization (k_1), as shown in equation 3.



I XVI



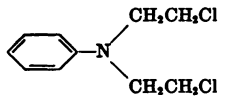
XVII

(3)

Therefore, little of the cyclic sulfonium (XVI) would accumulate and Cl^- and H^+ would appear to be released simultaneously (27). The transformation of XVII would be similarly characterised.

It should also be noted that the reactions of I in water are complicated, as in the case of VI, by interaction of products to form various sulfonium dimers, the physiological significance of which will be discussed below. Dimer formation is negligible in sufficiently dilute solutions of I (85, 240, 243).

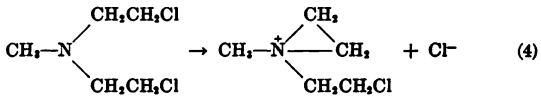
The pharmacological implication of the above hypothesis lies in the attractive proposal that the parallel biological actions of sulfur and nitrogen mustards may be related to the common formation of chemically reactive, cyclic ethylenonium cations. However, recent reports concerning aryl-*bis*(2-chloroethyl)amines, such as XVIII, have questioned whether the nitrogen mustard-like biological actions of these compounds can be related to formation of ethylenimonium intermediates (92, 224). Their reaction with water is slow and halide ions and H^+ are released



XVIII

simultaneously; thus no evidence for the intermediation of quaternary imines is provided. The further implications of this observation will be noted below. Nevertheless, it is pertinent to note that in keeping with the weakly basic character of aromatic amines it might be expected that aqueous reactions of aryl-substituted nitrogen mustards would be more akin to those of sulfur mustard than to those of aliphatic amines such as VI and XIV. It would seem important in future studies to compare in detail the kinetics of decomposition of sulfur and aliphatic nitrogen mustards with those of aromatic nitrogen mustards.

B. Reactions with inorganic ions and organic radicals. It has been established that ethylenimonium and, presumably, ethylensulfonium intermediates but not untransformed mustard molecules react with chemical radicals by condensation. This conforms with the fact that reactive solutes do not alter initial rates of cyclization of mustards in aqueous solutions. An example of the two-step displacement is the reaction of VI with alanine, as depicted in equations 4 and 5 (105).



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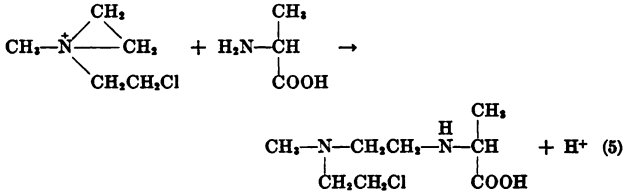


(5)

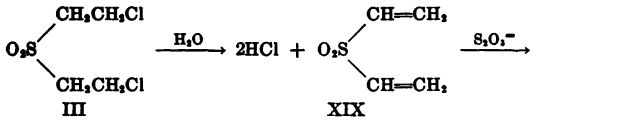
Reactivity of different substances with ethylenonium intermediates varies enormously. The range is exemplified by ions such as dithiophosphate, OH^- and Cl^- which vary in their sensitivity to sulfur mustard by factors of $10^4 : 10^2 : 1$, respectively (202, 203). The number of radicals sensitive to reaction is numerous, attesting to the alkylating propensities of the mustards.

Inorganic ions known to react with either sulfur or nitrogen mustards include thiophosphate, phosphate, pyrophosphate, sulfide, thiosulfate, hydroxyl and chloride (24, 74, 105, 202, 203, 218, 224). Among reactive organic radicals of amino acids are included α- and ϵ-amino, α-imino, imidazolyl nitrogen, sulfhydryl, thio-ether, carboxyl and phenolic hydroxyl (49, 104, 105, 127, 138, 194, 241, 242, 252). Esters are formed with carboxyl groups of organic acids and phosphate groups of organic phosphates (104, 105, 194). In regard to the latter, it is worthy of note that free sugars such as desoxyribose are not attacked (105). Compounds of biological interest which are alkylated include amino acids, di- and tri-peptides, nicotinic acid and amide, pyridoxine, thiamine, various second-

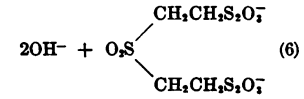
ary and tertiary amines, adenine, adenosine, adenylic acid, adenosine triphosphate, guanine, cytidine diphosphate, hexose and triose phosphates, succinate, salicylate, diethylbarbituric acid, etc. (70, 104, 105, 131, 194, 202, 263).

In an earlier attempt to explain tissue injury by sulfur mustard (see section II B), it was suggested that I might undergo intracellular oxidation to a more reactive sulphone (III). This proposal is obviously unnecessary since I itself readily interacts with compounds of biological interest. Nevertheless, the reactivity of III, of certain sulfonium dimers formed during hydrolysis of I, and of synthetic oxides of the nitrogen mustards remains of interest since they may exhibit considerable toxicity (49, 236, 237, 238, 243). Their chemical and pharmacological properties are also of interest inasmuch as these analogs do not interact via cyclic ethylenonium intermediates.

Bis(2-chloroethyl)sulfone (III) reacts initially in water at physiological pH with formation of HCl and divinylsulfone (XIX). The latter has been shown to condense readily with thiosulfate, amino and sulfhydryl groups of amino acids, and the N atoms of various nitrogenous bases. It is the probable intermediate in the reactions of the sulphone (III) as shown in equation 6 (49, 238).



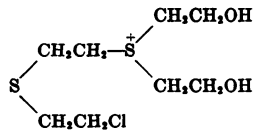
III XIX



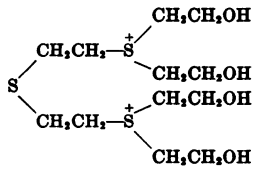
(6)

The derivatives of XIX containing quaternary nitrogen atoms in the β-position relative to the sulfur atom are unstable and decompose under physiological conditions with formation of reactive vinyl groups (238). In theory this property could assume importance in physiological actions of sulfur mustards. While the derivatives of sulfur mustard are stable, their hypothetical oxidation by tissues might convert them into unstable sulfone residues capable of further reactions with cell constituents. However, tissue oxidation of mustard derivatives has not as yet been demonstrated (238).

Certain sulfonium salts related to sulfur mustard, such as XX and XXI, have also been shown to be more or less potent alkylating agents. Their ability to form reactive intermediates in aqueous media under physiological conditions has been related to their toxicity *in vivo* (237). Finally, the oxides of some of the nitrogen mustards have been shown to have appreciable toxicity. This has also been related to decomposition in aqueous media into reactive intermediates, possibly, though not certainly, through loss of oxygen and formation of nitrogen mustards (236).



XX



XXI

C. Reactions with proteins and enzymes. In view of the high reactivity of the mustards toward relatively simple organic molecules, it is not unexpected for proteins in general to be sensitive to attack. Studies on susceptibility of proteins have, for the most part, been concerned with sulfur mustard (I). Extent of reaction has been determined by noting the increase in bound S atoms, either by elementary analysis or by use of I labelled with radioactive S (16, 19, 47, 50, 67, 141, 209, 245, 262, 263). Other methods of analysis of treated proteins have been based on serological evidence for acquisition of immunological specificity (47, 52), alterations in titratable groups (14, 86, 104 ,117, 138, 141, 143, 209) and changes in nutritive value (165, 166). In the last mentioned procedure it has been possible to repair nutritive deficiencies by simultaneous feeding of treated proteins with selected amino acids. Among proteins proven to react are included ovalbumin, ox fibrinogen, serum proteins, thymus and other nucleoproteins, insulin, hemoglobin, gelatin, egg albumin, casein, kerateine, collagen, salmine and others, in addition to enzymes which will be discussed below (16, 19, 47, 52, 86, 128, 138, 143, 165, 209, 215, 244, 245, 262, 263).

Esterification of free —COOH groups appears to account for the incorporation of the major portion of mustard residues. Nevertheless, —SH and —NH_2 radicals and imidazole groups of histidine have, in some instances, been susceptible to attack (86, 104, 138, 141, 143, 215). It should be emphasized, however, that binding of —SH is not an outstanding feature of the chemical reactions of mustards with protein (207). Thus, treatment of hemoglobin sufficient to block dissociation of free —COOH and imidazole groups fails to alter significantly titration of —SH groups in the pH region 8 to 11 (86). Furthermore, reduction in titratable —SH accounts for only small fractions of the total number of mustard residues incorporated during treatment of human serum-proteins, kerateine or egg albumin (19, 138, 141, 209). Such results are to be contrasted with the selective avidity for the —SH group of agents like arsenicals.

The inactivation of catalytic proteins *in vitro* has been the subject of intensive exploration for possible clues concerning the mechanism of the toxic action of mustards. An imposing number of enzymes have been treated *in vitro* with both sulfur and nitrogen mustards and found to resist significant alteration of catalytic activity (20, 87, 197). While such proteins cannot be considered unduly resistant to attack by the agents, their functional groups intimately concerned with catalysis presumably remain unaltered by mustard treatment 16). Such

insensitive enzymes include hematin proteins, flavoproteins, copper and zinc proteins, —SH proteins, enzymes requiring coenzymes I and II, various hydrolytic enzymes splitting proteins, carbohydrates and ribonucleic acid, etc.

Although the majority of enzymes are relatively insusceptible to inactivation *in vitro* by the mustards, the smaller group which is sensitive consists, for the most part, of "phosphokinases" concerned with transfers of phosphate, for example, hexokinase and adenosinetriphosphatase (79, 87, 143, 197). Chicken and swine pepsin (143), certain tissue peptidases and a pepsinase from kidney (34) are also exceptionally sensitive to sulfur mustards. Moreover, the nitrogen mustards are highly active against choline oxidase, acetylcholinesterase and cholinacetylase (1, 20, 79, 250), a fact which has been related to the close structural similarity between choline and the cyclic ethylenimonium intermediates (compare choline with VII in Fig. 1) (20, 250). The sensitive enzymes may owe their susceptibility to dependence for activity on essential groups uniquely reactive with mustards. Indeed, hexokinase when inactivated contains no more mustard residues than does ovalbumin or ox fibrinogen after similar treatment (16).

The disproportionate number of enzymes involved in phosphate exchange among the limited group of sensitive catalysts has provided substantial, though not conclusive, evidence that mustard intoxication involves primary cellular derangements of phosphate transfer (87, 197). This implication will be discussed further. It is, however, convenient at this point to exclude direct reaction with essential —SH of enzymes as responsible for their inactivation and, *pari passu*, for cellular intoxication. Sulfur mustard does not appear to react readily with —SH of large molecules, though it readily reacts with small molecules containing —SH (207). Although reaction with tissue —SH has been suggested as a mechanism of toxic action (14, 36, 96, 117), numerous examples of enzymes ordinarily sensitive to —SH reactive agents are included among the mustard insensitive group. In this connection may be mentioned succinoxidase, papain and urease (20, 87, 197). Conversely, the sensitive group is not composed exclusively of —SH enzymes. Such evidence fails to support the above contention; indeed, some investigators have denied that reaction with —SH is a significant factor in cell intoxication (100, 170, 172, 207, 208, 210).

D. Reactions with viruses and nucleic acids. Interest in susceptibility of nucleoproteins and nucleic acids has been fostered by the propensity of *bis*(2-haloalkyl) amines and sulfides to cause heritable mutations, affect chromosome structure and inhibit mitosis. Both nucleoproteins and nucleic acids are altered by mustard treatment and condense with significant quantities of the alkylating agents (19, 32, 67, 70, 91, 115, 142, 245, 262, 263). Indeed, yeast and thymus nucleoproteins and yeast nucleic acid react to a greater extent with sulfur mustard than do proteins such as serum globulin, salmine, keratin or kerateine (263). Titration of reacted nucleic acids reveals that condensation occurs primarily with phosphoryl and amino groups (91). Moderate treatment with sulfur mustard increases the viscosity of desoxyribose nucleic acid from thymus. It has been sug-

gested that viscosity is enhanced by formation of cross-linkages between polynucleotide chains, as indicated by the greater number of titratable groups blocked than mustard residues bound (91, 245). It should be stated, however, that other workers have shown nitrogen mustards to decrease viscosity of thymonucleate (115), a finding in apparent contradiction to the effects described above.

More related to the biological functions of nucleoproteins are studies concerned with inactivation of viruses by mustards. Among various mammalian, avian, bacterial and plant viruses, susceptibility to irreversible inactivation is high and, in fact, exceeds by far the sensitivity of such enzymes as chicken pepsin and hexokinase (142). Relative susceptibility among viruses appears directly related to the type of nucleic acid present. Thus, viruses in which the nucleic acids are known to be largely or exclusively of the desoxyribose type are more readily inactivated than those containing predominantly ribose nucleic acid. Confirming this relationship is the observation that the pneumococcus-transforming-factor, believed to be composed exclusively of desoxyribose nucleic acid (12), is the most sensitive of the virus-like molecules studied (142). Moreover, kappa factor, which is found in "killer" strains of *Paramecium aurelia* and which is associated with the ability of "killers" to excrete the antibiotic, paramecin, may be reduced by treatment of these organisms with VI (106a). Kappa factor is a self-reproducing, Fuelgen-positive, cytoplasmic particulate, presumably containing thymonucleic acid. It is apparent, then, that there may exist within nucleoproteins, possibly in their nucleic acid moieties, uniquely reactive groups which are essential for biological activity.

Practical application of virus susceptibility to mustards has been suggested by the demonstration that such agents may be useful in preparation of vaccines. Treatment of influenza virus *in vitro* with sulfur or nitrogen mustards renders it non-infective for mice without changing hemagglutinative properties (223). Thus, loss of infectivity may not be accompanied by loss of antigenicity. Vaccines prepared by treating Eastern equine encephalitis, fixed rabies, and hog cholera viruses with sulfur mustard successfully immunize test animals against challenging doses of the respective live viruses (249).

Virus inactivation is also of interest in a possible application of methyl-*bis*(2-chloroethyl)amine (VI) as a sterilizing agent. The instability of mustards in aqueous media at physiological pH and their eventual hydrolysis into non-toxic products are attractive properties recommending their use as antiseptics in stored blood or plasma. In human blood, serum and plasma, VI has proved to be viricidal and antibacterial in concentrations which cause slight changes in plasma proteins, complement, immune bodies, phosphatase and fibrinogen, and marked prolongation of prothrombin time. Fragility of red cells is also increased slightly (137). In regard to effects on normal constituents of blood, it should be recalled that mustards convert serum proteins into antigenically specific substances (33, 52) and inactivate complement (53, 259). Nevertheless, blood treated by VI is said not to sensitize either dogs or humans receiving repeated doses over long periods (137).

IV. EFFECTS OF *bis*(2-CHLOROETHYL) AMINES AND SULFIDES IN MAMMALS

A. Introduction. The manifestations of systemic mustard intoxication are numerous and may be conveniently described in terms of response to different doses of methyl-*bis*(2-chloroethyl)amine (VI, MBA). Supralethal doses of MBA elicit acute actions promptly and are usually fatal within the first 24 hours. Doses in the range of the MLD elicit few overt acute signs, but cause delayed manifestations and death after several days. Sublethal doses may be administered without serious consequences, but they induce profound transient alterations in hematopoiesis. Although discussion will center on the systemic actions of MBA, the various aspects of its pharmacology are, for the most part, qualitatively similar to those of sulfur mustard (I) and nitrogen mustards such as *tris* (2-chloroethyl)amine (V, TBA) and ethyl-*bis*(2-chloroethyl)amine (XIV, EBA).

The toxicity of MBA (hydrochloride) by intravenous administration is of the same order of magnitude in various species. The LD_{50} in the rat, mouse, rabbit, cat and dog is in the range of 0.005 to 0.01 mMol/kgm. (approximately 1 to 2 mgm./kgm.) (4, 126, 147, 148). Sulfur mustard and the hydrochlorides of TBA and EBA are somewhat more toxic than MBA in the rat (LD_{50} between 0.0025 and 0.005 mMol/kgm. or approximately 0.5 to 1.0 mgm./kgm.) (4, 5, 264). Variations in susceptibility of different species to the four mustards are considerably greater when other routes of administration are employed, such as subcutaneous or percutaneous (4, 5, 55, 119, 126, 264). Presumably, relative lethality is best compared when routes of administration are used which permit rapid entrance of these unstable agents into the circulation.

Before discussing the systemic pharmacology of the mustards it is convenient at this point to deal with problems associated with contact injury. Vesication and other sequellae arising from direct contamination by MBA are essentially similar to the classical, local effects of sulfur mustard. Several new aspects of vesication by mustards have been explored recently which are worthy of mention. Precise measurement of rates of penetration of the agents into human skin attests their escharotic potency. Total absorption of as little as $6\gamma/cm.^2$ of I or TBA causes vesication in 50% of white human subjects (196). The major portion of absorbed material probably finds its way into the circulation, and only minute quantities remain to induce local lesions (81). In the eye of the rat, topical application of $1\ \gamma/cm.^2$ of I causes permanent scarring and loosening of the corneal epithelium (100). In both skin and eye, unreacted mustard disappears within a few minutes after application, and the extent of vesication is proportional to the amount fixed to tissue constituents (13, 81, 100, 167, 230). Therefore, the initial step in damage is rapidly induced although the subsequent coagulation necrosis of skin may be delayed in onset and progresses slowly to a maximum over a period of 6 to 9 days (220). Decontaminants, presumably capable of penetrating cells, uniformly fail to prevent lesions when applied a few minutes later than the vesicants. This also affords evidence of the early genesis of irreversible derangements which lead ultimately to delayed necrosis (81, 100).

An essentially similar interpretation may obtain for systemic lesions caused

by mustards. Injected sulfur mustard (tagged with radioactive S atoms) is found widely diffused throughout all body compartments and is fixed to a maximum extent by reaction with tissue constituents within less than one hour after intravenous administration (48). Further proof for rapid fixation of I is shown by cross circulating blood of control dogs with that of animals receiving doses large enough to cause death within 5 hours. When the circulations are joined as early as 30 minutes after intoxication and maintained for 45 to 180 minutes, nonpoisoned members survive without evidence of untoward effect (37). Moreover, temporary occlusion of the arterial supply to the lower extremities of rats for less than 15 minutes after administration of lethal doses of MBA or I protects the bone marrow distal, but not proximal, to the site of clamping. Similar arterial occlusion protects respective portions of the small intestine. Nevertheless, lesions in bone marrow and small intestine are not manifested until some time after the initial fixation of mustard (161).

B. Acute actions. Overt responses to supralethal doses of mustards include central excitation followed by flaccid paralysis, depression, respiratory failure and signs of parasympathomimetic stimulation. TBA, EBA and I are more or less powerful convulsants, and cause intermittent paroxysms which begin within a few minutes after intravenous administration and continue until death (4, 5). More prominent in the case of MBA is a delayed neurologic disturbance resulting in progressive paralysis of the entire somatic musculature (4, 54, 98, 113, 148, 161). The flaccid paralysis is associated with gross incoordination, asynergia and kinetic tremor. Similar effects follow the other mustards but are complicated by concomitant convulsive activity (4, 5).

It has been suggested that the paralytic syndrome caused by MBA may be attributed to its transformation *in vivo* into methyl-2-hydroxyethyl-ethylenimonium (IX) (54, 98, 113, 148). Administration of IX in cats is followed shortly thereafter by manifestations of the syndrome, whereas paralysis is significantly delayed after MBA or its chlorimine (VII). The latency following MBA or VII may be equated to the time required for these agents to transform *in vivo* into IX. Moreover, IX causes paralysis in smaller dosage than either of its antecedents. It should also be noted that neither of the ethylenimonium derivatives of MBA (VII and IX) has convulsant properties (148). Loss of convulsant action also follows conversion of EBA into its two successively formed imine derivatives and, like IX, ethyl-2-hydroxyethyl-ethylenimonium causes paralysis (4).

The nature of the paralytic syndrome caused by MBA or its transformation products has been explored to some extent (98, 148). The disorder is reversible since animals surviving severe paralysis after IX are normal on the following day. It is not located in the myoneural junction or in muscle, inasmuch as contractile response to stimulation of motor nerves remains unimpaired throughout all stages of the paralysis. Placing, righting and spinal (knee-jerk) reflexes are not obtunded during paralysis, though some decrease in rigidity of decerebrate cats does occur. The neurological syndrome appears to be a disturbance of the motor system which remains to be more precisely localized. It accounts for the respiratory failure and terminal asphyxial convulsions (54) sustained by animals receiv-

ing either IX or methyl-2-hydroxyethyl-2-chloroethylamine (VIII) which readily undergoes transformation into IX (98, 148). Moreover, motor paralysis is the principal effect of VIII and IX in mammals (148).

Cholinergic activity is a characteristic effect of the four mustards under discussion (4, 5), a property possibly related to the choline-like structure of their cyclic onium derivatives (113). This relationship is most readily discerned in the structure of methyl-2-chloroethyl-ethylenimonium (VII). It is reasonable to attribute parasympathomimetic properties of MBA to its transformation *in vivo* into VII which, in fact, elicits cholinergic activity in lower dosage than does MBA (148). Overt manifestations of cholinergic activity following shortly after intravenous administration of MBA or VII include salivation, miosis, emesis, defecation, lachrymation, bradycardia and bronchorrhea (4, 99, 113, 148, 161). In addition, VII causes immediate transient prostration associated with flaccidity and widespread fasciculation of skeletal muscle (113, 148). If fatal respiratory paralysis does not ensue, animals rapidly regain normal locomotion only to be overtaken by the onset of the delayed paralytic syndrome described above. Thus VII is unique in its ability to elicit hypotonia of skeletal muscle by two pharmacologically different mechanisms (148).

The autonomic effects of MBA and VII encompass both stimulation of cholinergic effector cells and sympathetic ganglia. Vasodepressor responses are prevented in the atropinized animal and replaced by vasopressor responses (99, 113, 148). Vasopressor activity is in turn suppressed by prior administration of adrenergic blocking agents (Dibenamine). Injection of VII into the arterial blood supply of the superior cervical ganglion causes contraction of the nictitating membrane of the cat, an effect not elicited after crushing the ganglion. Isolated strips of small intestine are stimulated by MBA and VII, a response prevented by atropine. The hydroxyimine of MBA (IX) has similar though less potent nicotinic and muscarinic properties (148). When VII is injected into the artery supplying skeletal muscle, repetitive, asynchronous contractions ensue, followed immediately by a transient depression of contractile response to indirect stimulation. This is in keeping with the immediate paralysis and fasciculation of skeletal muscle noted in intact animals given VII. Cholinergic stimulation of the myoneural junction is not manifested after administration of hydroxyimine (IX) (148).

The cholinergic actions described above are reversible and may be elicited repetitively without evidence of tachyphylaxis. Therefore, stimulation cannot reasonably be regarded to involve irreversible chemical bonding of VII or IX to the receptors concerned. Rather the acetylcholine-like structure of these agents appears to be implicated. Nevertheless, the possibility of irreversible bonding is suggested in their actions on the submaxillary gland of the cat and in certain parasympatholytic effects of mustards to be described below. The close intraarterial injection of MBA or VII into the gland is followed immediately by salivation which persists for several minutes, then by a quiescent period with no secretory activity for 5 to 10 minutes, and finally by resumption of salivation which continues unabated for hours. Prior administration of atropine prevents

all signs of salivary stimulation; but its injection at any time after the initial burst of salivation, even during the quiescent period, does not alter the onset or extent of the second prolonged phase of secretion. Administration of hydroxyimine IX elicits only a single, prompt burst of salivation (148).

The mechanism of the diphasic response of the submaxillary gland to MBA or VII is obscure and may be related to two separate actions, one on the receptor mechanism and the other more delayed effect on the salivary cells directly without intervention of the receptor mechanism (99). However, it is tempting to propose that both phases of salivary stimulation are initiated at the same cholinergic receptor (148). If activation of the receptor depends on close approach of a molecule with choline-like configuration and bearing a strongly positive charge, then the initial effects of VII are understandable. Further, should VII interact with an adjacent radical it would become fixed to the receptor surface. Such interaction involves conversion of the imine N atom to the tertiary state and loss of strong positivity; hence, the temporary cessation of salivary flow. Subsequent cyclization of the free 2-chloroethyl radical would restore the quaternary state and strong positivity; this might account for resumption of salivary flow (217). Since, once initiated, the second phase of flow continues without interruption stabilization of the quaternary immonium structure would be required. Such stabilization is not unreasonable since reactivity of onium rings is influenced by the nature of other radicals attached to the N or S atom (26, 27, 74). Obviously, this proposal adequately explains the ability of atropine to block delayed flow when given before but not after MBA or VII. A similar mechanism may explain the effects of I on salivary glands (77).

Further evidence for possible irreversible reactions with cardiovascular receptors comes from observations that TBA, I and high doses of MBA are vagolytic after initially eliciting cholinergic cardioinhibitory arrhythmias (75, 76, 78, 148). Following TBA or I, arrhythmias progress in intensity until death of the animal; they are not prevented by vagotomy and are blocked only by prior administration of atropine (75, 76, 78). In addition to vagolysis, I and TBA elicit progressive obtundation of vasodepressor responses to acetylcholine or pilocarpine. Ultimately, the response to acetylcholine becomes vasopressor (75, 76, 78). Finally, in dogs, I causes progressive vasodepression and loss of responsiveness to test doses of epinephrine (78). This last phase appears to be the experimental counterpart of the refractory hypotonia noted in victims of the Bari disaster (section IIA).

C. Delayed lethal syndrome. In mammalian species, doses near or equal to the MLD give rise to a delayed syndrome characterized by death or severe debilitation within 3 to 7 days; animals which survive usually recover uneventfully. Anorexia is evident within the first 24 hours and continues until the crisis of the syndrome has been reached. Weight loss in poisoned rats is equivalent to that of starving controls, but in dogs it exceeds the effect of inanition alone. Intermittent and sometimes profuse vomiting is observed several hours after intoxication in dogs and cats and continues until the second or third day. Diarrhea, often hemorrhagic in dogs, appears early in the third day and continues until death inter-

venes or the crisis is passed. As debilitation progresses, animals become untidy in appearance and listless. Terminally, a more or less sudden deterioration sets in, and progressive hypothermia and hypotension are followed within several hours by coma and respiratory failure (4, 5, 88, 126, 147, 213).

The syndrome is complicated by an associated leucopenia. This becomes evident within 24 hours and progresses until maximal during the critical phase of the syndrome. Histopathological changes in fatalities are mainly limited to hematopoietic organs, intestinal tract and testis (see below). Except for adrenal cortical hypertrophy in rats, no consistent alteration of other organs has been reported. These pathologic manifestations invariably follow hypodermic administration of doses near the LD_{50} for sulfur mustard and all tertiary *bis*(2-chloroethyl)-amines which have been studied (66, 126, 163, 164, 177b, 177c). The syndrome as a whole is unique among the actions of toxic chemical agents in mammals, and resembles in many details the effects of total body x-irradiation (126). It is, however, not manifested *in toto* in chickens. After injection of an MLD of mustard, this species succumbs acutely within a few hours, or survives after exhibiting severe leucopenia and only moderate weight loss (126).

Attempts to explain the delayed lethal syndrome have not been wholly successful. There is no evidence of overwhelming sepsis at autopsy or by histological examination (126, 163, 164). Granulocytopenia may be contributory but is not essential. Indeed, damage to femoral bone marrow and granulocytopenia may be prevented in animals by occluding the abdominal aorta and inferior vena cava for a short period after intravenous administration of lethal doses. Animals so prepared succumb with typical manifestations of intoxication (161).

The possible role of the adrenal cortex in development of the delayed syndrome has been the subject of several studies. It is not surprising that hypertrophy and associated biochemical alterations of adrenal cortical tissue may occur as a result of the noxious effects of the mustards. Lethal doses cause depletion in ascorbic acid and ester cholesterol in rat adrenals (65, 182). Moreover, a number of non-specific changes appear in plasma constituents which may also be elicited by scalding, exposure to cold, or subcutaneous doses of turpentine. These include increased fibrin, cholesterol and α-globulin in dogs, and hypercholesterolemia and hyperglycemia in rats (71, 116). It is doubtful whether non-specific responses to injury contribute significantly to the fatal outcome in poisoned animals. Moreover, neither adrenalectomy nor administration of desoxycorticosterone or adrenal cortical extracts materially alters the course of intoxication (4).

Degenerative changes in the intestinal tract may be directly concerned with the mechanism of the delayed lethal syndrome. The majority of poisoned dogs exhibit marked anorexia, emesis and diarrhea which undoubtedly are factors in loss of body weight in excess of that due to inanition. In keeping with overt manifestations of intestinal damage and fluid loss are such findings as marked negative balances of extracellular electrolyte; increased fluid exchange; diminution of blood and plasma volumes, of total circulating plasma protein, and of plasma sodium and chloride; and decreased extracellular and intracellular fluid volume (147, 213). These changes may be considered to contribute ultimately to circulatory failure and fatal anoxia of the respiratory center (147).

Although the above explanation is compelling, it fails to account for the fatal course of a minority of poisoned animals which do not sustain significant changes in volume of extracellular fluid or blood (147). Indeed, the exceptional animals succumb at the same time as the majority of dogs and with similar manifestations of terminal collapse. Even among the majority the extent of fluid loss from all body compartments does not approach in magnitude the range associated with fatal shock following burns, trauma or hemorrhage (147). Neither do changes in volume of extracellular or intracellular fluid appear large enough to be uniformly fatal (213). Finally, fluid therapy prolongs survival to some extent but fails to alter mortality. Similarly, occlusion of the circulation to large portions of the small intestine for 15 minutes after injection prevents marked diarrhea and even decreases the extent of vomiting. Nevertheless, mortality in operated animals remains unaltered (147). It is apparent that the relation between oligemia and the delayed lethal effects of the mustards is not completely understood.

Other metabolic changes in poisoned dogs merit attention. Part of the enhanced excretion of extracellular electrolyte may be ascribed to a renal defect in reabsorption of sodium and chloride (213). This may be related to the observation that persistent renal hyperemia follows TBA intoxication; filtration fraction, however, remains unchanged (147). There is also an unexplained enhancement in the excretion of intracellular cation during fatal poisoning, in excess of that expected to result from tissue destruction. Although the excessive potassium excretion fails to cause lethal deficits in intracellular cation, it suggests a widespread type of cellular lesion (213). In this connection it is interesting to note that sub-lethal doses of I or MBA are said to cause prompt and maintained increases in the utilization of oxygen by dogs without other manifestations of toxic action (15, 17).

D. Lesions in hematopoietic tissues. Doses of mustards in the range of the MLD cause a singular depletion of all hematopoietic tissues, the details of which have, been described in a number of reports (39, 66, 72, 88, 125, 126, 152, 155, 160, 163 164, 177c, 193, 234). Cessation of mitosis and disintegration of formed elements become evident in all lymphoid tissues and bone marrow within 6 to 8 hours after intoxication. Necrosis progresses steadily, accompanied by prominent phagocytic activity in lymphoid organs. Within 24 hours, lymphoid tissues are largely devoid of lymphocytes and cellular debris and consist for the most part of condensed reticular and fibrous elements. Consequently the shrinkage of thymus, lymph nodes, and lymphatic plaques in intestinal wall and spleen is rapid and severe. Simultaneously, the more mature myeloid and nucleated erythroid elements in bone marrow and spleen are largely obliterated; this leaves a scanty population of basophilic erythroblasts, hematocytoblasts, plasma cells, sinus endothelial cells and bizarre megakaryocytes. Histologically, the bone marrow is aplastic. Atrophy persists in lymphoid tissues and bone marrow for 3 to 5 days until regeneration is initiated. Thereafter, recovery is rapid and complete within 1 to 2 weeks in bone marrow, though somewhat slower in lymphoid tissues (66, 126, 152, 163, 164).

Changes in hematopoietic tissues are paralleled by depletion of formed elements in circulating blood. The onset of depletion and its severity may be in-

versely related to the different life spans of various formed elements. Thus, maximal lymphopenia and reticulocytopenia are established at 1 to 2 days; maximal granulocytopenia, at 3 to 5 days; thrombocytopenia, at one week; in contrast, anemia, if it occurs, is more delayed in appearance and only moderate in degree. Recovery of formed elements in blood of surviving animals follows the initiation of regeneration in hematopoietic tissues.

Significant and transient hematopoietic injury may be obtained following administration of sub-lethal doses of MBA or TBA, such as those used therapeutically (39, 125, 155, 160). The usual course of therapy in patients with neoplastic disease produces moderate leucopenia in 7 to 10 days with recovery during the following 1 to 2 weeks. Hemoglobin levels and platelet counts are relatively unaffected. More radical therapy evokes a more severe leucopenia and thrombocytopenia. By itself, leucopenia has not proved unmanageable since few instances of associated generalized infections have been reported. However, in occasional cases, hemorrhagic tendencies develop with widespread purpura and bleeding from various orifices. Hemorrhagic manifestations are associated with prolonged clotting time and the appearance of a heparin-like anticoagulant in blood (232). A similar coagulation defect has been induced in experimental animals by toxic doses of MBA or roentgen radiation (3, 151). Increased urinary excretion of coproporphyrin is also reported to occur in patients receiving MBA (35). This finding may be related to possible hemolytic actions of the drug (152).

Considerable attention has been directed toward proving that the actions of mustards against hematopoietic tissues are direct and not secondary manifestations of generalized intoxication. The fact that fatal intoxication is complicated by indications of adrenal cortical hyperactivity (section II C) might suggest the possibility of involvement of corticosteroids in lymphoid involution (see review, 228). However, necrosis and atrophy following intoxication of adrenalectomized animals approximate in severity the degenerative changes elicited in lymphoid tissues of intact controls (126, 163). Direct actions on lymphocytes *in vitro* have also been described (163, 226). Moreover, various portions of bone marrow may be completely protected by local occlusion of circulation for a brief period during and after administration of lethal doses. Regions so protected are normal or hyperactive while unprotected bone marrow in the same animal is atrophic and aplastic (161). This is convincing evidence for the direct nature of mustard action on hematopoietic cells.

The susceptibility of hematopoietic cells to destruction by mustards is closely paralleled by their sensitivity to "total body" irradiation with roentgen ray. Distinguishing features become apparent during regeneration, since hematological recovery after toxic doses of radiation appears less rapid than following comparable intoxication with mustards. Otherwise, alterations in bone marrow and lymphoid organs are largely identical in both temporal and morphological appearance (126, 152, 154). Identity of action includes a similar selective destruction of specific cell types. Both agents spare the most primitive forms such as reticulum cells of lymphoid tissues and sinus endothelium, hemocytoblasts, and basophilic erythroblasts of bone marrow. While proliferation of such cell types is

inhibited during the atrophic phase of hematopoietic damage, nevertheless, regeneration eventually ensues from these earliest blood cell precursors. On the other hand, more mature precursors, as mentioned previously, rapidly disappear from hematopoietic tissues during intoxication (152, 154). The relative resistance of basophilic erythroblasts and hemocytoblasts may be readily demonstrated in rabbits previously rendered anemic by phenylhydrazine. Bone marrow in animals with phenylhydrazine anemia is abundantly populated by primitive blood cell precursors and accordingly severe depletion does not follow intoxication by mustard or roentgen ray. However, even in hyperplastic marrows, more mature myeloid and erythroid elements undergo typical dissolution (152, 154).

The parallelism between intoxication by roentgen ray and mustards extends to their common action on intestinal mucosa and testis. Both agents induce severe epithelial injury in intestinal mucosa with cellular hypertrophy, pyknosis, disintegration and desquamation (126, 164, 177c). The fact that mucosal damage may be prevented by temporary occlusion of circulation to portions of the intestine during and after administration attests to the direct nature of the epithelial lesion caused by mustards (161). Marked damage to spermatogenesis and atrophy of testis are also common effects of both roentgen ray and mustards (177b, 234).

E. Actions on neoplastic tissues. The resemblances between actions of roentgen ray and mustards which became apparent during early stages of recent wartime investigations sponsored a number of profitable attempts to duplicate radiation effects by means of the chemical agents. Applications to studies of immune mechanisms, genic mutation, and chemotherapy of cancer were anticipated and proved. None of these explorations achieved more widespread interest in laboratory and clinic than the positive demonstration that mustards, like "total body" roentgen irradiation, can destroy and retard neoplastic tissues just short of permanent eradication. It is deemed unnecessary to consider herein the abundant literature which has already accumulated on the chemotherapy of cancer by mustards; the initial announcements and several subsequent reviews are readily available (107, 112, 125, 155, 158, 159, 160, 221). However, a few remarks are in order insofar as they pertain to the mechanism of action of mustards.

Opinion generally does not relate anti-tumor activity to alteration of specific mechanisms present only in neoplastic cells. Rather it is pertinent to note that tumors susceptible to mustard therapy are, for the most part, lymphomas and other neoplastic diseases of hematopoietic organs. Accordingly, their sensitivity is probably related to the unique susceptibility of hematopoietic cells to mustards and "total body" irradiation. More generally, both normal and neoplastic blood-cell forming tissues may be liable to derangement by chemical and physical agents possessing propensities for damaging rapidly proliferating cells. Further consideration of the mechanism of action of mustards against proliferating cells will be given below.

Nitrogen mustards, like MBA and TBA (as hydrochlorides), may be administered with safety if due consideration is given to their potent actions against normal hematopoietic cells. In clinical dosage, a few additional undesirable side-

actions appear which are transient, *i.e.*, nausea, vomiting and anorexia. These may be reflex manifestations of moderate intestinal damage but no significant lesion of intestinal epithelium is reported to occur with clinical doses. Furthermore, patients receiving the usual therapeutic course fail to manifest the profound alterations in balance of water and intracellular and extracellular electrolyte which follow lethal intoxication in dogs (see section IV C) (82a). MBA "is an effective, temporary, palliative agent in Hodgkin's disease, lymphosarcoma, chronic leukemia, polycythemia vera, mycosis fungoides, primary lung carcinoma and, to a lesser degree, in other miscellaneous neoplastic disorders" (159). Nevertheless, it is not a curative agent and no evidence exists proving that it extends survival or otherwise alters the inexorable progress of neoplastic diseases. Its major use is confined to treatment of disseminated disease too extensive, inaccessible, or unresponsive for application of conventional roentgen ray therapy (107, 158, 159).

F. Effects on immune reactions. The destructive and suppressive actions of mustards on hematopoietic organs are apparently associated with untoward effects on immune mechanisms. Shortly after World War I, sulfur mustard, like other leucotoxic agents (benzol, roentgen radiation), was found to suppress antibody production (140). More recent investigations ascribe similar inhibitory properties to TBA and MBA (214, 235, 246). The fact that MBA retards antibody production has sponsored its use as an experimental tool in studies of tissue hypersensitivity. Development of local tissue reactivity (Shwartzman phenomenon) following intradermal injections of meningococcus endotoxin is prevented in animals which have previously received MBA, roentgen ray or benzol. It is suggested that a common action of the three agents on the reticuloendothelial system renders vascular endothelium anergic and incapable of reacting with foreign principles (28). In a comparable manner MBA suppresses development of circulating antibodies, cutaneous hypersensitivity, and vascular lesions in rabbits in response to horse serum or bovine gamma globulin given intravenously (62, 82, 156).

The above studies suggest trial of MBA in the control of disorders which may involve tissue hypersensitivity (28, 73, 82). Results of a preliminary nature have already indicated the heuristic value of this suggestion. Experimental nephritis in rabbits induced by administration of bovine gamma-globulin can be prevented by prior treatment with MBA (156). Therapeutic doses of MBA are reported to provide transient decreases in proteinuria in human glomerulonephritis (73). Finally, one instance of prompt remission in response to MBA therapy has been noted in a case of chronic disseminated lupus erythematosus (204). It is too early to conclude that such results are related to possible immunological alterations in tissues induced by MBA. Interpretation of effects in glomerulonephritis must take into account previous experimental studies which have impugned nitrogen mustards with direct renal actions (147, 213). Nevertheless, additional studies in glomerulonephritis seem indicated. Also observations might include trial of MBA in other disorders possibly based on acquired hypersensitivity, such as rheumatoid arthritis, rheumatic fever, periarteritis nodosa, drug sensitivities, etc. (28, 82).

V. ACTIONS AGAINST PROLIFERATION AND THE CELL NUCLEUS

A. Mitotic inhibition. Of the various manifestations of systemic injury elicited by mustards, damage to hematopoietic and neoplastic tissues appears to be effected by the smallest doses producing evidence of intoxication in mammals (Section IV D). A noteworthy feature is the prolonged period of atrophy, during which mitosis is significantly depressed. Presumably mitotic inhibition is not restricted to hematopoietic organs, for other germinal tissues such as the epithelium of the cornea and of the intestinal mucosa may be shown to share suppression of cell division caused by hypodermic administration of lethal doses (100). Inhibition of mitosis is found in regenerating liver, although structural and functional impairment has not been reported to occur in normal hepatic cells (177d, 188). The singular sensitivity of the mitotic mechanism in mammalian cells has been studied in the response of corneal epithelium to various doses applied directly to the eye (100). In rats, 0.01 γ/cm.^{2} of sulfur mustard is a threshhold dose which causes only suppression of division in epithelial cells. By daily repetition of this dose mitotic inhibition may be maintained for as long as a week. During this period epithelial cells undergo hypertrophy and the resulting increments of cellular substance formed keep pace with the normal rate of growth of corneal epithelium. In spite of mitotic block, wound healing remains unimpaired in the enlarged epithelial cells. Mitotic inhibition is manifested in some pre-mitotic phase of cell division since division figures do not accumulate and cells in mitosis at the time of intoxication complete reproduction normally. When dosage is discontinued, epithelial cells recover proliferation and eventually normal size without evidence of permanent damage. It is important to note that doses higher than threshold cause additional lesions, *i.e.*, scattered nuclear fragmentation at 0.1 γ/cm.^{2} and uniform pyknosis of all cells at 1 γ/cm.^{2}. The latter dose induces permanent scarring and is 100-fold greater than the amount required to block mitosis (100).

Mammalian cells are not uniquely susceptible to selective interference of proliferation by mustards. Reproduction of yeast cells is sensitive to both sulfur mustard and TBA (168, 169, 173, 187, 251). Brief exposure to threshold concentrations of sulfur mustard reduces rates of proliferation for several cell generations without inducing significant mortality (168, 169). After exposure to higher concentrations, yeast cells continue growth for considerable periods without evidence of budding. Hypertrophied cells eventually succumb. Hypertrophy and reduced rates of cell division are also observed in unicellular plants treated with low concentrations of TBA (149). Inhibition of mitosis occurs in root tips of higher plants exposed to minimal concentrations of MBA which do not elicit other cellular changes (201).

The propensity of nitrogen mustards to cause selective damage to proliferating cells has been studied intensively in amphibian embryos (41, 42, 110). Exposure of embryos in various developmental stages produces arrest of mitosis in the interphase throughout all tissues. Nevertheless, regions in which cells have completed mitotic activity at the time of exposure continue to differentiate histologically in a normal manner. For example, it is possible, by exposure of embryos in a suitable stage, to permit undisturbed differentiation of cranial cartilage and

cranial ganglia while the growth of trunk cartilage and spinal ganglia is completely suppressed.

Two different responses to mustard treatment are noted in proliferating tissues of the amphibian embryo. Cells such as those of the early retinal cup disintegrate rapidly after an initial nuclear fragmentation. This type of response occurs in cells which, if undisturbed, eventually differentiate into non-proliferating adult tissues. On the other hand, a different response is given by proliferating cells which remain primitive in character in the embryo or which, like epidermis or gut epithelium, differentiate into permanently germinal tissues in the adult. After treatment, such germinal cells fail to divide but undergo a remarkable nuclear and cytoplasmic hypertrophy. Moreover, the gigantic forms differentiate without further mitosis into more adult cell types in pace with analogous cells of untreated embryos (42).

The cellular lesions obtained in amphibian embryos appear to be analogous to changes noted in mammalian hematopoietic tissues (section IV D). Thus, more differentiated elements of bone marrow and lymphoid organs rapidly disintegrate in response to intoxication. On the other hand, the reticulum of lymphoid organs and sinus endothelium, hemocytoblasts, and basophilic erythroblasts of bone marrow, all relatively undifferentiated, germinal cells, resist destruction and remain non-proliferative during the prolonged periods of atrophy which characterize hematopoietic lesions following mustard poisoning.• During periods of mitotic arrest, hypertrophy of primitive forms occurs in bone marrow and also in the epithelium of the cornea (see above) and the intestinal mucosa. The nature of the response to mitotic inhibition by mustards, whether it be arrest with prompt disintegration or with continued cell growth, is cell-specific and at present without explanation (42).

Specific interference with development of particular embryonic organ systems has been elicited in both mammals and insects. Fetal deformities may be produced by administration of MBA to pregnant rats (139). Profound modification of adult morphology occurs after treatment of embryonic fruit flies (43). Presumably such alterations result from retardation of specific cell systems during critical periods of their development (43, 139).

B. Effects on cell nuclei. In conjunction with mitotic inhibition or in response to doses somewhat higher than those required to arrest cellular reproduction, proliferative cells manifest cytological evidence of alteration in chromosomal structure and function. Multipolar mitoses, "lagging" and "stickiness" of chromosomes during anaphase, and chromosomal fragmentation, deletions, deficiencies and interchanges may be elicited in a wide variety of plant and animal cells (6, 8, 11, 84, 90, 93, 94, 95, 174, 181a, 201, 231). If aberrations are not so extensive as to cause non-viable chromosomal deficiencies in subsequent cell generations, they may be reproduced indefinitely as inherited mutations.

The mutagenic properties of both sulfur and nitrogen mustards, initially investigated in classical experiments with the fruit fly, *Drosophila melanogaster*, represent the first conclusive evidence that chemical agents (6, 8, 9, 10, 11) can duplicate hitherto exclusive effects of penetrating radiations (195). It is of in-

terest to note that the initial experiments for assessing mutagenic activity in fruit flies were suggested by the observed parallelism of effects of roentgen ray and mustards in mammals. This, indeed, is a tribute to the validity of comparative pharmacology! Subsequent studies have proved the general nature of mutagenic properties since mustards are reported to increase mutation rates not only in *Drosophila* but also in bacteria, fungi and higher plants (46, 61, 109, 132, 133, 146, 162, 185, 239, 247, 248, 261). A preliminary report is available indicating the possible induction of a visible mutation among the progeny of male mice treated with MBA (7).

The mutagenic action of mustards is of further interest in relation to their possible carcinogenicity (248). Inasmuch as carcinogenesis may involve primary somatic mutation of normal into neoplastic cells, it has been of recent interest to test mustards for tumor-inciting properties (56, 58). Studies antedating the recent war found sulfur mustard to inhibit production of skin cancers when applied to sites simultaneously receiving repeated doses of potent carcinogens (29, 30). Current reports show that repeated application of small doses of sulfur mustard to skin of mice fails to incite tumors although, many alterations are caused which resemble the precancerous effects of 3,4-benzpyrene (94). More positive evidence for carcinogenicity is reported following prolonged, chronic hypodermic administration of MBA and TBA in mice (58). Mice so treated exhibit a higher incidence of tumors at various sites than is found in uninjected controls. However, this study is not convincing since the strains of mice used and the incidence, time of onset and nature of tumors found normally in these strains are not indicated. It has also been reported that single doses of MBA cause more rapid onset and greater incidence of pulmonary tumors in strain A mice in which lung cancer is readily elicited by other chemical agents (145). It does not appear warranted at present to draw significant conclusions concerning the relative carcinogenicity of mustards. Accordingly, relations between carcinogenicity and mutagenicity remain to be established.

C. Mechanism. The results of a number of recent investigations converge toward a reasonable explanation of the primary site of action of mustards in proliferating cells. Cogent to this explanation are observations described above proving nucleoproteins and nucleic acids to interact readily with mustards *in vitro* (section III D). Even more pertinent is the fact that nucleoprotein-containing bodies like viruses and a nucleic acid like the pneumococcus-transforming factor (12) are highly susceptible to inactivation by mustard treatment (142) (section II D). Furthermore, among these bodies the capacity to reproduce intracellularly is most readily destroyed in those containing nucleic acids of the desoxyribose type. In view of the cytological actions of mustards it seems more than coincidence that self-duplicating structures containing thymonucleates should be outstandingly sensitive. Thymonucleic acids are uniquely associated with chromosomes in cell nuclei and have assumed prime importance in conceptions of the structure, function and reproduction of chromosomes (59, 69, 83, 130, 192). In fact, "the great accumulation of desoxyribose nucleoproteins in the chromosomes strongly suggests that these substances either are the genes them-

selves or are intimately related to genes" (192). It follows, then, that chromosomal derangements elicited by mustards may result from a primary interaction of the agents with thymonucleates in cell nuclei.

Disturbances in the synthesis of thymonucleic acids have also been proposed as the primary factor in mitotic inhibition by mustards. Treatment of the amphibian embryo with MBA causes an immediate cessation of synthesis of desoxyribose nucleic acid in conjunction with prompt suppression of cellular reproduction. At the same time synthesis of ribonucleic acid keeps pace with the normal increase of this constituent in untreated embryos (45). The fact that concentrations of MBA sufficient to inhibit both cell reproduction and synthesis of thymonucleate fail to prevent development of ribonucleic acids suggests an exquisite selectivity of cytological action. This selectivity is further demonstrated in bacteria which may be rendered non-proliferative by brief exposure to threshold concentrations of sulfur mustards. When such non-proliferating cells are subsequently infected with bacteriophages, which are largely thymonucleoproteins, they are found to support intracellular multiplication of the bacterial viruses (144). Presumably subsidiary cellular mechanisms necessary for the duplication of thymonucleate are left unimpaired by concentrations of mustard which prevent cell proliferation. Accordingly there is some indication that mustards may act directly on thymonucleates to render cells non-proliferative. However, this plausible interpretation must be accepted with reservation. Inhibition of mitosis from any cause could be expected to prevent synthesis of nuclear components. Thus the demonstration that synthesis of desoxyribose nucleic acid is inhibited by mustards does not constitute final proof that this specific metabolic process is the primary site of attack of the agents in the proliferating cell (100a).

VI. EFFECTS ON TISSUE METABOLISM

The respiration and glycolysis of mammalian tissues in general are inhibited *in vitro* by M/1000 of either sulfur or nitrogen mustards (21, 31, 87, 157, 197, 198, 227). Inhibition is immediate and increases progressively with time. On the other hand, a latent period of several hours ensues after application of vesicant doses of mustard to intact skin before metabolic changes can be detected. These consist of a progressive fall in anaerobic and aerobic metabolism of glucose while oxygen uptake remains relatively unimpaired. Since the anaerobic conversion of hexosediphosphate to lactic acid is also unaltered, the primary effect of mustard in causing vesication has been considered a specific inhibition of the initial phosphorylation of glucose to glucose-6-phosphate. This is supported by the fact that hexokinase, which catalyzes phosphorylation of glucose, is unusually sensitive to inactivation *in vitro* by treatment with sulfur mustard (section III C). Further support comes from the observation that, of 25 vesicants studied, all readily inactivated hexokinase whereas a large number of non-vesicant structural analogs of mustard proved ineffective against the enzyme at test concentrations employed (87, 197).

The "enzyme inactivation" theory of vesication by mustards has met with

serious objection. Hexokinase and, indeed, all sensitive enzymes undergo immediate and maximal inactivation when treated with mustards *in vitro*. Nevertheless, hexokinase activity in contaminated skin remains unimpaired for several hours and only begins to decrease when early signs of injury become evident (87, 197, 207, 208). On the other hand, studies with radioactive mustard and with penetrating decontaminants indicate that irreversible interaction with skin constituents must occur within the first few minutes after contamination [(81) and section IV A]. The delayed inactivation of hexokinase *in vivo* appears then to be a secondary manifestation of cell injury. Actually the decreased activity of hexokinase in injured skin may result from its leaching out of injured cells into subcutaneous edema fluid which collects beneath mustard lesions. Other enzymes, irrespective of their *in vitro* sensitivity to mustards, have been found to escape from damaged skin cells and have been recovered with undiminished activity in edema fluids (79, 81, 197, 207). It is pertinent to note that changes in permeability of cells and capillaries in injured areas of skin occur quite rapidly after contamination (81, 207, 264).

The difficulties attending association of systemic lesions with primary enzyme inactivation are apparent in mammalian cornea (100) and yeast (173), in which concentrations of mustards below those which affect either respiration or fermentative action have been found to alter mitotic activity. Following hypodermic administration of lethal doses of sulfur mustards to rabbits, glycolysis or oxygen consumption of bone marrow and spleen is inhibited but only after a delay which coincides with the time required for morphological changes to become evident in these organs (197, 198). Decrease in enzyme activity of intestinal tissues after intoxication coincides with the development of intestinal pathology. Apparent inactivation occurs simultaneously in various intestinal enzymes irrespective of their *in vitro* susceptibility. Accordingly, loss of activity appears related to death and sloughing of intestinal mucosa (79). Conversely, tissues like lung or skin which sustain no visible injury after hypodermic lethal doses exhibit no change in activity of either sensitive or insensitive enzymes (79). Furthermore, administration of overwhelming doses of sulfur mustard which are lethal within 2 to 3 hours fails to alter glycogenolysis, glyconeogenesis, or glycolysis in rats before death ensues (38). Such results do not offer evidence for direct *in vivo* inactivation of hexokinase or other "phosphokinases" concerned with carbohydrate metabolism in spite of the fact that such enzymes are sensitive to treatment *in vitro*. Moreover, the P^{32} uptake of regenerating rat liver is not altered by intravenous doses of sulfur mustard which cause marked reduction of cells in mitosis (188).

A general objection to the "enzyme inactivation" theory may be raised on the grounds that the sensitivity *in vitro* of most enzymes is not great enough to implicate their inactivation as a primary factor in systemic intoxication (20, 21). Most sensitive enzymes are not appreciably inactivated by concentrations of mustards below 10^{-4}M. On the other hand, after hypodermic administration of median lethal doses, assuming equal diffusion throughout all tissues, sulfur mustard and nitrogen mustards like MBA and TBA would exist momentarily in

concentrations not greater than 10^{-6}M. Actually, studies of the distribution of sulfur mustard tagged with radioactive S atoms indicate that mustards become fixed rather uniformly throughout all tissues after intravenous injection. Therefore, preferential penetration into and retention by tissues like bone marrow and spleen cannot account for tissue susceptibility (48, 51).

Of the group of sensitive enzymes, only choline oxidase is sufficiently susceptible to warrant consideration as a possible factor in intoxication by nitrogen mustards (20, 21). Choline oxidase is half inactivated by 10^{-6}M MBA or TBA *in vitro*. Following lethal doses of MBA in rats there is a rapid decrease in choline oxidase activity in kidney but not in liver. However, choline oxidase is not inactivated by sulfur mustard *in vitro* (197) and, therefore, inhibition *in vivo* of this enzyme by nitrogen mustards would appear to have little bearing on the mechanism of mustard intoxication.

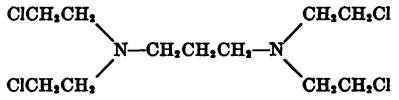
The fact that mustards inhibit cholinesterase *in vitro* has been associated with their parasympathomimetic activity (197, 250). There is little other evidence to support this contention. Brain, red cell and plasma cholinesterases are at least 1000-fold less sensitive to MBA or TBA than to physostigmine or diisopropylfluorophosphate (1). Cholinesterase activity of submaxillary glands of cats is not depressed by intracarotid administration of sufficient MBA to cause irreversible salivary flow (148). Moreover, the prolonged cholinergic manifestations of mustard intoxication, once established, are not suspended by subsequent injections of atropine (section IV B). Thus, it is difficult to consider mustards to be cholinergic by virtue of anticholinesterase activity.

Although efforts to correlate alterations in activity of specific enzymes with biological actions of the mustards have not been successful, certain metabolic changes in synthetic reactions occur in intoxicated cells and tissues which may reflect primary enzyme damage. Inhibition of both ammonia utilization and protein synthesis follows exposure of a unicellular plant to concentrations of TBA which also retard cell division (149). Urea synthesis is inhibited in livers of rats receiving lethal doses of MBA. *In vitro* synthesis of carbohydrate, creatine and urea in rat tissue slices is sensitive to nitrogen mustards (21). Finally conjugation of various substances by rat liver slices is retarded by MBA (186). It has been suggested that inhibition of synthetic reactions may be a vital factor in the various manifestations of the delayed lethal syndrome described previously (21).

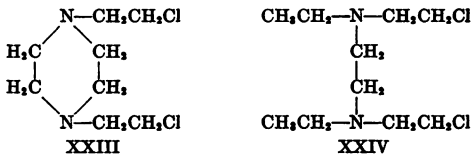
VII. CHEMICAL CONSTITUTION AND BIOLOGICAL ACTIVITY

A. Actions against proliferating cells. Regardless of the criteria employed, whether derangement of mammalian hematopoiesis or of tumor growth, cessation of cell reproduction in embryos, or mutation in *Drosophila*, the configuration essential for selective actions against proliferating cells is the association of at least two 2-haloethyl groups per mustard molecule (4, 5, 10, 44, 55, 63, 64, 92, 119, 134, 177a, 177c, 181a, 224). Analogs of nitrogen or sulfur mustard containing only single 2-haloethyl groups do not selectively affect hematopoietic or other multiplying tissues. The halogen may be either chlorine, bromine or iodine, but must be in a position beta to N or S atoms. *Bis* (2-halopropyl) derivatives, how-

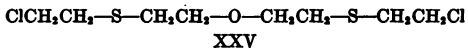
ever, act like mustards. Among nitrogen mustards the secondary amine, *bis* (2-chloroethyl)amine, is active but less potent than tertiary amines like MBA or TBA. Quaternary amines are uniformly devoid of the characteristic properties of mustards, for example, the close relative of MBA, dimethyl-*bis*(2-chloroethyl)-ammonium chloride. Typical activity may be found among congeners of nitrogen mustard containing a wide variety of aliphatic or aromatic groups attached to *bis*(2-haloethyl)amine moieties through C-N linkage. The third substituent has considerable influence on the potency of derivatives and, if too complex, may render analogs inactive (102, 134). Polyamines like XXII have typical mustard-like properties. Both haloethyl groups need not be bonded to the same N or S atom. For example, XXIII and XXIV are potent nitrogen mustards (64, 118, 214a). Moreover, XXV is an effective mutagen in *Drosophila* (10).



XXII



XXIII XXIV



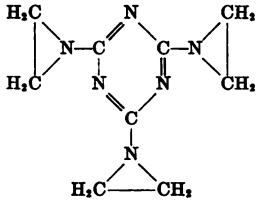
XXV

The pharmacology of mustards can best be interpreted in terms of the capacity of 2-haloethyl amines and sulfides to transform *in vivo* into reactive, cyclic ethylenonium moieties. Where this transformation is hindered as in the case of quaternary amine analogs of mustards, typical activity is absent. Moreover, synthetic ethylenimonium transformation products have been found to exhibit almost all of the pharmacological properties of their parent amines (sections IV B, C, D). Primary convulsant activity appears to be the only action of tertiary nitrogen mustards not shared by quaternary ethylenimonium derivatives. This suggests that the quaternary imines are excluded from some regions of the central nervous system readily penetrated by parent substances. Presumably, after penetration into these areas tertiary nitrogen mustards undergo intracellular transformation into reactive imines. The fact that nitrogen mustards are distributed somewhat differently than their ethylenimonium derivatives is in keeping with findings that the imines may be more toxic than the respective tertiary amines (4, 54, 98, 148).

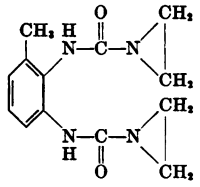
On the assumption that cells might be relatively impermeable to cationic

ethylenimonium derivatives, it has been suggested that nitrogen mustards gain access to intracellular sites only in the form of the parent, tertiary amines. After administration synthetic ethylenimonium ions are presumed to undergo back-reaction with extracellular chloride and thus give rise to permeable tertiary amine mustards (4). However, such a proposal seems untenable in view of evidence that synthetic ethylenimonium derivatives are more toxic than their respective parent amines.

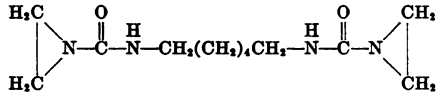
The importance of reactive ethylenimine moieties in the pharmacological actions of *bis*(2-haloethyl)amines has recently been reaffirmed by the finding that compounds such as XXVI, XXVII and XXVIII elicit effects in mammals which closely resemble those of nitrogen mustards. Doses of the three ethylenimine derivatives near the MLD cause a delayed lethal syndrome, marked pancytopenia and severe diarrhea.



XXVI



XXVII

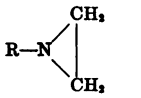


XXVIII

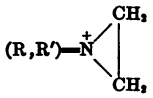
Necrosis and atrophy of all hematopoietic tissues and damage to intestinal epithelium occur. Supralethal doses of XXVI, 2,4,6-*tris*(ethylenimino)-s-triazine, like those of aliphatic *bis*(2-chloroethyl)amines, are convulsant and paralytic. However, unlike MBA, XXVI is not outstandingly parasympathomimetic. Presumably this may be related to the tertiary state of the ethylenimine moieties of XXVI in contrast to the quaternary state of the ethylenimonium transformation products of MBA (249a). The ethylenimine derivatives, XXVI, XXVII, and XXVIII, are active against animal tumors which are sensitive to the chemotherapeutic effects of nitrogen mustards (62b, 62d, 62e, 179a, 245a). Furthermore, XXVI appears to be as effective as MBA in the palliation of clinical neoplasms (159b).

The demonstration that derivatives, such as XXVI, exert specific actions against proliferative cells and tissues may provide further evidence for the nature of the molecular configuration essential to this pharmacological property

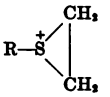
of the mustards. Compounds, or their active intermediates, which contain the unstable 3-membered heterocyclic radicals, XXIX, XXX, and XXXI, are now known to elicit the unique features of mustard intoxication in mammals. To this group of active 3-membered heterocyclic radicals may be added the ethylenepoxy configuration, XXXII. Ethylenepoxy derivatives such as XXXIII and XXXIV



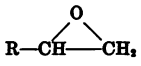
XXIX



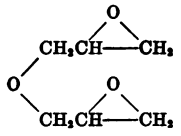
XXX



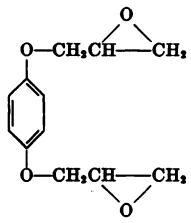
XXXI



XXXII



XXXIII



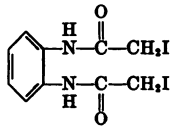
XXXIV

have recently been found to evoke characteristic mustard-like derangements in the chromosomes of dividing cells (181a).[2]

[2] It has recently been stated that attempts to improve the specificity of action of amine mustards against neoplastic tissues have been based on "limited knowledge of the chemical basis for the desired action and have met with little success" (199, 200). In contrast, the highly specific adrenergic blocking action of Dibenamine analogs, which are also 2-haloethylamines, can be accurately predicted on the basis of specific requirements of chemical structure. It was further suggested that studies comparable to those carried out successfully in analysis of structure-activity relationship among Dibenamine analogs might lead to improvements in specificity against tumors (199, 200). This judgment might be tempered by observations presented herein which show that destruction of tumors by mustards is actually related to a highly specific mechanism associated with the *bis*(2-haloethyl) configuration. This specific mechanism operates generally in proliferating cells, a factor unfortunately limiting the chemotherapeutic potentiality of nitrogen mustards in treatment of neoplastic diseases. Thus, attempts to select mustard analogs with proportionately greater activity against neoplastic cells than, for example, against normal hematopoietic cells have in the opinion of the present reviewer not been retarded by ignorance of a rational mecha-

An interesting hypothesis has been offered to account for the relation between the molecular configuration of mustards and their propensity to upset functions of cell nuclei. It is suggested that the presence of two alkylating groups per molecule permits reaction at two distinct points in fibrous molecules or at single points on each member of pairs of contiguous fibers. Cross-linkages formed between sister chromatids could lead to breakage of chromatids during their extension in interphase. During the subsequent prophase and metaphase, chromosomes formed from broken chromatids would appear aberrant. Cross-linking alkylation could thus give rise to the typical cytological lesions noted after mustard intoxication (118, 181a).

It is further proposed that the unique activity of mustards may not depend on capacity to form cyclic ethylenonium derivatives but rather on bifunctional properties which permit cross-linking alkylation between vital fibrous molecules (118, 181a). This generalization is based in part on the lack of evidence for formation of cyclic ethylenimonium derivatives during hydrolysis of aryl-*bis*(2-haloethyl)amines, which, nevertheless, act biologically like nitrogen mustards (however, see section III A). Presumably other types of bifunctional molecules such as XXXV might be found to duplicate mustard effects (92, 118, 134, 181a, 224).



XXXV

It is interesting to note that XXXV produces cytological changes in amphibian skin resembling those caused by mustards; yet it fails to affect the growth of mustard sensitive tumors in rats (92). Some pertinence must also be attached to the pharmacology of certain close analogs of sulfur mustards, *i.e.*, its sulfoxide, sulfone and sulfonium derivatives, XX and XXI, as well as divinyl sulfone. Although several of these agents are highly toxic, none of them severely damages bone marrow in mammals and their pharmacology has to some extent been differentiated from that of true mustards (5, 171; however, see 14a, 181a). With respect to the validity of the generalization under discussion, it is important to emphasize that the various analogs of sulfur mustard listed above are bifunctionally reactive compounds (section III B).

B. Delayed lethal syndrome. In general, *bis*(2-haloethyl) amines and sulfides and their ethylenonium derivatives which exert selective action against hemato-

nism of action. Rather it has been difficult to predict molecular configurations causing selective damage in only one class of proliferative cell, *i.e.*, the neoplastic cell. Such a problem might also plague the investigator who attempts to select from Dibenamine analogs those compounds blocking only responses to thoracic sympathetic outflow while leaving those to lumbar outflow unchecked.

poietic tissues also manifest the characteristic delayed lethal actions of sulfur mustard or MBA (section IV C). Conversely, analogs and transformation products of amine and sulfur mustards which do not derange proliferative tissues usually fail to cause delayed deaths. This close parallelism between cytotoxic action and delayed lethality implies a causal involvement of lesions in hematopoietic organs and intestinal mucosa in the death of mammals. However, such an implication has not been wholly susbstantiated (section IV C). Furthermore, a highly toxic sulfonium derivative of sulfur mustard, XX, causes delayed deaths without evidence of marked involvement of bone marrow (5). One must presume that the nature of the delayed lethal syndrome is too obscure at present to permit ready understanding of molecular configurations essential to its evocation.

C. Acute pharmacological actions. The variety of prompt responses which may be elicited in mammals receiving supralethal doses of amine and sulfur mustards have been described in an earlier section (IV B). As stated previously, cholinergic properties have been ascribed to a suggestive structural resemblance between cyclic ethylenonium transformation products and choline. The most interesting aspect of certain of the parasympathomimetic features are their irreversibility. It is reasonable to attribute the prolonged nature of cholinergic stimulation (also atropine-like actions when these exist) to fixation of active derivatives to receptor sites through reaction with cyclic ethylenonium moieties. Advantageous employment of this chemical property in the development of compounds having a variety of selective irreversible pharmacological actions might be anticipated. Actually such a possibility has been realized in analogs of dibenzyl-2-chloroethylamine which are irreversible adrenergic blocking agents (199, 200). Furthermore, derivatives of α-naphthylmethyl-ethyl-2-chloroethylamine are, in addition to being adrenergic blocking drugs, antihistaminic in action (181). Finally, compounds related to dimethyl-2-chloroethylamine have been found to elicit a prolonged neurological disturbance in a number of mammalian species, which suggests possible damage to cerebellar centers. After intoxication, animals may exhibit permanent ataxia, incoordination and derangement of righting and landing reflexes (120, 175).

VIII. MUSTARD ACTION AND EFFECTS OF IONIZING RADIATION

In previous sections, reference has been made to evidence ascribing to mustards and roentgen radiation certain similarities in biological action (sections IV D, E and F and V B). These may be briefly reiterated. Many of the clinicopathological changes which characterize the delayed lethal syndrome (section IV C) are manifested in mammals receiving fatal doses of penetrating radiation (126, 219). Outstanding are parallel hematological changes (89, 126, 151, 153, 154, 225, 226) and lesions in intestinal mucosa (101, 126, 164). Both roentgen radiation and mustards suppress antibody production (28, 140, 156) and induce fetal abnormalities in pregnant mammals (139). Certain neoplastic diseases exhibit a common susceptibility to both the physical and chemical agents (57, 60). The proclivity to inhibit cell division is a classical property of ionizing radiation (108) and a prominent cytological effect of mustards (section V A). Finally, both agents

appear to be relatively unique in their potent actions against chromosomes (84, 174, 181a) and in their capacity to induce genetic mutation (6).

The parallelisms existing between actions of mustards and those of ionizing radiation have engendered the use of "radiomimetic" as a summary term descriptive of the relevant biological properties of the chemical agents (55, 90). While "radiomimetic" is a compelling characterization of certain features of mustard action, its use should be accepted only with due regard for certain differences between effects of mustards and those of penetrating radiation which have already come to light. The temporal course of mitotic inhibition and cytological changes in corneal epithelium following mustard application are to some extent different than after exposure to radiation (100). Thymic lymphocytes also respond dissimilarly to the physical and chemical agents (225, 226). The various types of chromosomal changes involved in the mutagenic actions of mustards are found to occur in different statistical proportions than after roentgen radiation (6). Finally the toxicities of roentgen radiation and mustards appear not to be strictly additive and the fatal effects of combined doses seem dependent on the order in which the agents are administered (159a).

While these preliminary indications of differences in action between penetrating radiation and mustards remain to be fully explored, it seems cogent to remark that the continuation of comparative studies of the biology and biochemistry of both agents may lead "to a better understanding of the processes involved and to the discovery of means of neutralizing some of the adverse effects" (55). Furthermore, the fact that reactive chemical agents can duplicate so many of the cytological actions of ionizing radiation may have some bearing on current considerations of the mechanisms involved in radiation effects, to wit, whether ionization produced in cells exposed to radiation is directly effective in vital cellular constituents or indirectly through interaction with intracellular water and formation of reactive radicals (179, 233). It is interesting to note a speculation which associates the proposed cross-linking alkylation of fibrous molecules by mustards (section VII A) with possible union of similar molecules through disulfide linkages (—S—S—) resulting from irradiation (118). Presumably there would be oxidation of —SH groups by radicals arising from radiation of intracellular water. Indeed, it has been reported that enzymes dependent for catalytic activity on essential —SH radicals are inactivated by oxidation attending their irradiation (22, 23).

Note Added in Proof. Recent findings, obtained subsequent to the preparation of this review, may further elucidate the nature of chemical configurations essential for mustard-like action against proliferative cells. It has been shown that ethylenimine and several mono-ethylenimine derivatives, such as 2,4-dimethoxy-6-ethylenimino-s-triazine, cause pancytopenia in rats and typical nitrogen mustard-like damage in all hematopoietic tissues (214a). Among dividing myelocytes and metamyelocytes in the bone marrow of rats given mono-ethylenimine derivatives, aberrant anaphases can be found which resemble the cytological abnormalities characteristic of the effects of radiation, *i.e.*, chromosomal bridging and fragmentation (118, 179, 181a). The mono-ethylenimine derivative mentioned above has also been shown to inhibit the growth of and cause typical mustard-like damage in mouse sarcoma 180 (62a) and to lower the leucocyte count in blood of leukemic mice

(62c). Moreover, ethylenimine and the mono-ethylenimine derivatives readily elicit radiation-like and mustard-like chromosomal derangements in cells of the onion root-tip and in tissue cultures of mouse embryonic skin and in sarcoma 180 (35a). Finally, another monofunctional compound, glycidol or 2,3-epoxy-1-propanol, has also been shown to evoke these nuclear lesions in tissue cultures of mammalian cells (35a).

One must presume from the above evidence that polyfunctional activity is not an essential requirement for mustard-like derangements in proliferative cells. By the same token it becomes unnecessary to explain mustard damage to chromosomes on the basis of a possible cross-linking alkylation. Nevertheless, it is pertinent to note that in intact animals doses of the mono-ethylenimine derivatives, which are effective against hematopoietic organs or tumor tissue, are 50- to 100-fold greater than those of MBA or a polyfunctional ethylenimine such as XXVI. It would appear at present that duplication of 3-membered heterocyclic moieties increases the selective action of molecules against proliferating cells. Indeed, ethylenimine in effective dosages causes marked damage to renal collecting tubules (90a), an action not shared by ethylenimine derivatives or nitrogen mustards (214a).

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