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13. ABSTRACT *(Maximum 200 words)*

Sulfur mustard is a poisonous chemical agent which exerts a local action on the eyes, skin and respiratory tissue with subsequent systemic action on the nervous, cardiac, and digestive and endocrine systems in man and laboratory animals causing lacrimation, malaise, anorexia, salivation, respiratory distress, vomiting, hyperexcitability, cardiac distress, and death. Sulfur mustard is a cell poison which causes disruption and impairment of a variety of cellular activities which are dependent upon a very specific integral relationship. These cytotoxic effects are manifested in widespread metabolic disturbances whose variable characteristics are observed in enzymatic deficiencies, vesicant action, abnormal mitotic activity and cell division, bone marrow disruption, disturbances in hematopoietic activity and systemic poisoning. Indeed, mustard gas readily combines with various components of the cell such as amino acids, amines and proteins.

Sulfur mustard has been shown to be mainly a lung carcinogen in various test animal species; this effect is highly dependent of size of the dose and the route of exposure. In the human, there is evidence of cancers of the respiratory tract in men exposed to mustard gas. Mutagenicity of sulfur mustard, due to the strong alkylating activity, has been reported to occur in many different species of animals, plants, bacteria, and fungi. There is no strong evidence that sulfur mustard is a teratogen but much further research, with particular emphasis on maternal and fetal toxicity, is needed and recommended.

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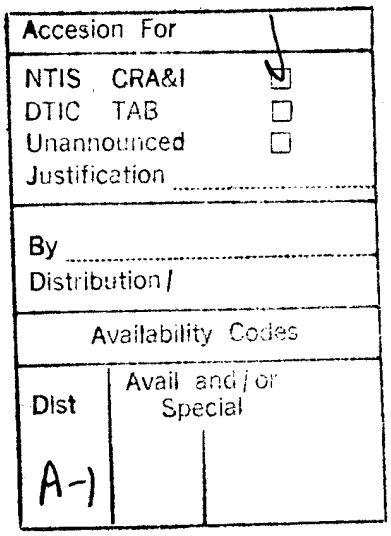

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INTRODUCTION

Chemical warfare which is cost effective is present today. The efficacy of producing large numbers of casualties through use of weapons made from cheap and readily available chemicals has given rise to a new designation for chemical weapons, "the poor man's atomic bomb." All countries regardless of size or economic condition, capable of producing petrochemicals, pesticides or detergents have the potential and capability of conversion to production of a wide variety of chemical warfare agents of which mustard gas is the most accessible.

Chemical attacks, in which mustard may have been used on Iranian soldiers and civilians during the Gulf War of 1984-85 (Marshall 1984; Dickman 1988) and heavy use of chemical weapons in Afghanistan by the Soviet military is a recent innovation in military tactics which has been highly successful and may ensure further use in future military conflicts and terrorist attacks as a profitable adjunct to conventional military arms (Marshall 1984; Segal 1987). This weapon has been used recently by Iraq to attack her own Kurdish population in the Iranian occupied village of Halbja in 1988, which resulted in many civilian casualties (Dickman 1988; *NY Times* 1988; *Japan Times* 1988).

Today, many nations have the ability to produce and use chemical agents. Nations that do not have the expertise to build factories for production of these chemicals have been eagerly aided by European nations which are eager to share their own scientific expertise in these matters. The means of production, the means of delivery and the stockpiling of such chemicals make their use against armies and civilian populations only a matter of time. Concurrently in the United States, recommendations are being made to provide safe measures and appropriate health standards for handling the national stockpiles of all chemical agents that are mandated for demilitarization. To resolve questions of public concern about possible exposure and potential health hazards resulting from destruction of these stockpiles of chemical agents, scientific data are being accumulated concerning potential adverse effects including toxicology, carcinogenicity, mutagenicity and teratogenicity. The "need to know," applicable to the scientific community as well as to an enlightened and educated public, implies that evidence be scientifically scrupulous and verifiable.

Although this review is exclusively on sulfur mustard, on occasion, references and illustrations will be made to the nitrogen mustards, intentionally, because of the similarity of structure and mode of action. This is done in order to maintain a clarity and continuity in our survey where evidence is sparse or is lacking regarding the involvement of sulfur mustard in specific instances.

CHEMISTRY OF MUSTARD GAS

Although there are presently more highly effective chemical warfare agents which are more toxic, mustard gas has not lost its military usefulness because of its special characteristics: it is very toxic and difficult to treat, versatile, persistent, cheap and easy to produce industrially and difficult to protect against. Moreover, mustard gas is toxic as droplets, liquid, vapor and most of all as "a poisonous cloud " in the form of an aerosol (Gates *et al.* 1946).

Physical Properties

Pure sulfur mustard is a transparent liquid with a slight odor of castor oil while technical sulfur mustard is a dark liquid with an unmistakable odor of mustard or garlic. It is barely soluble in water (0.07% at 10°C) and very soluble in organic solvents, fuels and lubricants (Aleksandrov 1969).

It is easily absorbed by many foodstuffs, porous materials, paint and varnish coatings and rubber articles which will remain contaminated for long intervals (Aleksandrov 1969; Rosenblatt *et al.* 1975).

Terrain and all objects present will become contaminated by sulfur mustard for very long periods of time because of its stability and persistence. Norwegian samples of snow analyzed for the presence of sulfur mustard attested to its persistence two weeks after its initial presence, but was no longer detectable four weeks later (Johsen and Blanch 1984).

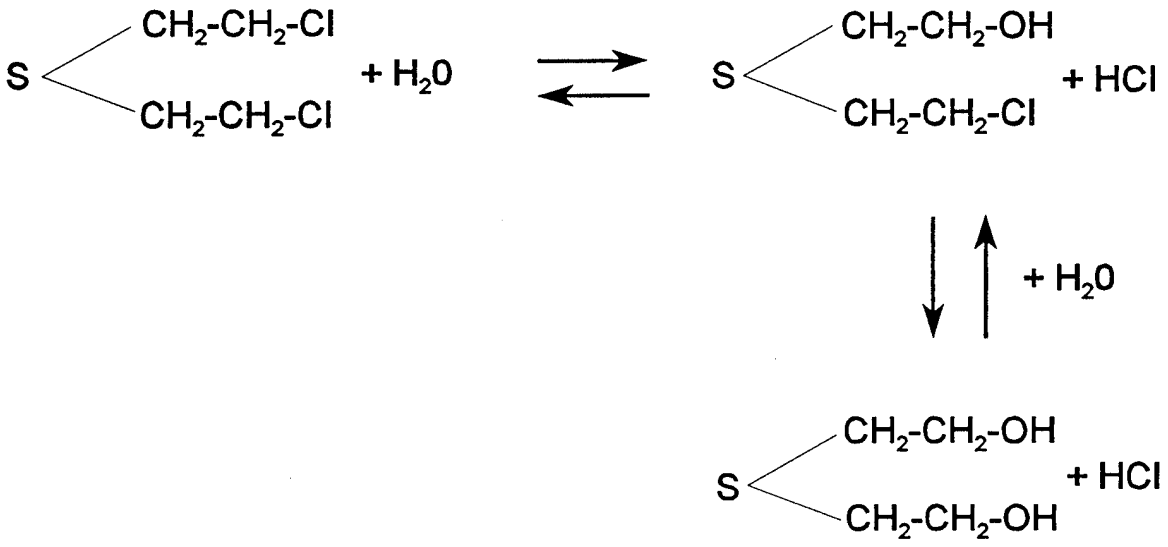
Chemical Properties

Mustard gas because of its alkyl properties displays nucleophilic substitution of its chlorine atoms by hydroxyl groups on interaction with water and alkalies. This hydrolysis proceeds in two stages with gradual substitution of the chlorine atoms by hydroxyl groups and is reversible; the second hydrolytic reaction is more rapid than the first hydrolysis (see Figure 1).

See Dacre and Burrows (1988) for further information about the solubility and hydrolysis of mustard gas (see Tables 1 and 2).

Preparation of Mustard Gas

Mustard gas was first synthesized in 1882 by Despretz and since then it has been produced by several different methods: the Vector Meyer process in which 2-chloro-ethanol reacts with sodium sulfide to produce thiodiglycol which then will react with hydrogen chloride; the Levinstein process in which ethylene reacts with sulfur chloride; and the American process in which ethylene oxide reacts with hydrogen sulfide to yield thiodiglycol which will react with hydrogen chloride (Rosenblatt *et al.* 1975; IARC 1975). A Military Specification (1969) for Mustard Gas has been approved by the U.S. Departments of the Army, the Navy and the Air Force.



Overall Reaction:

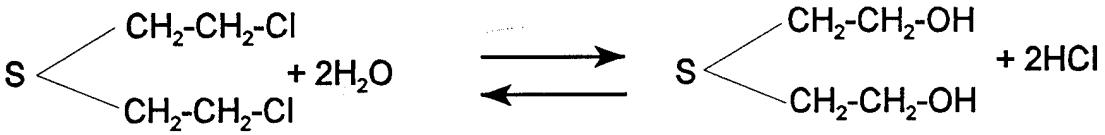


Figure 1. Hydrolysis of Sulfur Mustard.

GENERAL TOXICITY

Man

Sulfur mustard was first used in World War I as an offensive weapon by the Germans during an attack against the British at Ypres' in July 1917 (Jackson 1936). The British called it mustard gas because of its peculiar odor; the French called it Yperite because it was first used at Ypres; the Germans called it Lost, an acronym of the first letters of the names of two German chemists (Lommel and Steinkopf) who were associated with the pioneer industrial development of the agents, and everyone alluded to it as "yellow cross" since the German shells containing the agent were marked with a yellow cross (Vedder 1925; Aleksandrov 1969). The codedesignation H.S., is generally believed to stand for Hunstoffe (Vedder 1925). In the United States mustard gas is commonly called sulfur mustard and is designated HD. Indeed, the national stockpiles of mustard actually consist of sulfur mustard (HD), Levinstein mustard (H), bis[2(2-chloroethylthio)ethyl]ether (T), and a mixture of 60% HD and 40% T (HT). It was

a devastating weapon in World War I and is still regarded as a very dangerous chemical warfare agent: "The Poisonous Cloud" (Haber 1986).

Table 1. Physical and Chemical Properties of HD

Physical and Chemical Data

Synonyms and trade names

Chemical abstract name:

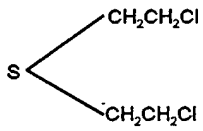
Ethane, 1,1'-thiobis(2-chloro) [after 1971]

Sulfide, bis(2-chloroethyl) [before 1971]

Other names:

Bis(2-chloroethyl)sulfide; 1-chloro-2-(2-chloroethyl-thio)ethane; 2,2'-dichlorodiethyl sulfide; di-2-chloroethyl sulfide; 2,2-dichloroethyl sulfide; Schwefel-Lost; 5-lost; 5-mustard; sulfur mustard; mustard; mustard gas; Levinstein mustard; Yellow cross mustard; Yperite

Structural Formula:



Other data:

Chemical Abstracts Registration No. (CAS No) 505-60-2

Defense Department Symbols: H, HD

Edgewood Arsenal No. EA 229

Registry of Toxic Effects of Chemical Substances (RTECS) No. WQ 0900000 (1983-84 Supplement)

Wiswesser Line Notation: G2S2G

Molecular formula: $C_4H_8Cl_2S$

Molecular weight: 159.1

Mustard is a poisonous chemical agent which exerts a local action on the eyes, skin and respiratory tissues with subsequent systemic action on the nervous, cardiac, and digestive systems in man and laboratory animals. It causes lacrimation, malaise, anorexia, salivation, respiratory distress, vomiting, hyperexcitability and cardiac distress (Lynch *et al.* 1918, Marshall and Williams 1920; Vedder 1925; Smith 1943; Anslow and Houck 1945; Gates and More 1946; Anslow *et al.* 1948). Under extreme circumstances, dependent upon the dose and length of exposure to the agent, necrosis of the skin and mucous membranes of the respiratory system, bronchitis, bronchopneumonia, intestinal lesions, hemoconcentration leucopenia, convulsions with systemic distress and death occur (Lynch *et al.* 1918; Warthin and Weller 1918; Warthin and Weller 1919a; Smith 1943; Morgenstern *et al.* 1947; Lohs 1975; Department of the Army 1974). Severe mustard poisoning in humans is associated with systemic injury which is manifested as headache, epigastric distress, anorexia, diarrhea and cachexia (Marshall 1919; Lohs 1975; U.S. Army 1974). Damage to hematopoietic tissues with progressive leucopenia

Table 2. Selected Physical Properties of HD

Property	Value
Melting Point, °C	14.4
Boiling Point, °C	215-217 at 760 mm Hg
	108 at 14 mm Hg
Flash Point, °C	105
Vapor pressure (20°C), mm Hg	0.72
Heat of vaporization Kcal/mol	15.0
Heat of fusion, Kcal/mol	4.3
Heat of combustion, Kcal/mol	708
Heat of formation, Kcal/mol	32
Viscosity (20°C), poise	0.046
Liquid density d^{20}_4	1,274
Specific heat, liquid, cal/g-°C	0.330
Refractive index n^{20}_D	1.531

occurs with mustard doses of 1000 mg-min/m^3 (Krumbhaar 1919; Krumbhaar and Krumbhaar 1919, Bodansky 1945; Philips 1950).

Recently, drinking water criteria have been derived for the chemical agent sulfur mustard, HD [bis(2-chloroethyl)sulfide] for the protection of human health and specifically for the health of the soldier in the field. Criteria have been calculated for a daily intake of both 5L and 15L over a maximum period of seven days, using the basic formula proposed by U.S. Environmental Protection Agency. The no-observable-effect-level (NOEL) of 0.1 mg/kg/day was determined from the available toxicology data base for a 90-day study in the rat given the compound by oral administration (Sasser *et al.* 1988; Sasser *et al.* 1989a, Sasser *et al.* 1989b). The recommended drinking water criteria, based on an uncertainty factor of 100, are 28 μg/L (5L/day) and 9.3 μg/L (15L/day intake) (Dacre and Burrows 1988).

Mustard Intoxication Studies

Four human subjects who were injected intravenously with 0.1 mg tris(2-chloroethyl)amine developed headaches and nausea within nine hours. These symptoms persisted for some time; and while only two out of four subjects displayed dizziness and weakness, all four developed thrombophlebitis. While exposure to nitrogen-mustard vapors incurred nausea, vomiting and

headaches, neither vomiting nor anorexia were observed after i.v. injection. Although lymphocytopenia developed, the normal count falling from 2500 cells/mm^3 to 700 cells/mm^3 on the third to sixth days, the lymphocyte counts returned to normal by the fourteenth to twentieth days (Anslow and Houck 1945).

Oral ingestion of two to six mg tris(2-chloroethyl)amine dissolved in tap water caused anorexia, recurrent nausea, vomiting, fullness and tenseness in the epigastric region with lassitude, depression, occasionally diarrhea and gaseous eructations. These symptoms were associated with two mg doses, pronounced with four and six mg doses, and absent with one mg after ingestion. Repeated oral ingestion, three times per day with one mg of the nitrogen mustard, for five days, resulted in moderate symptoms of discomfort with prompt recovery once oral intake ceased. The ingestion of a total of 15 to 18 mg caused a moderate leucopenia in five out of seven cases within seven to nine days which was persistent in all instances for seven to 25 days and continued for as long as seven weeks. Consumption of a total of seven to nine mg in those individuals on a one mg dose schedule caused a definite leucopenia. All subjects were asymptomatic 18 months later (Jager 1946; Anslow and Houck 1945). When solutions of tris(2-chloroethyl)amine were hydrolyzed and the dominant product, bis-β-hydroxyethyl-ethylenimonium chloride was given, only those subjects that had ingested 24 mg displayed nausea and vomiting but no alterations in leukocyte count (Anslow and Houck 1945).

Mustard Intoxication - The Bari Incident

Destruction of 16 cargo vessels carrying several thousand tons of high explosives and 100 tons of mustard gas by German bombers on December 3, 1943, in the harbor of Bari Italy, led to the formation of a giant oil slick mixed with mustard gas that coated the surface of the water for several days. While one thousand men were killed or missing and 800 men were hospitalized, 617 of the latter suffered from exposure to mustard. Indeed, eighty-three deaths occurred which were attributable to mustard contamination. Some of the casualties of mustard contamination displayed first and second degree burns, in some cases, involving up to 90% of the body surface. Those casualties who had floundered in the oil slick for many hours before rescue and who had endured additional waiting for medical aid, while wrapped in blankets, displayed shock and clinical symptoms consequent to systemic intoxication by mustard. In addition, low arterial pressure and hemoconcentration which resisted treatment, was associated with depression and apathy that persisted from 18 hours to three days, and was followed either by slow recovery or death. By the second day, the few men who removed the blackened oil from their bodies appeared well, while those covered with the black fuel and mustard gas mixture were dying. A severe conjunctivitis, and a generalized brawny edema of the skin and subcutaneous tissues were observed in the majority of the rescued men. Additional deaths occurred and were ascribed to the decreasing leukocyte counts which continued to show a disappearance, first of lymphocytes and later of polymorphonuclear cells; in some cases, the leucopenia was extreme, reaching levels as low as 50 to 100 cells per cm^3. In such cases, where there was a failure in the granulocyte response, death occurred (Rich and Ginzler 1944; Rhoads 1947; Alexander 1947; Philips 1950).

In this instance, the lungs, kidneys and skin were the major tissues sustaining the most damage probably as a consequence of the inhalation of mustard vapor, aspiration of foreign material and mustard-in-oil into the lungs and stomach, blast injury and subsequent infection. The clinical features associated with the casualties of this disaster were a reflection of systemic intoxication by mustard absorbed through the skin from the oil-mustard gas-in water mix on the surface of the harbor water (Rich and Ginzler 1944; Alexander 1947; Rhoads 1947; Philips 1950).

Colonel Stewart F. Alexander, a medical officer, who had been trained by the Chemical Warfare Service was stationed in North Africa, and on being informed of the Bari tragedy, confirmed the findings and immediately realized that the casualties were dying from mustard gas poisoning and not from blast or immersion shock (Alexander 1947). He was able to obtain some tissue specimens from the casualties which were sent to the Medical Laboratories of the Chemical Warfare Service where the tissues were reviewed at Edgewood Arsenal and confirmed his diagnosis (Rhoads 1947). Interestingly, skin burns due to mustard exposure do not have the same toxic properties that thermal burns have (Sollmann 1957).

Mustard Intoxication - An Early Incident

There have been a number of incidents where ignorance and naivety of the hazards of mustard gas have led people to suffer injury and death from mustard gas intoxication. In 1919, a large can of alcohol that had been used to clean out Yperite from a shell filling apparatus was stolen and kept in a room for several days. Since the can leaked, the alcohol contaminated Yperite saturated the floor of the room and subsequently dripped down below into a room occupied by a family consisting of a man, his wife and two children, who all died of Yperite poisoning. Apparently the house was heated and rapid evaporation of the Yperite was effected (Zanetti 1919).

Mustard Intoxication - Recent Incidents

A more recent report states that 197 people were contaminated with mustard gas from handling 50,000 to 150,000 pound bombs that had been netted by fishermen off the coast of Borholm, Denmark. These bombs had been previously sunk in the Baltic Sea in 1946-1947. About 90% of the hospitalized people were fishermen who had caught the bombs in their nets and had become contaminated with mustard from the corroding steel casings. Twenty-seven men showed skin lesions, erythema, vesication, necrosis and eye lesions; there were two deaths (Aasted *et al.* 1985; Jorgensen *et al.* 1985).

On March 2, 1984, 15 Iranians were brought from Teheran to Vienna suffering from burn injuries due to a gas attack in the Iraqi-Iranian War. Clinical examination and presence of mustard gas in the urinary specimens of two of the patients by the University of Vienna provided evidence that the injuries were a consequence of a mustard gas attack. This was confirmed by Heyndrickx's laboratory in Ghent which found mustard gas present in urine, feces and blood (Heyndrickx and Heyndrickx 1984; Pauser *et al.* 1984; Mandl and Freilinger 1984; Vycudilik 1985), urinary excretion of thiodiglycol (Wilis *et al.* 1988) and inhibition of serum

cholinesterase (Vojvodic *et al.* 1985). Indeed, careful scrutiny of the chemical records of 65 Iranian patients who were casualties of the Iraqi-Iranian War affirmed the conclusion that they had been poisoned by sulfur mustard (Willems 1988; Willems 1989).

Animals

Single intravenous injections of 0.5 mg/Kg of sulfur mustard in young male rats caused widespread degenerative damage to all hemopoietic tissues such as spleen, thymus and bone marrow and resembled the hematological condition which prevails after X-ray irradiation (Kindred 1947). This was also observed by Graef *et al.* (1948) in rats, mice, rabbits and dogs where the most dramatic and pervasive feature of mustard poisoning was the marked effect on the hemopoietic system. Within 12 hours after subcutaneous injection of three mg/Kg of sulfur mustard, granulocytosis was observed, quickly followed by leukopenia with bone marrow depletion 24-48 hours later. Damage to the spleen, thymus, small intestine and injury to the testes with inhibition of spermatogenesis were also observed. Associated with these destructive changes was a widespread and prevalent systemic poisoning of the gastrointestinal, pulmonary and nervous systems (Kindred 1949a; Kindred 1949b). There was a loss in control of body temperature, a fall in respiration, diarrhea, muscular weakness with tremors and convulsions (Anslow and Houck 1945). Although most animals recovered from such intoxication, some were not able to sustain the sequence of damaging events and shock and succumbed, particularly, if great fluid loss and anoxia had been incurred (Graef *et al.* 1945; Graef *et al.* 1948) (see Tables 3 and 4).

Table 3. LD_{50} Values (mg/Kg) for Sulfur Mustard in Various Animal Species

	Route of Administration		
Animal Species	Subcutaneous	Intravenous	Dermal
Rat	1.5, 2.0	0.7, 3.3	9
Mouse	26, 4 (LDLo)	8.6	92
Dog		0.2	20
Rabbit		Ca 1.1, 2.7	Ca 100
Guinea Pig			20
Goat	40		50

Subcutaneous injection of 0.625 mg of mustard gas/Kg in young male rats which was 50% of the LD_{50}, caused injury to the thymus gland with inhibition of cell division on the fifth day, and while recovery was slow it was gradual (Cataline *et al.* 1971). Friedberg *et al.* (1983) injected 15 mg of mustard gas/Kg i.p. which depressed the activity of bone marrow cells in the

Table 4. LCT_{50} Values for Sulfur Mustard in Various Animal Species

Animal Species	LCT_{50}	Time Range
	$mg/min/m^3$	min.
Mouse	860-4140	2-360
Rat	840-1512	2-360
Rat	420	2
Guinea Pig	1700	10
Rabbit	900	10
Cat	700	10
Dog	600	10
Goat	1900	10
Monkey	800	10
Human	1500	1

femur three to eight days after administration of the agent. The depression persisted for three days after which recovery was observed eight days later. In dogs, nausea, vomiting and anorexia were observed a few hours after mustard intoxication with diarrhea on the second and third day. Weakness with diminution in body temperature followed so that the extremities were cold and the animals sank into coma and death from respiratory failure (Anslow and Houck 1946).

The LD_{50} for mustard, whether administered topically, subcutaneously or intravenously was rather high in mice and rabbits compared to that observed in rats, which appeared to be the more sensitive species examined (Anslow *et al.* 1948).

Soon after introduction into body tissues, hydrolysis of mustard occurs to form thiodiglycol and semi-mustard which are relatively non-toxic, having no effects on heart rate, blood pressure or vagus nerve irritability in dogs or rabbits. However, 2-chloroethyl-2(bis(2-hydroxyethyl)-sulfonium)ethyl sulfide chloride was toxic and caused enteritis in mice and rabbits, necrosis of the liver, damage to lymphoid tissues and adrenal congestion but no bone marrow injury; in rats, large doses of this hydrolytic product, as the picryl sulfonate produced diarrhea, reduction in body weight, in addition, to the previous noted effects; both the picryl sulfonate and chloride given i.v. caused a slight leucopenia. Despite the toxicity of this compound, it was not considered a likely candidate in contributing to the overall-toxicity of mustard since thiodiglycol was necessary for its formation. However, the concentration of thiodiglycol that would actually be present following i.v. injection of mustard gas would be inadequate for transformation of

mustard into the bimolecular sulfonium salt. Moreover, the concentration of radioactively labeled sulfonium derivative actually extracted from pig skin, after injection of labeled mustard, was very small, 2.5% of the radioactive material present (Anslow and Houck 1946; Anslow *et al.* 1948). In addition, incubation of labeled 0.0008M mustard with blood plasma for 30 minutes at 37°C resulted in 2.4% of labeled mustard being converted into the labeled sulfonium derivative (Anslow and Houck 1946). The investigators concluded that the active toxicant after mustard gas administration was mustard gas itself or its cyclical form, β-chloroethyl-ethylenesulfonium chloride, which was the first reaction product in the hydrolysis of mustard gas (Anslow *et al.* 1948).

The LD_{50} determined by the investigators varied widely and may have been influenced by the solvent used for dissolving the mustard; intravenously injected mustard gas for rats was about five times lower for mustard dissolved in propylene glycol than for "neat" mustard. In fact, neat mustard caused severe pulmonary necrotic lesions with associated damage to neighboring tissues, while mustard dissolved in propylene glycol resulted in diffuse pulmonary congestion (Anslow *et al.* 1948).

Injection of a lethal dose of mustard intravenously in dogs elicited the following course of action within 10 to 20 minutes: salivation which increased in flow followed by a diarrhea which persisted until death of the animal. Rapid respiration, muscle spasms, tetanic muscular contractions, extension of the hind legs, arching of the neck and back with subsequent convulsions were also observed. The pulse became irregular, the blood pressure fell slowly, the cardiac rhythm became anomalous with more auricular beats than ventricular (3:1); and the vagus became unresponsive. The convulsions ceased eventually, and the animals died in coma less than 24 hours after injection of the mustard. Autopsy revealed an intense congestion of the intestinal mucosa from pylorus to anus with hemorrhaging into the lumen (Lynch *et al.* 1918; Warthin and Weller 1919b).

RESPIRATORY SYSTEM

Animals

Next to eye lesions, the greatest discomfort produced by exposure to mustard gas is that resulting from irritation and injury of the respiratory system (Giraud 1917; Mandel and Gibson 1917; Warthin and Weller 1919a). Indeed, Victor Meyer (1887) who discovered mustard gas showed experimentally that irritation and inflammation of the upper respiratory tract resulted from exposure to the agent and terminated eventually in pneumonia.

When rabbits were gassed in special gassing chambers, symptoms of nasal irritation were observed immediately, such as rubbing of the nose and turning about of the animals to present their backs to the in flowing gas. Photophobia and lacrimation together with increased nasal secretion and inflammation of respiratory membranes appeared two to three hours after exposure. Rabbits exposed to low concentrations of mustard gas such as dilutions of 1:100,000 for 10 to 15 minutes or to higher concentrations for one to several minutes displayed some

degeneration and necrosis of the epithelium of the mucous membrane with congestion, edema and mucus secretions. The lesions were mild and limited to the anterior nares, pharynx, larynx, and upper portions of the trachea; however, some pulmonary congestion and edema with increased bronchial secretions were often noted. Recovery without any secondary infection usually followed (Warthin and Weller 1919a).

Rabbits exposed to higher concentrations for short periods of time, such as one to several minutes, or to prolonged exposures of mustard gas showed the usual respiratory clinical symptoms but somewhat later than the occurrence of conjunctivitis. Respiration became difficult and coughing, rales, exudation and death ensued. The clinical symptoms were associated with laryngeal and tracheal edema attended with congestion and marked necroses of the mucosa of the respiratory tract which generated the formation of diphtheritic membranes (easily detached) in the anterior nares, neopharynx, larynx, trachea and bronchi as a consequence of the fibrous inflammatory exudation. Secondary infection with staphylococci followed in severe exposures and led to purulency of the lesions in the larynx, trachea and bronchi within four to six days. The lungs displayed congestion, edema, hydropic and mucoid degeneration of the epithelia in mild cases of exposure. Necrosis of the epithelia which extended into the smaller bronchioles accompanied by secondary infection was observed in severe cases of exposure; edematous and hemorrhagic atelectatic areas due to plugging of the bronchioles with exudates alternated with emphysematous areas and resulted in hemorrhagic bronchopneumonia. In most cases, death was due to an infective purulent bronchopneumonia which was secondary to the gassing; however, in severe cases of exposure, death occurred quickly due to diphtheritic or purulent laryngitis in absence of pneumonia. Under some circumstances, where bronchopneumonia was not extensive, recovery ensued when the localized diphtheritic patches in the nose, mouth, larynx and trachea healed and cicatrization with resulting cicatricial contraction present in the trachea and larynx occurred (Warthin and Weller 1919a).

No permanent effects could be demonstrated in dogs intoxicated by a single intravenous injection of one mg/Kg of methyl-bis(β-chloroethyl)amine or tris(β-chloroethyl)amine, although some dogs did die of anoxia of the respiratory centers due to peripheral circulatory failure promoted by a reduction in blood volume (Houck *et al.* 1947). Exposure to mustard gas at a concentration of either 0.001 mg/m^3 for 16, 32 and 52 weeks or 0.2 mg/m^3 for 4, 8, 16 and 32 weeks did not alter the normal respiratory rate or values in dogs (McNamara *et al.* 1975).

Man

War

Clinical descriptions of men exposed to mustard gas on the battlefield during World War I emphasized the initial effects of mustard on the mucous membranes of the respiratory system (Sollmann 1957; Blewett 1986; Haber 1986). Early symptoms included sneezing and coughing, and with increasing irritation of the nose and throat, a discharge of mucus from the nose with loss of the sensations of smell and taste within 12 hours. Longer periods of exposure to mustard gas caused laryngitis, aphonia, incapacitation, bronchitis and pneumonia within 36 to 48 hours.

Dysphagia or difficulty in swallowing appeared on the second or third day, lasting from four to six days or longer and was associated with local white diphtheritic necroses in the mucosa of the pharynx that covered a great part of the oropharynx and laryngopharanyx. As a result, tickling, dryness, burning sensations were experienced in the larynx and in the sternum for several days or even weeks depending on the extent of the lesion present in the mucosa. There was constant coughing which was painful, dry, paroxysmal and troublesome, particularly, at night and occasionally a bloody and even purulent expectoration was also present. Aphonia was sometimes present, but more usually the voice was merely rough or husky and was associated with changes in the mucosa of the larynx. The larynx displayed a marked hyperemia, swelling and local necroses which developed into whitish or grayish eschars that tended to form pseudomembranes. The most frequent lesions were the isolated eschars associated with the arytenoids, epiglottis and vocal cords which healed very slowly, taking several weeks. Tracheal lesions were similar to those described for the larynx and were associated with subjective symptoms of bronchitis. Inflammation of the airways of the pharynx, larynx, trachea and bronchi led to bronchitis, bronchopneumonia and death (Warthin and Weller 1919a; Buscher 1944; Sollmann 1957; Blewett 1986).

Lesions of the respiratory tract in man due to mustard gas exposure did not appear to be different from those observed experimentally in laboratory animals. Hyperemia and a slight necrosis led to inflammation of the nose, laryngeal huskiness, cough and sore throat which were commonly observed in human beings who had been exposed to either light or moderate exposures of mustard gas; and although recovery was rapid, some irritation, cough and huskiness might persist for some time. Severe exposure caused more degeneration, necrosis and exudation in the epithelia of the respiratory tract, resulting in eschars on the palate, back of the tongue, pharynx and larynx associated with tracheal inflammation, bronchitis, pulmonary congestion and edema. Indeed, severe exposure as may be encountered on the battlefield may produce diphtheritic lesions of the larynx, trachea, and bronchi leading to bronchopneumonia (Warthin and Weller 1919a; Sollmann 1957; Haber 1986).

Chemical Factories

People employed in factories manufacturing mustard gas may become partially or totally disabled because of injury to the mucosa of the respiratory system after protracted exposure to small quantities of mustard gas vapor. Typically, such workers develop an aggregate of several or many symptoms: eye problems, such as "red" eyes, photophobia, lacrimation, impaired vision, and blepharospasm; respiratory problems, such as loss of sensation of taste and smell, bleeding from the nose, sore throat, hoarseness, difficulty in swallowing, chest pain, wheezing and dyspnea; gastrointestinal problems such as anorexia, vomiting, weight loss; and nervous problems, such as insomnia and irritability. Treatment of these sick people is difficult and takes a very extensive course of time and invariably results in total or partial disability (Morgenstern *et al.* 1947).

In another study, Brown (1949) reported that a large number of employees, 20-60 years of age, working at the Huntsville Arsenal in Alabama, exposed to continuous concentrations of

mustard gas vapor for long periods of time displayed coughing, chest pain, shortness of breath and fatigue. Eventually the coughing contained foul sputum and the workers developed bronchiectasis with progressive emphysema and narrow attenuated bronchioles. Out of 224 workers at the plant who were considered as having some degree of disability as a consequence of mustard gas exposure, 80% displayed disabilities that were rated from 25 to 100%; 25% of these were considered 100% disabled.

Weiss (1958) has reported such delayed effects as changes in the mucous membrane which led to swelling and formation of polyps in the paranasal sinuses and Lohs (1975) has provided a summary of such slowly evolving effects.

SKIN

Man

Ordinarily, the skin serves as a bulwark, a vital and important defense system against penetration of potentially toxic and even life-threatening chemical agents. However, on exposure to mustard gas, the skin itself becomes a target which is vulnerable and defenseless against the rapid penetration and fixation of sulfur mustard. This leads rapidly to a sequence of reactions which result in severe damage to the basal cells which are vital for the replacement of epidermal tissue thereby, attenuating the healing process so that lesions take several months to heal. In general, 80% of the mustard making contact with the skin will evaporate and the 20% remaining will penetrate the skin, but only 2% of the mustard becomes fixed so that 18% is absorbed into the circulation to cause systemic intoxication (Pruit 1987).

Humans exposed to mustard gas vapors display simultaneous irritation to the eyes, skin and respiratory system evidenced by lacrimation, acute conjunctivitis, sneezing, running nose, burning in the throat, coughing, hoarseness and erythema of the skin (Marshall and Williams 1920; Smith 1943; Morgenstern *et al.* 1947). Soldiers exposed to mustard gas vapors or mists complained mostly about injury to the eyes, respiratory tract, scrotum, face and anus as body areas that were most vulnerable (Blewett 1986). Such exposure is usually not associated with any immediate discomfort or pain because of mustard's long latency period which thereby adds to its insidious nature; later discomfort and pain are due to the lesions themselves and not to the causative agent, mustard (Warthin and Walker, 1919a; Daily *et al.* 1944). While Lewisite is a vesicant which provokes the immediate initiation of protective measures because the agent gives adequate warning through its acute irritation to the eyes and respiratory passages (Daily *et al.* 1944), the severity of skin lesions as a consequence of exposure to mustard is dependent upon the dose of the agent, the humidity and the length of exposure (Renshaw 1946; McNamara *et al.* 1975). Under hot and humid conditions, mustard gas contamination may cause maceration and desquamation in certain dry and most moist areas of the body without prior development of vesicles, particularly in the skin of the scrotum and penis and in the axillae (Renshaw 1946). Indeed, mustard contamination may cause an erythema resembling sunburn or widespread vesications (McNamara 1960).

Mustard gas applied to the forearms of humans, as droplets by a capillary pipet (0.002 ml), spread rapidly to occupy an area of 3-4 mm in diameter with erythema and edema appearing two to three or even eight to twelve hours later (Warthin and Weller 1918). On the other hand, the erythema following Lewisite contamination appeared rapidly, spread immediately over a wide area and diminished just as quickly (Daily *et al.* 1944). Then, 16-24 hours after contact with mustard, a vesicle formed which increased in height to four mm, measured 25 mm in diameter and was filled with a clear, pale yellow fluid; it was at this time, that some pain was first perceived. Absorption of the fluid within the vesicle caused folding and wrinkling of the epidermal covering of the vesicle 46 hours after application of the mustard. Vesicle collapse occurred within 72 hours and the delicate wrinkled epidermis rubbed off within four days. The brown crust at the base of the vesicle became loose, came off and was accompanied by itching during the following 10 days. The excavated area began to fill-in with a yellow brown crust and came off as a scab during the ensuing 10-18 days. Healing was completed with formation of a scar 50 days later (Warthin and Weller 1918 ; Warthin and Weller 1919b). A characteristic brown pigmentation may be observed during healing and may persist for a very long time (Chiesman 1944).

Vesicles may even appear on different parts of the body as late as 7-12 days after exposure. Australian studies held in the tropics have reported that most men were incapacitated 10 to 12 days after exposure to mustard rather than at either earlier or later times. Men wearing tropical Australian battle-dress together with respirators were subjected to mustard gas vapor for several successive days and rated on their ability to carry out standard physical performance tests. Exposure to high doses of mustard led to an inability to perform these tests due to the prevalence of a combination of systemic intoxification, surgical shock and severe generalized burns. Moderate doses of mustard resulted in the incapacity to perform these tests because of severe skin burns on the genitalia, axillae and buttocks which became severe with maximal disability eleven days after exposure (Chiesman 1944; Sinclair 1950; SIPRI 1973). While the skin of the genitalia and buttocks required effective protection against exposure to moderate doses of mustard, the rest of the body did not appear to be as sensitive under these conditions when ordinary tropical Australian battle-dress was worn (Nagy *et al.* 1946; Cullumbine 1947; Sinclair 1950; Medema 1986). The severity of skin lesions consequent to mustard contamination was influenced not only by the degree of exposure but also by the prevailing weather conditions: hot humid weather intensified the severity of the lesion so that the healing process took longer (McNamara 1960; SIPRI 1973).). Indeed, certain regions of the skin were more sensitive to the action of vesicants, so that hot sweaty skin (Smith *et al.* 1919) accelerated the fixation of mustard in skin (Cullumbine 1947, Sollmann 1957).

The severity of injuries produced by mustard gas or nitrogen mustard (HN-1) vapors in contact with the skin of forearms of human volunteers, two days after exposure, was markedly increased in skin having a thin continuous layer of water. Evidently, hot sweaty human skin incurred greater damage from these agents because its sensitivity to the injurious vapors were enhanced by a film of moisture on the skin surface (Renshaw 1945).

Fairley (1943) has reported the occurrence of prolonged vomiting which was frequent and violent in four men wearing respirators but whose skin was exposed to a concentration (Ct) of 660 mg/min/m^3 under tropical conditions; no leucopenia was observed in these men.

Vesicant Action of Mustard and Lewisite

The addition of one drop of mustard to the forearm and simultaneous addition of a drop of Lewisite (2-chlorovinyl-dichloroarsine) to the other arm by eye-droppers evinced a sequence of events leading to skin lesions by the two vesicants that could be compared. Immediately after application, a slight burning sensation was evident with Lewisite but no sensations at all were noted in the response to the mustard. The Lewisite drop spread over a wide area and was absorbed within five minutes, while the mustard was absorbed slowly over 20 to 30 minutes. After 15 minutes, the Lewisite site showed some erythema which developed, spread and was associated with pain. The erythema became intense after 50 minutes and stood out, being sharply demarcated from nearby skin 100 minutes later. It was extremely severe two hours later when only a very light and spotty erythema was present at the mustard site. It took five hours for an edema, similar to that observed earlier at the Lewisite site, to develop at the site where mustard had been applied. The Lewisite inflammation was three times greater than that of the mustard lesion and faded off into nearby tissue, and was associated with a severe sensation of burning. Nine hours later, both the Lewisite and the mustard sites were swollen with edema fluid. After 13 hours, a cherry sized blister was present, surrounded by a corona of small blisters at the Lewisite site, while a well demarcated area of inflammation was present at the mustard site. Fifteen hours later, the small vesicles had coalesced at the Lewisite site, while the lesion at the mustard site was yellowish-white in appearance and sharply delimited from the rest of the skin nearby. After 24 hours the small coalescing vesicles had formed a single large bulla or blister which is a characteristic feature of Lewisite burns. All of the tissue injured by Lewisite now had become elevated and had separated at its base from the epidermis below. At the mustard site the border of the inflamed area formed a sharp red line after 20 hours, and small vesicles began to appear in the marginal zone to give the appearance of a string of beads that sharply demarcated the healthy surrounding skin. After 26 hours, the Lewisite vesicles contained a large amount of whitish yellow clear fluid which bathed a gelatinous soft cushion-like mass. While the Lewisite site displayed an area which was inflamed and encircled by a string of bead-like vesicles, the area inside the circle was pale and lacked any blisters; the tissue outside the circle in contact with healthy tissue displayed an intense erythema (Buscher 1944).

The inflammatory process was rapid, brief and abrupt for Lewisite and regressed rapidly after peaking on day two, while in the case of mustard the process was slower, very long, peaking on day 14, then regressing very slowly. Three days later the swelling was down and the blisters at the Lewisite site had shriveled up; in absence of secondary infection, healing which was four times faster than that at the mustard site was completed in 14 days; secondary infections, however prolonged the healing process to 26 days. At the mustard site, the destruction was more severe and profound with no sloughing of necrotic tissue and infections were more likely because of the protracted healing time. The forearm with the mustard was still

swollen and displayed a conspicuous pigmentation around the area of inflammation which was never associated with injuries due to Lewisite. After five days, the Lewisite site either displayed an intact but gradually shrinking bulla or an open bulla containing healthy granulation tissue in the margins with an oily coating in the center. Between the sixth and seventh days, a loss of neurotic tissue occurred at the Lewisite site with sporadic bleeding and absorption of the waxy exudates in the open bulla, while the mustard area showed a deep bluish red discoloration which became copper colored and grew still darker to eventually form pigmentation spots typical of mustard exposures. By the ninth day, the Lewisite region showed healthy granulation tissue everywhere, with skin growing over the margin of the wound, while blisters at the mustard site broke easily and patches of pigmentation appeared over the entire arm. Between the tenth and twelfth days, necrotic tissue was still being sloughed off from the open bulla as more healthy granulation tissue appeared at the Lewisite site, while the epidermis and the skin covering the blisters at the mustard site were both so thin that they tore easily, revealing extensive masses of viscous adherent necrotic material below the epidermis (Buscher 1944).

Comparison of the two lesions on the fourteenth day after exposure, showed that the Lewisite region had healthy skin everywhere as the healing process continued, while the mustard area revealed an open wound showing severe destruction everywhere without any signs of healing so that the tissue in these lesions were moribund. Indeed, if the bulla in the Lewisite region was intact and remained in such condition, the lesion healed by the fourteenth day, while the mustard lesion at this time had just reached its peak in the inflammatory process. If a secondary infection is contracted at the Lewisite lesion, 26 days may be necessary for complete healing.

On day 15, removal of an intact bulla from a Lewisite region showed that the skin beneath was completely healed, while an open bulla displayed considerable healing with a few islands of necrotic tissue in the middle. The mustard region, however, showed open wounds with thick adherent masses of necrotic tissue. Twenty days later, the healed portions of the Lewisite region were dry and rough with a tendency to form furuncles, while the mustard region revealed a very severe necrosis. By the twenty-fifth to twenty-sixth day even those Lewisite regions that had contracted secondary infections were healed; however, sloughing of necrotic tissue still had not occurred in mustard lesions (Buscher 1944).

Instillation of blister fluid, obtained from either Lewisite blisters or mustard bulla, into the conjunctival area of rabbits caused no discomfort or untoward reactions; thus, the blister fluids did not appear to be toxic (Buscher 1944).

Another study confirmed the finding that lesions caused by sulfur mustard were more severe than those due to Lewisite and resulted in third degree burns that not only exposed but also damaged the corium (Daily *et al.* 1944).

Moreover, Nagy *et al.* (1946) has estimated that as little as six $\mu g/cm^2$ of liquid mustard will cause lesions in human skin in most exposed sites.

Models for Studying Vesication

A recent study using electron microscopy to investigate the effects of mustard gas on human skin grafts, borne by Athymic nude mice, did not reveal any new information that prior investigations had not reported using light microscopy (Papirmeister *et al.* 1984a). Electron microscopy reemphasized the finding that mustard gas poisoning of human skin, using either moderate doses of mustard, such as 60 $\mu g/cm^2$ or higher doses such as 635 $\mu g/cm^2$, was initiated at the basal cell layer of the epidermis and that injury to these cells led to separation of the epidermis from dermis with the formation of a subepidermal microblister. Although the collagen fiber bundles remained intact, they separated from one another; however, the fibroblasts and endothelial cells in contact with the dead basal cells were damaged (Papirmeister *et al.* 1984b).

An *in vitro* model has been developed recently for investigating the degree of injury and the role of the various components participating in the early phase of the inflammatory response provoked by addition of different concentrations of sulfur mustard to the skin (Dannenberg 1988). This model, called the paranuclear vacuolization test, consists of full-thickness 1.0 cm^2 human skin pieces to which are added topically various dilutions of sulfur mustard and incubated in organ cultures for 24 hours at 37°C. The explants are examined histologically to determine the number of paranuclear vacuoles and the organ culture fluids assayed for various lysosomal enzymes and other factors released by the epidermis exposed to sulfur mustard. The usual markers of cell death and early inflammatory events such as lysosomal enzymes, trypsin and chymotrypsin-like proteases, angiotensin-converting enzyme are assayed from the culture fluid. Dannenberg (1988) has reported that the culture fluids of both normal and sulfur mustard-treated explants displayed no significant difference in any of the enzyme concentrations except for one mediator of early inflammation, the plasminogen-activating processes which were significantly increased in the organ culture fluid of sulfur mustard-treated human skin explants. The plasminogen-activating proteases are released by the basal epidermal layer of human skin (Justus *et al.* 1987) may play an important role in blister formation, since they have also been associated with activity of alkylating agents (Brdar 1986).

Another recent model for studying sulfur mustard toxicity of skin is the use of human lymphocytes. Human lymphocytes are incubated with either $1x10^{-3}M$ sulfur mustard and $1x10^{-3}M$ niacinamide or with sulfur mustard alone for 24 hours at 37 C. Light microscopy revealed a loss of viability and various phases of cytotoxicity such as fragmented cells and ghosts of necrotic cells present among surviving cells (30%) that had been treated with mustard alone. The surviving cells showed condensed nuclear chromatin, pyknosis with paranuclear vacuolation surrounding the nucleus while cells treated with both mustard and niacinamide had a viability of 87% with normal cytology. While scanning microscopy showed the normal surface features of numerous microvilli and an intact plasma membrane in those lymphocytes treated with both mustard and niacinamide, lymphocytes incubated with mustard took on a rounded shape because they had lost their microvilli and revealed numerous perforation of the plasma membrane. Finally, transmission electron microscopy showed that those lymphocytes that survived treatment with mustard had nuclear pyknosis, condensation of nuclear chromatin with loss of euchromatin, paranuclear vacuolation, cytotoxic organelles, extensive blebbing of the nuclear envelope with

perforation and fragmentation of the plasma membrane; but simultaneous treatment with mustard and niacinamide resulted in the absence of any cytotoxic effects and presence of an essentially normal ultrastructure. The pathology observed in lymphocytes treated with mustard is very similar to that which has been found occurring in epidermal basal cells of human skin grafted onto Athymic nude mice and which leads to cytotoxic effects and ultimately cell death. This suggests that human lymphocytes may be used as a model for studying mustard toxicity (Petrali *et al.* 1988).

Animals

The application of 25-250 $\mu g/cm^2$ mustard gas to rabbit or guinea pig skin provoked an erythema within 30 to 60 minutes. This spread over the ensuing four hours so that in five to eight hours, the erythema had progressed beyond the site of application. Edema, necrosis and an exudate were present during the subsequent 48 to 72 hours (Vogt *et al.* 1984). After three to five days, attenuation of the erythema and edema occurred, with formation of a scab which sloughed off and was replaced with a new epidermis within 10 to 14 days. The developing inflammatory response was evident as noted by activation of the lysosomal enzymes in the basal epidermal cells at early times with increased activity of mesenchymal elements sometime later (Dannenberg *et al.* 1987; Higuchi *et al.* 1988). The reaction of the skin to the mustard insult revealed a biphasic response (Dannenberg and Vogt 1981; Vogt *et al.* 1984), characterized by an early phase in which damage to the basal epidermal cells, superficial small capillaries and venules resulted in limited vascular leakage. This was followed by a massive infiltration of granulocytes which were dominated by basophils into the area of injury; this was termed the intermediate phase. The delayed phase occurred eight hours later and was characterized by the death of the epidermal cells, increased lysosomal enzyme activity, muscular leakage and edema. Massive infiltration of heterophils (equivalent to neutrophils in humans) with an ensuing reduction of basophils into the area below the dying epidermal cells occurred over the next 24 hours with consequent ulceration. The inflammatory response achieved its maximal activity between 27 to 72 hours and was followed by the formation of a crust over the ulcerated skin with repair and healing. Subsequent shedding of the scab exhibited the presence of a new skin some 10 days later. The above investigation was an extremely thorough undertaking in which light and electron microscopy, histochemistry and injections of Evans blue dye were used to study the pathogeneses of skin lesions by sulfur mustard. Intramuscular injection of 25 mg cortisone acetate/Kg alone, or together with one topical application of 1.0% hydrocortisone, to the multiple sites where lesions were developing as a consequence of the application of 100 μg sulfur mustard/cm^2 to rabbit skin, reduced the degree of edema developing during the immediate phase but had no influence on the rate of healing (Vogt *et al.* 1984).

Penetration of the Skin by Mustard Gas

The mustards are oily lipophilic liquids which easily penetrate skin and mucosal surfaces within 3-5 minutes after contact to provoke local discomfort both to the respiratory tract and to the skin. The injury resulting may be transitory or may lead to permanent damage to the more

sensitive proliferating tissues such as bone marrow, the reticuloendothelial system and the intestinal tract (Anslow and Houck 1945; Cullumbine 1947; Rudin 1953).

The initial step in the development of injury to the skin appears to be the fixation of mustard molecules at the site of contact where they react with the proteins of the skin. No free mustard can be detected within the skin even when the skin has been massively contaminated with mustard, while the mustard that has become fixed cannot be physically removed without causing further injury (Renshaw 1946). A large portion of the mustard that penetrates the skin is carried away by the circulation to produce systemic injury (Anslow and Houck 1945). Although the reactions between the mustard and the systemic tissues are completed within a few minutes (Rudin 1953), there is a latency period of many hours before the appearance of overt cellular damage as a consequence of the disruptions of nucleic acid and protein metabolism at the cellular level (Cullumbine 1947).

Thirty minutes after topical administration of five mg of radioactive mustard to the skin of a rat, 0.17 mg of the material was found distributed throughout the animal. The concentration of labeled ^{35}S reached maximal activity two to six hours after application and attained a steady activity between 24 and 72 hours. Although the activity was about the same in all tissues, kidney tissue activity was consistently higher. The radioactivity in the blood increased during the first six hours, fell and then rose again as a consequence of hemoconcentration at 48 and 72 hours. The plasma radioactivity was higher than that in the cells during the first six hours, but 12 hours later, the cells showed a higher activity than the plasma. Fifty percent of the radioactivity applied topically was excreted into the urine by 72 hours with the greatest fraction excreted during the first 24 hours (Anslow and Houck 1945).

In human skin the penetration rate of saturated vapor or liquid mustard gas is 1-4 $\mu g/cm^2/min$ (Dutra *et al.* 1944; Cullumbine 1947; Rudin 1953). Using mustard gas labeled with radioactive sulfur, it was found that penetration of the skin by mustard gas occurred within 10 minutes and 12% of the labeled mustard gas became fixed (Dutra *et al.* 1944; Cullumbine 1947). The unfixed fraction of labeled mustard (88%) remaining in the skin during the first 10 minutes disappeared rapidly, being carried away by the circulation.

In man and pig, there appears to be a correlation between the amount of labeled mustard fixed per unit area of skin and the severity of the injury. Fixed mustard disappears from the skin slowly, remaining constant for about a week, then disappearing slowly at a rate that parallels the rate of healing and the rate at which the epidermis-desquamates; apparently the body is not able to metabolize the fixed mustard. In pig skin exposed to liquid mustard *in vivo*, about 80% of the fixed sulfur is present in the epidermis and about 70% of that is in the cornified layer, while only 20% is present in the dermis. The presence of about 0.25 μg of mustard per cm^2 of skin surface that has been fixed in the Malpighian layer is associated with exposures that will produce vesication in man (Dutra *et al.* 1944; Renshaw 1946).

Apparently mustard vapor is more toxic than are mustard aerosols in mice. Approximately 50% or more of ^{35}S-labeled mustard vapor was fixed to the skin of mice than was ^{35}S-labeled

mustard as an aerosol. Moreover, less labeled mustard, as an aerosol, was retained in the respiratory tract or blood, since it was not transported very far into the respiratory tract (Southern Res Inst 1953).

Alkylation of DNA and Inhibition of Glycolysis

Addition of mustard either to minced tumor tissues or rabbit brain tissue depressed glycolysis markedly, while thiodiglycol addition was without effects (Berenblum *et al.* 1936). Marked reduction in glycolysis of rat skin by mustard was also demonstrated by Barron *et al.* (1948). It is now known that mustard inactivates those enzymes which are involved in the transfer of phosphate groups: the phosphokinases, such as hexokinase and adenosine triphosphatase (Dixon and Needham 1946). As a matter of fact, both oxygen uptake by pyruvate oxidase and anaerobic oxidation of glucose are inhibited, particularly, hexokinase phosphorylation of glucose (Dixon and Needham 1946). Moreover, poisoning of hexokinase plays an important role in vesication and the features associated with the vesicant's action on skin appear to be correlated with the inactivation of the glycolytic system. Indeed, there appeared to be a correlation between the ability to inactivate hexokinase and manifest vesicant properties that certain chemical compounds such as mustard gas displayed (Dixon and Needham 1946). Accordingly, Wheeler (1962) concluded that there was a correlation between the ability of an alkylating agent to inhibit glycolysis and its vesicant, antineoplastic, mutagenic and carcinogenic activities.

However, the mechanism of action of mustard on enzyme activity unlike that of Lewisite (Peters *et al.* 1946; Johnston 1963) was not through combination with sulfhydryl groups essential for enzymic activity (Dixon and Needham 1946; Peters 1947). An important objection to the "enzyme inactivation" hypothesis of vesication by mustard was that while many enzymes, particularly hexokinase, are inactivated immediately by mustard *in vitro*, there is a delay time of several hours before hexokinase activity in contaminated skin begins to diminish. By that time, there is usually adequate evidence of tissue damage so that the delay in inactivation of hexokinase in vivo may be secondary to cell injury (Peters 1947; Cullumbine 1947). In addition, very large doses of mustard which are lethal within 2-3 hours do not appear to inhibit carbohydrate metabolism, since neither glycogenolysis, gluconeogenesis, nor glycolysis display any alteration until the animal is moribund (Black and Thomson 1946). Such evidence does not appear to support the position that mustard directly inactivates hexokinase or other phosphokinases.

DNA-Alkylation-Damage Hypotheses of Papirmeister

A recent hypothesis of Papirmeister *et al.* (1985) attempts to elucidate the phenomenon of vesication in human skin in biochemical terms, emphasizing the damage incurred due to alkylation of DNA and inhibition of glycolysis. This causes an alteration in metabolism which results in a sequence of pathological events that terminates in vesication.

Fixation of mustard in human skin occurs rapidly by alkylation of DNA in the keratinocytes of the basal layer in the epidermis (Renshaw 1946; Papirmeister *et al.* 1984a, Papirmeister *et al.* 1948b). This results in monofunctional adducts at N-7 in guanine (60%), N-3 in adenine (16%) and bifunctional adducts at N-7 in two guanines (16%) adjacent to one another on the same strand, or in two guanines of opposite strands of DNA, with trace amounts of monofunctional adducts on other DNA purines and pyrimidines (Brookes and Lawley 1961). The resultant apurinic sites are rapidly cleaved by apurinic endonucleases; the N-3 alkyladenine adducts being most sensitive to these enzymes, with further degradation of the DNA being generated by exonucleases. The DNA breaks activate the chromosomal enzyme poly(ADP-ribose) polymerase (Berger *et al.* 1979) which uses NAD^+ as its substrate. As a consequence, the levels of coenzyme are depleted below a critical concentration (Roitt 1956) which depresses glycolysis in the skin (Renshaw 1946; Dixon and Needham 1946; Peters 1947; Wheeler 1962). The levels of alkylation by ^{14}C-labeled sulfur mustard and the reduction in concentration of NAD^+ (Rankin *et al.* 1980) are correlated with the severity of lesions in human skin implants in Athymic nude mice four hours after exposure to the agent (Gross *et al.* 1985). Indeed, fixation of 0.1 to 1.0 μg mustard/cm^2, of 1.0 to 2.5 μg mustard/cm^2 and of 2.5 μg mustard/cm^2 caused mild erythema, vesication and necrosis, respectively, and correlated well with proportionate reductions in levels of NAD^+ in human skin implants of mice, four hours after exposure. The resultant reductions in NAD^+ levels in mildly injured skin are reversible, those in vesicant skin are partially reversible while those in necrotic skin are irreversible. Moreover, 3-aminobenzamide which is an inhibitor of poly(ADP-ribose)polymerase (Purnell and Whish 1980) increased the four-hour NAD^+ levels from 45 to 58% in mildly injured skin, from 37 to 68% in vesicant skin and from 23 to 50% in necrotic skin after mustard exposure.

The diminution in NAD^+ levels in the basal epidermal keratinocytes inhibits glycolysis and causes an accumulation of various intermediate metabolites such as glucose-6-phosphate which stimulates the activation of the $NADP^+$-dependent hexosemonophosphate shunt. Additionally, DNA damage also causes the release of several proteases which may be responsible for the sequence of pathological events which result in vesication in human skin. The reduction in NAD^+ levels is associated with the presence of pyknotic nuclei in basal cells which is first seen six hours after exposure to mustard. The number of basal cells showing pyknosis increases by 12 hours and becomes maximal 18 to 24 hours later (Papirmeister *et al.* 1984a; Papirmeister *et al.* 1984b). The epidermis separates from the basement membrane at the epidermal-dermal junction forming small clefts, which fill with debris and cells accompanied by the formation of extracellular vacuoles below the moribund basal cell layer. The vacuoles increase in number, fill with fluid and debris, coalesce and cause widening of the clefts to form subepidermal blisters consisting of necrotic basal cell tissue to cause vesication (Papirmeister *et al.* 1984a; Papirmeister *et al.* 1984b; Papirmeister *et al.* 1985).

Protein Thiol Depletion Hypothesis of Orrenius

A variety of cellular defense mechanisms exist for the detoxification of many different toxic metabolites produced intracellularly, once cells are penetrated by toxic molecules (Orrenius 1985; Orrenius and Nicotera 1987). One such cellular mechanism is the glutathione and protein

thiol systems which play a critical role in preventing an increase in intracellular oxygen radicals such as hydrogen peroxide and organic hydroperoxides produced by redox active substances for example, quinones, paraquat, etc (Jones *et al.* 1981; Eklow *et al.* 1984; Orrenius and Nicotera 1987). If these systems are overwhelmed and the pools of glutathione and protein thiols exhausted in coping with an influx of toxic molecules, disruption in Ca^{++} homeostasis occurs leading to a continual rise in cytosolic Ca^{++} which results in plasma membrane blebbing in isolated hepatocytes with subsequent cell killing as a result of the oxidative stress (Schanne *et al.* 1979; Jewell *et al.* 1982; Thor *et al.* 1982; DiMonte *et al.* 1984; Thor *et al.* 1985). Surface blebbing of membranes has also been induced by the presence of calcium ionophores such as A23187 and is preceded by changes in intracellular thiol levels (Jewell *et al.* 1982; DiMonte *et al.* 1984). Ordinarily, a rise in cytosolic Ca^{++} is prevented by intracellular compartmentalization of Ca^{++} by mitochondrial and microsomal Ca^{++} pumps and binding to proteins such as calmodulin. Active transport of Ca^{++} through the endoplasmic reticular and plasma membranes is mediated by a Ca^{++} stimulated Mg^{++} dependent ATPase which in turn is dependent on the presence of free thiol groups (Moore *et al.* 1975; Bellomo *et al.* 1983; Thor *et al.* 1985). Thus, an oxidative stress leading to depletion of glutathione and protein thiol levels disrupt those mechanisms regulating Ca^{++} sequestration and results in blebbing of the plasma membranes of hepatocytes with subsequent cytotoxicity (Orrenius 1985; Orrenius and Nicotera 1987). Exposure of isolated hepatocytes to reducing agents such as dithiotreitol (DTT) or precursors for glutathione synthesis to prevent thiol depletion results in protection against cytotoxicity (Moore *et al.* 1985). Thus, calcium homeostasis appears to be involved in the mechanism of cytotoxicity (Orrenius 1985; Orrenius and Nicotera 1987).

It has been reported recently that the glutathione concentrations in mouse skin are 0.75 and 0.32 μmoles/g in epidermis and dermis, respectively, (Wheeler 1986) which were about one-tenth of the levels found in mouse liver, 6.74 μmoles/g (Burton and Aherne 1986). If it turns out that human skin also has very low glutathione and protein thiol levels, this would explain why skin is so very sensitive to alkylating agents such as sulfur mustard.

The two hypotheses discussed here offer some glimmer of hope for understanding the complicated pathological phenomenon of vesication by putting in some order a multitude of different findings which occur on first contact with a vesicant such as sulfur mustard and at later stages so that the data takes on some coherent perspective for researchers. Neither hypothesis is contradictory or exclusionary, and each may be operating in some sequence not yet known or perhaps, simultaneously, which is yet to be discovered (Medical Chemical Defense 1989).

EYES

Man

Of the three immediate target tissues of chemical warfare agents, namely, skin, eyes and respiratory system, the one that is immediately susceptible to the toxic action of mustard are the eyes (Mandel and Gibson 1917; Warthin *et al.* 1918; McNamara 1960). Since mustard gas

penetrates the cornea more rapidly than it does skin, the eyes must be cleaned rapidly, within two to three minutes after contact in order to minimize damage (Friedenwald and Woods 1948).

The acute effects of mustard incurred by the eyes of humans with exposures of 12 mg-min/m^3, 50 mg-min/m^3, 100 mg-min/m^3 and 200 mg-min/m^3 were respectively: mild reddening with no effect at lower doses; mild reddening with no injury but some erythema; onset of eye effects such as grittiness, photophobia, lacrimation, discharge and staining initialed six to 24 hours after contact and persisting for one to three days; and temporary blindness due to blepharospasm (spasmodic winking) beginning three to twelve hours after contact and persisting for two to seven days (Mandel and Gibson 1917; Warthin *et al.* 1918; McNamara *et al.* 1975). The latter condition may become severe and lead to conjunctivitis, corneal opacity and blindness (Morgenstern *et al.* 1947; Sollmann 1957; McNamara *et al.* 1975; Geeraets *et al.* 1977; Dahl *et al.* 1985).

When men were subjected to mustard gas in chambers where the concentration of the vapor was 0.0005 mg/L of air for less than one hour, eight out of 13 men displayed definite conjunctivitis while three out of 13 men also suffered from erythema which persisted for several days (Reed 1920).

Contact with mustard gas vapor caused lacrimation, pain, temporary blindness due to blepharospasm, photophobia, edema and a discharge within 3-12 hours which persisted for 2-7 days. This might be followed later by conjunctivitis, opacity and ultimately blindness (Morgenstern *et al.* 1947; McNamara *et al.* 1975).

Approximately 60-90% of the workers in a plant producing mustard gas at the Edgewood Arsenal who had been on the job for 15 months or longer displayed a range of corneal effects such as conjunctival changes, reduced corneal sensitivity, superficial punctate staining of the corneal epithelium and pigmentation of the corneal epithelium (Laughlin 1944a). In another study performed at the Huntsville Arsenal, Alabama, men who had been exposed to long term low level mustard gas vapors displayed acute but not serious eye conditions which cleared up, when they absented themselves from the work place, even for a short time (Brown 1949).

Besides the usual reports of eye lesions and blindness incurred during World War I exposure to mustard gas, Weiss (1958) quoted in Delayed Toxic Effects of Chemical Warfare Agents (Lohs 1975), has reported studies of people employed in plants producing mustard gas during World War II. These people developed delayed effects such as chronic conjunctivitis and other severe eye diseases many years later. Although most eye damage incurred by soldiers exposed to mustard gas during World War I were not permanent, keratitis or delayed keratopathy, which is associated with corneal ulceration and gradual erosion of the cornea, eventually led to blindness or vision impairment. This condition may have a latency of eight to 40 years and has been noted in several cases (Berens and Hartmann 1943; Mann 1944; Atkinson, 1948; Geeraets *et al.* 1977; Dahl *et al.* 1985; Grant 1986).

On exposure to fairly high concentrations of mustard gas vapor, the eyes began to water immediately, the conjunctiva became erythematous and the sensation that a foreign body was present in the eyes produced great discomfort. There were also complaints of pressure exerted on the eyelids so that eyelids feel too heavy to open while the eyes cannot endure bright light for many weeks thereafter. Inflammation progressed very rapidly and within a few hours, very severe swelling in the lids was present and the eyes became completely closed. Nevertheless, the prognosis was often good, without much permanent damage, even if severe keratitis with some opacity was initially present; however, severe ulceration of the cornea leads to blindness (Mann 1944; Atkinson 1948).

Mustard Gas Dump at Breloh

When empty mustard gas shells were burned at a gas dump in Breloh, Germany, after World War I, in order to destroy any residue of the agent so that the casings could be used for scrap metal, a great many workers incurred eye damage from mustard vapor, ranging from slight irritation to very severe injuries to the cornea. Eyes that were severely inflamed and caused severe headaches which were localized in the forehead seemed to indicate that the supraorbital nerves were affected. Such patients groaned and complained of pain; their eyes were swollen shut, their faces swollen beyond recognition, their coughing hoarse, their voice toneless and the eyes could not be opened for several days. The possibility of secondary infection was always imminent, especially, when there was continual itching and scratching. Usually, after a few days, the inflammation of the conjunctiva and swelling of the eyelids receded sufficiently so that the patient could see. Although the corneal injuries might improve with time, scar formation became a complicating factor. The symptoms of pain and pressure in the frontal region occasionally continued for several weeks (Buscher 1944).

If the inflammation caused by mustard vapor from the shell casings was treated early, corneal changes often disappeared in three to four weeks and even corneal opacities cleared up. Although the physicians attending these patients injured at the gas dump at Breloh did not know the concentrations of vapor that the patients had been exposed to, eye damage due to mustard vapors frequently cleared up while contact with liquid mustard caused destruction of the eye with subsequent blindness (Buscher 1944).

Animals

Exposure to mustard gas vapors causes discomfort, pain, redness and a mild edema in laboratory animals (Anslow *et al.* 1918; Warthin *et al.* 1918), followed by lacrimation, photophobia, conjunctivitis, blepharospasm, edema with a discharge observed 24 to 48 hours after exposure. Complete destruction of the eye and blindness, subsequently, depended upon the severity of exposure (Mann and Pullinger 1942; McNamara *et al.* 1975).

A single drop of mustard gas, 0.0004 ml applied with a fine pipet to the center of the cornea of a rabbit caused the animal to blink and rub its eye with its fore paws. After one to two minutes exposure, irritation of the conjunctiva with lacrimation was observed, and was

followed within 30 minutes by hyperemia and edema after 60 minutes. The edema progressed for approximately 12 hours at which time chemosis was definitely present. These effects are identical to those obtained by a 15-minute exposure to mustard vapor at a concentration of 1:20,000; consequently, the drop method appeared to be much more convenient and safer to handle experimentally. In animals, the edema developed first and most markedly in the palpebral conjunctiva when a drop of liquid mustard was applied, in contrast to the vapor which produced edema first in the bulbar conjunctiva. Within 15 minutes after eye exposure, a milky white discharge began to flow from the nose, and the eye became swollen and completely shut. The edema became severe and obscured the eye so that when the eyelids were first opened, a profuse exudation was seen with badly swollen tissues around the eye. While the edema persists for several weeks in animals, it is more irregular and less severe in man where the hyperemia is more marked. Necrosis of the cornea was manifested by a cloudiness which developed five to six hours later and by the eighth hour the cornea had acquired a porcelain like appearance with a bluish-white opalescence. In mild lesions, only a slight cloudiness was observed. Frequently, an opaque band or line was observed running horizontally across the cornea, immediately below its transverse diameter. At this time, the eyelids contained a seropurulent exudate which increased in flow and sealed the eyelids 10 hours later; the eyelids remain closed until the inflammatory process diminished three weeks later. By the fifth or sixth day when a diminution of the edema had occurred, a kinking or ruffling of the upper eyelid appeared at the same time that the lower lid displayed some aversion. Both facial hair near the eye and eyelid hair were lacking at this time. During the second week, changes in corneal curvature were observed with clouding of the anterior chamber. After the third week, the lesions began to undergo repair very slowly and progressive vascularization of the cornea was observed with vessels reaching the center of the vertex by the end of the sixth week. Consequently, corneal cicatrization, that is, fibrous tissue which form scars as it contracts, was noted at this time, accompanied by marked impairment of vision and thickening of the eyelids and nictitating membrane. Even in mild cases of exposure in man, the edema and hyperemia of the conjunctive, showed a chronic progression towards visual disturbances and reduced vision. In animals, whose eyes were treated medically, purulent panophthalmitis did not develop, however, when larger doses of mustard were given than could be delivered with the drop method or with vapor concentrations of 1:20,000 and no medical treatment, suppurative panophthalmitis occurred and terminated in the complete destruction of the eyeball (Warthin *et al.* 1918; Buscher 1944; McNamara *et al.* 1975).

When rats, rabbits, guinea pigs and dogs were subjected to mustard gas, in a chamber, either to a concentration of 0.001 mg/m^3 or 0.1 mg/m^3 for 52 weeks, only the dogs receiving the mustard gas vapors at the higher concentration, 0.1 mg/m^3, displayed corneal opacity, keratitis, vascularization, pigmentation and granulation after 16 weeks of exposure; these manifestations persisted until termination of the project (McNamara *et al.* 1975).

Laughlin (1944b) has developed a bioassay for mustard in which the eyes of a rabbit are exposed to mustard vapors for 30-60 minutes and observations made for 24 hours. He found that the same dose of mustard was less toxic when given over a longer period of time such that

a Ct delivered in two minutes of exposure produced more severe eye effects than the same Ct delivered in 30-60 minutes.

GASTROINTESTINAL SYSTEM

Man

Mandel and Gibson (1917) reported that nausea, vomiting and epigastric distress were frequent symptoms displayed four to 16 hours after mustard gassing. Canalli (1918) reported that the stomach was distended with gas, had a cloudy mucosa with many minute ulcerations, and a thickened congested mucosa and a colon which was necrotic and covered with a purulent exudate at autopsy. The duodenum also displayed a cloudy and congested mucosa covered with minute ulcers, while the jejunum and ileum were anemic with a desquamative epithelium. Canalli (1918) diagnosed the findings as acute gastroduodenitis with hemorrhagic erosions, acute desquamative enteritis and severe hemorrhagic necrotic colitis, and called attention to the selective action of mustard gas on the gastrointestinal tract.

There is a report of violent, frequent and prolonged vomiting in four cases of experimental mustard burns in which the skin only had been exposed to the agent. These men wearing respirators were subjected to a Ct of 660 mg/min/m^3 under tropical conditions; no leucopenia was observed (Fairley, 1943).

A young man who was drunk, drank five ml of mustard gas in attempt to commit suicide; he vomited, collapsed, and became unconscious within eight minutes; this was also associated with urination and defecation. On recovering from unconsciousness, his stomach was lavaged and twice rinsed with permanganate, but he died five hours later when his heart ceased beating. Although no mustard was demonstrable chemically in the blood or gastric contents, gauze strips soaked with either his blood or gastric contents when applied to the skin of rabbits caused hyperemia with subsequent development of a crust. Autopsy revealed the presence of hyperemia of the oral, laryngeal, tracheal and pharyngeal mucosa, but only slight inflammation of the intestinal mucosa, swollen kidney tubules and a fatty liver. The brain, spinal cord, meninges and sympathetic nervous system showed congestion, degeneration and loss of cells with pyknotic nuclei. Sympathetic ganglia of the neck and chest, Purkinje cells of the cerebellum and olivary nucleus also showed pathological changes (Jankovich, 1938).

Animals

Rabbits and guinea pigs were given capsules containing 0.06-0.24 ml of mustard gas in olive oil, butter and lard while other capsules containing mustard were mixed with meat and given to dogs. Vomiting, irritation of the mucous membranes of the mouth, profuse salivation, foul discharges from nose and mouth, diarrhea, tarry stools, depression and refusal of food and water occurred within one to twelve hours. Anorexia and weakness persisted so that six days later the animals were weak and prostrate. Death followed 12 days later from ingestion of 0.03

to 0.06 ml doses and three to five days following a 0.24 ml dose for dogs; animals such as guinea pigs and rabbits died sooner (Warthin and Weller 1919a)

At autopsy those animals that had died of mustard given orally showed: emaciation, congested liver, dilation of the gall bladder and anemic spleen; dilation of the right side of the heart with clots in the right ventricle; congestion of the lungs but no pneumonia; and congestion of the kidneys. Very prominent lesions were observed in the gastrointestinal tract such as distention of the stomach and duodenum which were filled with a green or black fluid. Necrosis of the mucous membrane of the stomach with occasional congestion and thin necrotic areas were present but very little hemorrhaging was observed. Peritonitis occasionally was noted and ulcers were observed later, six to 12 days after ingestion of the mustard. However, stomach lesions did not display the characteristic edema that one associates with mustard contamination of the skin and conjunctiva nor hemorrhagic lesions. The intestines showed patches of necroses and congestion with slight hemorrhages and some inflammation (Warthin and Weller, 1919a; Young 1947).

Air, saliva, or secretions from the upper portion of the respiratory system, if contaminated with mustard, may upon swallowing cause some irritation and necrosis of the elementary mucosa, varying from inflammation to eschar formation; these may develop into gastric ulcers and cause perforation (Warthin and Weller, 1919b).

Extensive intestinal injury was incurred by rats and rabbits that had received one or two LD_{50} doses of either sulfur mustard or any of the nitrogen mustards. The intestinal lesions involved the entire intestine starting just below the pylous, being most severe in the region of the ileum, while the stomach itself was unaffected when the mustards were given i.c., i.v., i.m. or i.p. The mucosa showed degeneration and inflammation with marked erosion and injury to the epithelium with edematous villi within 24 hours of injection. The most severe lesions were found 72 to 96 hours after dosing when the glandular tissue appeared cyst-like with denuded mucosal surfaces; the epithelial tissue displayed hypertrophy and hyperplasia; maximal sloughing, fluid distention and mucous diarrhea were also observed (Mayer *et al.* 1920; Smith 1943; Graef *et al.* 1948).

Histomorphological examination of the small intestine, liver, kidneys, and spleen 1,3 and five days after i.v. injection of ½ LD_{50} of sulfur mustard revealed that the most severe necrotic damage was observed on day three. Tissue renewal was found on day five in the glandular epithelium of the small intestine, liver and kidneys and in the histiocytic reaction of the stroma in the myocardium but no recovery was seen in the lymphatic follicles of the spleen. Rats that had been injected previously with sulfur mustard were injected with 140 μCi/animal of sulfur mustard labeled with ^{35}S. Historadiographic examination of the small intestine, liver, kidneys, myocardium and spleen showed that the distribution of the labeled sulfur was concentrated in fragments of intact tissue but are irregular in necrotic tissues (Andrzejewski and Scianowski, 1978).

NERVOUS SYSTEM

Man

In man, large doses of mustard gas (2 mg/Kg) injected i.p. that affect the neuro-muscular system provoked convulsions simulating an epileptic crisis. While weak doses of mustard gas caused stupor, large doses, in addition to inciting convulsions also resulted in a fall in body temperature to 35°C at the time of death (Mayer *et al.* 1920).

In 1961, Spiegelberg reported on the psychopathological and neurological lesions incurred by workers in German plants producing war gases during World War II, which resulted in delayed effects, following an earlier association with mustard gas. Likewise, U. Hellmann (1970a) and also Lohs (1975) reported on the development of delayed lesions in those workers, previously examined by Spiegelberg (1961) in 1958-1960, such as debility, loss of vitality, impaired concentration, sensory hypersensitivity, diminished libido, weakened potency, neuralgic complaints, and disorders in autonomic regulation of the heart.

There appears to be one report of the occurrence of the Guillain-Barre Syndrome in an American enlisted man, a "toxic gas handler," employed in the Chemical Warfare Service in India who was accidentally exposed to mustard gas. Neurological examination showed absence of deep reflexes, generalized weakness of the arms and legs with ataxia. He recovered several months later, although absence of deep reflexes was still a clinical feature (Chusid and Marquardt 1946).

Vomiting follows exposure, even to mild doses of mustard, and may reflect cholinergic activity or excitation of the vomiting center in the CNS as a consequence of nasopharyngeal irritation (Buscher 1944). This is followed by less rapid gastrointestinal disturbances such as anorexia, epigastric pain, constipation and diarrhea which may persist for some time (Warthin and Weller, 1919b; Velden 1921; Buscher 1944). The return of one's appetite was considered as one of the most desirous of diagnostic signs (Velden 1921; Buscher 1944).

The literature on the effects of mustard on the nervous and neuro-muscular system is sparse. Although some behavioral changes are occasionally mentioned, no concerted effort has been expended on investigating behavioral modifications associated with mustard insults.

Animals

Injection of mustard gas into dogs resulted in hyperexcitability followed by unsteadiness of gait, muscular weakness and defecation (Lynch *et al.* 1918). Indication of central stimulation was observed in mice 20 minutes after injection of 500 mg/Kg i.p. when convulsions, seizures, hind feet and leg flexion, Straub-tail, progressive weakness, respiratory distress and death ensued (Philips and Thiersch 1950). Rats given 15 mg/Kg of nitrogen mustard displayed weakness, ataxia within 60 minutes and diminution in righting reflexes. Marked activation of both

cholinergic and sympathetic activities were observed in cats and dogs (Philips and Thiersch 1950).

Smith (1943) found that the cells of the cerebral cortex, basal ganglia, pons and the medulla had degenerated and the Purkinje cells of the cerebellum were reduced in number in a cat that had received a total of 10 drops of mustard gas over a period of five months, and suggested that mustard gas had selectively exerted a destructive action on ganglia cells of the CNS and may have been mediated through skin absorption. The salivation, vomiting, defecation and diarrhea following intoxication with mustard may be a reflection of parasympathetic stimulation (Smith 1943).

Rabbits, guinea pigs and dogs display a marked depression and refusal to eat after exposure to mustard gas contained in capsules given orally (Warthin and Weller 1919a).

Cardiovascular System

While a lethal dose of mustard gas (2 mg/Kg) injected i.p. caused a significant fall in blood pressure within 5-10 minutes (Mayer *et al.* 1920), mustard gas (1 mg/Kg) injected i.p, i.v. or given percutaneously showed no effect on the activity of the heart, blood pressure or vagal activity in dogs or rabbits (Cordier and Cordier 1950). Delayed effects on impaired regulation of the heart by the autonomic system in human beings have been reported by Weiss (1958).

Immune System

Because of the special affinity of the sulfur and nitrogen mustards for injuring hematopoietic tissues, it is not surprising to find that mustard gas and bis(2-chloroethyl)methyl amine suppress and retard antibody production (Philips 1950). This action of the mustards was put to use as an experimental tool in studying tissue hypersensitivity (Birenbaum 1962).

An attempt was made to sensitize rabbits by applying different concentrations of liquid mustard to the right eye at a rate of 0.1 ml, three days/week for three weeks. Concentrations of 0.2% caused redness, chemosis and corneal opacity, 0.02% caused redness and a discharge while 0.002% caused redness in only one rabbit after the first application. A challenging dose of 0.002% was then applied to the other eye (left) two weeks after the last sensitizing dose. Since the challenging dose was without effect another challenging dose at the same concentration was given but without effect (McNamara *et al.* 1975).

When the eyes of rabbits were exposed to a Ct of 400 mg/min/m^3 for a second time, two weeks later, the eyes displayed a more severe reaction as noted by an increased conjunctival reaction and discharge (Laughlin 1944b). There have also been a few reports of sensitization to mustard in people who work in mustard gas filling plants (Marshal 1919; Morgenstern *et al.* 1947).

McMaster and Hogeboom (1945) have reported that human skin contaminated with mustard became very sensitive to repeated treatment with BAL (63% of volunteers) and that the rate of

sensitization was many times greater in mustard contaminated skin than that observed when BAL was repeatedly applied to normal skin or skin contaminated by Lewisite or by arsenical compounds.

ENDOCRINE GLAND

Adrenals

Male rats injected i.v. with several different agents such as 0.4 mg/Kg bis(β-chloroethyl)-sulfide, 0.04 mg/Kg ethyl bis(β-chloroethyl)amine, 1.2 mg/Kg methylbis(chloroethyl)amine and 0.6 mg/Kg tris(β-chloroethyl)amine showed marked hypertrophy of the adrenal glands (Chanutin 1944; Ludewig and Chanutin 1946; Chanutin and Ludewig 1947). Total lipid and ester cholesterol were decreased in the adrenal glands and replaced by water. The investigators interpreted these findings as indicating a conversion of adrenal cholesterol into adrenal cortical hormones such as corticosterone and other adrenocorticosteroids and pointed out the parallelism between the alteration in adrenal lipid and cholesterol and the alarm reaction of Selye which is a response to stress (Ludewig and Chanutin 1946; Chanutin and Ludewig 1947). Indeed, Anslow *et al.* (1947) reported that mice and rabbits given mustard showed some adrenal congestion which was more marked in mice. Male dogs injected i.v. with several different nitrogen mustards also showed evidence of damage to the nuclei of cells located in the adrenal cortex while the nuclei of cells in the adrenal medulla were not affected. The fasiculata and reticularis zones of the cortex displayed characteristic necroses and damage, implying reduced production of cortical hormones (Kindred 1949b). Intravenous injection of 100 mg/Kg of mustard gas in rats resulted in the death of all animals within 2-3 hours, including intact and adrenalectomized rats receiving cortin as replacement therapy. Those adrenalectomized rats unsupplemented with cortin survived for only about half that time (Black and Thomson 1947). Kindred (1947) likewise reported that adrenalectomized rats that received several different doses of tris(2-chloroethyl)amine i.v. which were not lethal caused damage to the lymphoid tissues of the animals. Consequently, it was suggested that mustards appeared to exert a direct action on lymphoid tissues, although part of this action might be mediated through the adrenal cortex (Karnofsky 1948b; Kindred 1947; Philips 1950).

While corticosteroids did not influence the healing of lesions provoked by sulfur mustard application to rabbit skin, they did appear to have a general effectiveness in reducing the degree of edema and so may influence some important phase in vesication. Vogt *et al.* 1984 and Vojvodic *et al.* (1985) have suggested that corticosteroids used alone or together with other drugs be utilized in treatment of the local and systemic effects induced by mustards.

Gonads

Testes

Intravenous injection of ether nitrogen or sulfur mustard in male mice results in damage to the testes with inhibition of spermatogenesis (Graef *et al.* 1948). Indeed, it has been reported

that the administration of any one of nine different nitrogen mustards injected into young mice caused testicular damage (Landing *et al.* 1949). A study was performed in which three different nitrogen mustards were injected intraperitoneally: 4.0 mg/Kg of bis(2-chloroethyl)methyl amine, 1.5 or 2.8 mg/Kg tris(2-chloroethyl)amine, 150 mg/Kg 2-chloroethyl morpholine as single injections and 0.6 mg/Kg bis(2-chloroethyl)methyl amine for seven injections. Histological examination 24 hours after the last injection, revealed that disruption of spermatogenesis had occurred with many mitotic figures showing abnormal metaphases or anaphase divisions, dark swollen chromosomes and pyknotic nuclei with cell degeneration and lysis. At 48 and 72 hours, there was a marked disorganization of the layers of spermatogenic cells, damaged tubules filled with abnormal appearing cells and fusion of spermatids into multinucleate giant cells; marked acellularity with disorganization and fragmentation of spermatogenic tissue and loss of sloughed cells through the epididymis were also prominent (Landing *et al.* 1949). Repeated injections of small doses of bis(2-chloroethyl)methyl amine caused more severe damage than did a single injection of the same total dose. Nevertheless, the damage was usually transient, since testicular recovery was observed in two weeks with the formation of mature sperm four weeks after exposure (Landing and Eisenberg 1949; Landing *et al.* 1949).

Female rats never exposed to mustard gas were mated with male rats that had been exposed to either 0.001 mg/m^3 or 0.1 mg/m^3 mustard gas continuously for 52 weeks. At the end of the gestation period, the pregnant rats were sacrificed, but no evidence of fetal abnormality was found. In addition, pregnant rats exposed to either 0.001 mg/m^3 or 0.2 mg/m^3 mustard gas during the first, second or third week of gestation or throughout the gestation period as well, did not display any fetal abnormalities (McNamara *et al.* 1975).

Ovaries

Administration of nitrogen or sulfur mustard did not affect the reproductive potential of female mice since the fertility of the mice was not altered and no injurious effects were observed in the ovaries (Graef *et al.* 1948).

Pregnant rats subjected to low levels of exposure to sulfur mustard vapors revealed no increase in fetal mortality or fetal abnormalities (McNamara *et al.* 1975)

Recently, a two-generation reproductive study was completed by Battelle Pacific Northwest Laboratory. Male and female Sprague-Dawley rats of each generation were distributed into several groups and gavaged with either 0, 0.3, 0.1 or 0.4 mg/Kg sulfur mustard for 13 weeks prior to mating, and continued throughout gestation, parturition and lactation for 42 weeks. The treatment did not affect the course of reproduction nor that of pregnancy in any of the generations. The highest dose did depress growth of adult male and female rats of the F_1 generation, and growth of the F_1 and F_2 offspring during lactation. The presence of a gavage-related hyperplasia was noted in both sexes in the forestomach and was dose-related (Sasser *et al.* 1988; Sasser et al 1989a).

Other Effects

An interesting but rather peculiar phenomenon of mustard gas is its ability to induce acetylcholinesterase activity and axonal growth in mouse neuroblastoma cells, however, the mechanism involved is still obscure (Turnbull *et al.* 1973, Lanks *et al.* 1975). The addition of 0.5 μg/ml of mustard gas to the culture medium provoked a 5-fold increase in enzyme activity four days after exposure to the agent, with a 25-fold increase in the rate of reappearance of acetylcholinesterase activity following an essentially irreversible inhibition, which implied the induction of new enzyme. The very low concentrations required to induce axonation and acetylcholinesterase activity in neuroblastoma cells suggested to the investigators that the site of action might be DNA (Turnbull *et al.* 1973). Since a monofunctional mustard such as semimustard required much larger concentrations to produce the same effects as the difunctional mustard gas, it was concluded that cross-links were necessary to produce these two remarkable activities (Turnbull *et al.* 1973).

Gille (1969) reported that mustard inhibit cholinesterase activity. More recently, it has been reported that cholinesterase activity determined, four days after s.c. injection of lethal doses of sulfur and nitrogen mustards ($3LD_{50}$), showed a profound depression in several tissues such as diaphragm, intercostal muscle, liver and serum but no inhibition was observed in either brain or erythrocyte cholinesterase; the cholinesterase activity was 36%, 43%, 44% and 52%, respectively, in the depressed tissues and 96% in brain, 100% in erythrocytes and controls (Vojvodic *et al.* 1985).

Following exposure to mustard given intravenously, dogs showed a moderate hyperglycemia for two days succeeded by a reduction to normal or below normal levels by the fifth or sixth day (Dziemian 1945). Although Chanutin (1944) showed a progressive hyperglycemia following intravenous administration of mustard in dogs and rats, Ball (1944) was not able to find any alteration after intravenous injection of mustard in rabbits. Indeed, Dziemian (1945) was not able to report any significant or consistent alternation in blood glucose levels in dogs after intravenous injection of mustard and concluded that the action of mustard on carbohydrate metabolism, incurred by the two different routes of delivery, i.v. or whole body gassing, lead to different effects; this was noted by the dissimilar influences on certain parameters of carbohydrate metabolism such as the glucose tolerance test, gastrointestinal motility and blood glucose concentrations. Black and Thomson (1947) suggested that the alterations in carbohydrate metabolism were a reflection of the anoxic conditions which developed during mustard intoxication.

Sulfur mustard administered i.v. to adult male Wistar rats at ½ LD_{50} resulted in the depression, for several days, of lactate dehydrogenase (LDH) total activity in homogenates of heart and skeletal muscle, liver, spleen, kidney cortex and ileum. Serum LDH activity rose on day 1, fell on day three and returned to normal levels on day five after administration of the agent. The investigators suggested that sulfur mustard caused a temporary inhibition of LDH synthesis which was reflected as a fall in LDH activity and that the LDH increase in activity in the serum was a consequence of increased membrane permeability with leakage of enzymes from

the cytosol (Andrzejewski and Scianowski 1976, Scianowski 1977). These findings appeared to be contrary to the findings of Needham (1947) who reported that sulfur mustard inhibited only the phosphokinases and several proteinases.

When tissues were exposed to X-ray irradiation, lysosomal damage resulted in the release of hydrolytic enzymes which play an important role in tissue injury (Watkins 1975). Although sulfur mustard and 2-chloroethyl ethyl sulfide did result in a slight release of three lysosomal enzymes: aryl sulfates, β-glucuronidase and acid phosphatase, their release occurred only at very high concentrations of the agents and so lysosomal release of enzymes that could play a role in the cytotoxic activity of sulfur mustards does not appear to be relevant (Gross *et al.* 1981).

The destruction of cells of bean root plants, *Vicia faba*, occurred after 10 minutes immersion in liquid mustard gas, however, the destruction of such cells occurred more rapidly and was more severe if the roots were immersed in Lewisite (Milovidov 1949).

METABOLISM OF MUSTARD

Intravenous injection of sublethal doses of five mg/Kg ^{35}S-labeled mustard gas into rabbits resulted in a rapid diffusion of most of the radioactivity from the circulation to the urine and bile within 20 minutes after injection, while the remainder of the ^{35}S was distributed to most of the tissues including the liver, kidneys and lungs where it became firmly fixed or bound to protein complexes (Boursnell *et al.* 1946a).

A major portion of the radioactivity of ^{35}S-labeled mustard gas injected intravenously into mice was excreted within 24 hours, with an additional 10% on the following day, while a considerable amount of radioactivity was once again found in the bile. There was no evidence of any intact mustard being present due to its rapid hydrolysis and only small amounts had been oxidized completely to sulfate. While 25% of the urinary substances excreted were the hydrolytic product of mustard, thiodiglycol, the major portion of the remaining radioactivity was associated with an unidentified anionic component (Davison *et al.* 1957).

In cancer patients i.v. injection of labeled ^{35}S mustard gas resulted in the disappearance of 90% of the radioactivity, almost as soon as the injection ceased. The portion that remained in the plasma turned over very slowly, and remained constant for the entire period of observation. The radioactivity was excreted to the extent of 25% in 48 hours; however, the greater fraction of that (2/3) was excreted during the first 12 hours (Davison *et al.* 1957). The portion of labeled mustard gas that remained in the plasma showed a very slow turnover and remained constant through the period of observation.

In another study of the fate of ^{35}S-labeled mustard gas, 1-5 mg/Kg of the agent was injected into mice, rats and humans. A major fraction of the radioactivity was excreted in the urine within the first 24 hours in rodents, while a considerable amount of radioactivity was retained for very long periods of time in humans (Davison *et al.* 1961). The metabolites excreted in the urine after injection of labeled mustard gas were present mainly as conjugates, especially

conjugates of glutathione. This suggested that these products of metabolism were probably the result of alkylation reactions rather than a consequence of enzymatic alterations (Davison *et al.* 1961). Among the major products excreted in the urine were thiodiglycol, conjugates of thiodiglycol with glutathione and other substances; bis-β-chloroethyl sulfone was present also as conjugates with either glutathione or cysteine (Davison *et al.* 1961). In terminal patients, the labeled urinary metabolites isolated after injection of ^{35}S-labeled mustard gas were the same as those found in the urine of mice and rats (Davison *et al.* 1961).

Injection i.p. of one mg/Kg ^{35}S-labeled mustard gas into rats resulted in the urinary excretion of labeled bis-cysteinylethylsulfone and small amounts of thiodiglycol metabolites (Roberts and Warwick 1963).

In the mouse and rat, almost all of the ^{35}S of the labeled mustard injected was excreted within three days: about 50% during the first six hours and 90% for the first day, while only 50% was excreted in humans within two days. Chromatographic analysis revealed the presence of six substances in the urine, all of which were either neutral or anionic; 20% of these urinary products was associated with the neutral peak. The same pattern was observed in mouse, rat and man. Isotope dilution showed that 10% of the neutral peak was identifiable as thiodiglycol. Traces of two sulfone acids were also found, isethionic and sulfoacetic acids. The suggestion was made that thiodiglycolic acid could be a logical intermediate between thioglycol and sulfoacetic acid and thereby account for about 1.5% of the radioactivity in the urine (Smith *et al.* 1958). There was no evidence that sulfur mustard was excreted as a conjugate of taurine or taurocholate (Rozman *et al.* 1957, Smith *et al.* 1958.

Injection of ^{35}S-labeled oxidation products of mustard gas, bis-β-chloroethyl sulfoxide and bis-R-chlorethyl sulfone, i.v. into rabbits resulted in the distribution of the ^{35}S in the urine and bile in the same manner as ^{35}S labeled mustard (Boursnell *et al.* 1946b).

Whole body autoradiography of mice given ^{35}S-labeled mustard gas by i.v. or percutaneously showed that the highest levels of radioactivity were mainly located in the nasal region, kidneys and liver with some activity in the CNS and in fetuses of pregnant animals (Clemedson et al 1963).

Perfusion of isolated lungs of dogs with ^{35}S-labeled mustard revealed that equilibrium between the blood and tissues occurred within five minutes. Moreover, 14% of the radioactivity was fixed to the lung tissue, while 74% of the labeled sulfur remained in the perfusate. The remaining radioactivity which could not be accounted for was assumed to have been thiodiglycol which had left the lung when it had been washed free of blood and had been distributed into the intracellular fluid. Other than the radioactivity fixed to lung tissue, negligible amounts of radioactivity were found in lymph, blood, spleen, liver, brain, thoracic duct and kidney tissue (Pierpont and Davison 1962).

DECONTAMINATION AND ANTIDOTES

Decontamination of Mustard

Skin

The high toxicity of sulfur mustard requires rapid and very effective removal or decontamination to prevent cutaneous absorption with consequent vesication (Jelenko 1974; Trapp 1985). Liquid mustard must be removed from the skin in less than 3-5 minutes, that is, as rapidly as possible but the efficacy of this is also affected by weather conditions since hot, humid weather aggravates the effects of mustard (SIPR 1973). An additional consideration is quick, effective removal without spreading the vesicant. This may be performed in a number of different ways such as prolonged washing with soap, frequent changes of water and chemical neutralization to minimize penetration and absorption to preclude local and systemic intoxication (Chiesman 1944; U.S. Army 1974; Lindsten and Schmitt 1975; van Hooidonk 1983).

There is no universal skin decontamination procedure which is effective and safe. During World War I a bleach cream was used which had to be removed by washing since it was a severe skin irritant. This was followed by application of anti-gas ointments which could be readily used in the field; anti-gas ointment No. 1 (obsolete, no longer used) was an excellent antidote against mustard but needed to be washed off because of its skin irritancy; this was replaced by anti-gas ointment No. 2 (composition not given) which was very effective but became an irritant when covered by bandages and clothes. Hair contaminated with mustard was also treated with a bleach cream and washed out or rubbed off with anti-gas ointment or removed by kerosene (Chiesman 1944).

An effective decontaminant which neutralized free mustard on skin during the initial critical exposure interval was S-330 or M-5 (Army designation) 7,8-diphenyl-1,3,4,6-tetrachloro-2,5-diminoglycoluril. If used during the critical period, it could reduce the harsh effects of large liquid splashes of mustard to a mild erythema (Rudin 1953). The U.S. Army also used M-5 as a protective ointment after removal of the mustard or soap and water to wash the area. However, decontamination is of little value against vapor exposure (McNamara 1960). Washing the area with oil, kerosene or gasoline followed by copious washing with soap and water has also been recommended (Jelenko 1974) as well as neutral hypochlorite (1% chlorine) (Chiesman 1944).

The system used in the U.S. is the Personal Decontamination Kit, M258A1, which is used against nerve agents and blister agents but consists of chemicals that are both irritating to the skin and eyes which has been confirmed in rabbit tests (Hayes *et al.* 1984; Harrington 1987). This system has been recently placed by resins, such as Ambergard XE-555 and Ambergard XE-556, which readily absorb and detoxify the agent without themselves being toxic or irritating (Harrington 1987).

Effective methods of decontamination used by the military are not available to civilian population. However, a recent report suggests that the use of common every day household products by ordinary people during a mustard gas attack might be a very effective means of decontamination. Household products such as flour, talcum powder, salad oil, Dutch powder, dry tissue paper and wet tissue paper removed more than 97% of the sulfur mustard from guinea pig skin contaminated with the agent. Thus, simple household means are available and can be used by civilian populations to protect themselves. Any excess powder may be removed from the skin by copious amounts of water. Indeed, more important than the type of decontaminant used is the time delay between contamination and irritation of the decontamination process (van Hooidonk *et al.* 1983).

Aspiration of blisters with subsequent cleaning with mild soap and water or a bland antiseptic, such as 8% dithol, followed by washing with saline, has also been advocated (Chiesman 1944). Since there is a question as to the toxicity of the fluid within mustard-induced vesicles, it has been suggested that blisters or bulla larger than two cm be debrided or opened while being lavaged and treated topically with sulfamylon cream (mafenide acetate) or silvadene cream (silver sulfadiazine) for its antibacterial activity (Pruitt 1987).

Repeated application of 1/4 to ½ ml of a 5% BAL and petrolatum ointment on gauge dressings for 48 hours after contact with 2.0 mg liquid mustard gas, or its saturated vapor half an hour after contamination, or after the appearance of erythema two to 2-1/2 hours later, was very effective in inhibiting vesiculation. Although BAL ointment does not accelerate healing, pain and tenderness are reduced during the first week. Moreover, both BAL and chlorinating decontaminants may be used simultaneously without interference of each others action. However, there are a number of disadvantages which outweighs the efficacy of BAL treatment of mustard gas lesions; BAL ointment tends to trap any residual mustard still on the skin surface and in so doing prevents further evaporation and thus results in worse lesions. In addition, skin contaminated with mustard displays a higher degree of sensitization to repeated treatment with BAL, then does normal skin or skin contaminated with Lewisite or other arsenical compounds (McMaster and Hogeboom 1945).

The Federal Republic of Germany and the USSR have included sodium thiosulfate and Unithiol (2,3-dimercapto-propane-1-sulfonate, DMPS) for detoxification of mustard in pretreatment and or early treatment of skin and systemic mustard exposures, however, recently they have been found ineffective in reducing the severity of cutaneous mustard incurred injuries (Papirmeister 1987).

Eyes

Contamination of the eyes by liquid mustard requires immediate decontamination by the individual who has been splashed (Chiesman 1944; SIPRI 1973). BAL ointment which is rubbed gently into the eye followed by irrigation with copious volumes of water has been used, however, this treatment may be effective within seconds of contamination but is of little aid after two minutes of contact (McNamara 1960). Ainsworth (1945) reported that the BAL ointment

was very effective in removing surface mustard from the eyes of rabbits, if applied within one minute of contact with the agent; however, the application of an irritant ointment, containing carbon disulfide, to the eyes of rabbits contaminated with a drop of mustard was equally effective. Since administration of anesthetic to reduce pain also inhibited the normal blink reflex which would ordinarily retard the rate of absorption of mustard by the eye, the implication, evidently was that the effective agent was not the BAL ointment but the involuntary opening and shutting of the eye caused by any substance in contact with the eye. Indeed, BAL ointment will reduce the severity of lesions caused by mustard contamination of the eyes, to allow recovery because the ointment will remove surface mustard by taking it up into the greasy base of the ointment and thereby stimulate the blank reflex; lacrimation will then expel most of the resulting fluid solution. The use of BAL in such a procedure is problematical and may be eliminated in the future (McNamara 1960). It has been suggested that since ointments contain fatty substances, they should be avoided because of mustards solubility in lipids and so the eyes should instead be irrigated, immediately with large volumes of water or with 1.5% sodium bicarbonate solution within 15 minutes, before most of the mustard is absorbed (Berens and Hartmann 1943).

Eyes may also be washed with alkaline solutions such as 2-5% $NaHCO_3$ or with neutralizing solutions such as 0.5% dichloramine-T dissolved in chlorinated paraffin or chlorinated diphenylether with prior instillation of a local anesthetic (Hughes 1942). Flushing the eye with dichloramine-T for the first 15 minutes after contact with mustard gas may prevent eye damage (Berens and Hartmann 1943).

Hypertonic solutions have also been recommended for inducing drainage of eyes that have been contaminated by mustard using saturated solutions of sodium sulfate or magnesium sulfate in water containing syrup termed Bonnefon solution (Bonnefon 1939). Zinc or boric acid may also be instilled during convalescence (Berens and Hartmann 1943).

To relieve pain or to permit the separation of the eyelids for examination, 0.5% pontocaine may be instilled into the eyes once or twice, however, cocaine and atropine are to be avoided (Berens and Hartmann 1943). Additionally, mercurial salve has been applied to eyelids contaminated with mustard in order to prevent the formation of small tumors, styes and multiple small abscesses of the eyelids (Pickard 1919).

Rabbits were injected i.v. with 500 mg of ascorbic acid every day for six days. When the first injection was given 20 minutes prior to the application of a small drop of liquid mustard from a fine glass pipet into the eyes of rabbits, the spread of keratitis and lid inflammation were prevented. When 10% sodium thiosulfate was injected intravenously in 500 mg doses, in the same manner as the ascorbic acid, it was concluded that treatment with the thiosulfate was effective, but the results obtained were not as good as that found with ascorbic acid (Livingston and Walker 1940). However, Mann and Pullinger (1940) reported that i.v. injected ascorbic acid had no effect on mustard gas lesions of the eyelids, cornea and conjunctiva.

There doesn't appear to be any effective decontamination procedures for eyes contaminated by mustard vapor since damage is initiated on contact, however, it does take several hours for the final outcome to be determined. Treatment requires analgesics to ease pain and irritation, antiseptics to prevent infection and sterile petrolatum to keep the eyelids patent. Secondary infections may increase scarring and so is prevented by 10% solutions of sodium sulamyd every two hours or penicillin solutions every four hours (McNamara 1960; Rim and Bahn 1987).

If sterile solutions or drops for cleansing eyes exposed to mustard gas vapors is not available, no treatment is preferred in order to avoid incurring devastating secondary infections (Berens and Hartmann 1943). Although eyes that have been exposed to mustard vapors may appear to be seriously damaged at first, recovery has occurred even without any treatment, if secondary infections can be avoided; thus no treatment at all is preferred if sterile solutions are not available.

Systemic Intoxication - Antidotes

Systemic intoxication by mustard has been treated with atropine to reduce gastrointestinal activity, morphine or barbiturates to reduce discomfort and restlessness, and whole blood, plasma, dextrose and saline to replace lost fluids and electrolytes and maintain good nutritional status (McNamara 1960).

Sodium Thiosulfate

Sodium thiosulfate is relatively non-toxic and can be injected intravenously in large doses without incurring any morphological or chemical changes in the circulation (Schultz *et al.* 1962; Bonadonna and Karnofsky 1965). When sodium thiosulfate is introduced into the circulation it becomes rapidly distributed throughout the extracellular fluid with little of the thiosulfate entering the cells because of its negative charge and the absence of a specific cell membrane transport system (Cardozo and Edelman 1952); however, glomerular filtration clears the body of thiosulfate very rapidly (Gilman *et al.* 1942; Gilman *et al.* 1946) even though a large concentration of thiosulfate is necessary for long periods of time (Gilman and Philips 1946; Gilman *et al.* 1948; Cardozo and Edelman 1952; Fasth and Sorbo 1983).

Sodium thiosulfate reacts with mustards when these agents are in the cyclized form, but offers no protection against the systemic effects of mustard intoxication if thiosulfate is injected after prior injection of cyclized mustard because of the very rapid reactions of the cyclized species with the body cells (Litwins *et al.* 1943; Gilman 1963; Connors *et al.* 1964 Connors 1966). Pretreatment with sodium thiosulfate or simultaneous injection with mustard indicates that thiosulfate is a systemic antidote which neutralizes the mustard and so prevents or reduces systemic intoxication (Hatiboglu 1960; Owens and Hatiboglu 1961; Foster *et al.* 1962; Lawrence *et al.* 1964).

Thus, sodium thiosulfate is an effective antidote to systemic intoxication by mustard, if taken prior to mustard exposure, and so is of practical significance only when there is advanced

notice or warning of a pending chemical attack. Additionally, skin lesions produced by mustard cannot be medicated by thiosulfate present in the extracellular fluid (McKinley *et al.* 1982).

Injection of 300 mg/Kg thiosulfate i.p. 10 minutes before 6.27 mg/Kg sulfur mustard offered protection to 80% of the rats treated, but no protection if the thiosulfate was infused s.c. over a 33-minute interval, immediately after injection of the mustard; oral administration of thiosulfate was not very effective (Callaway and Pearce 1958).

It has been suggested that sodium thiosulfate may act as a "mustard scavenger" in the extracellular spaces to reduce the lethal effects of mustard (Callaway and Pearce 1958). Apparently, a scavenger can be anything; an enzyme, antibody or low-weight compound that specifically binds or chemically reacts with an agent to effectively lower the active toxicant levels in the body.

Sodium Thiosulfate in Combination With:

- Cysteine

Thiosulfate has been used in combination with several other drugs in order to increase the effectiveness of thiosulfate (McKinley 1982). Thiosulfate in combination with cysteine or together with an additional drug such as methenamine (1g/Kg of each) decreased the mortality in mice from 100% to 10% when injected i.p. 30 to 40 minutes prior to receiving a lethal dose of 10 mg/Kg nitrogen mustard (Scarborough and Thomas 1962). Interperitoneal injection of two g/Kg thiosulfate and one g/Kg cysteine together, or separately before i.p. injection of an LD_{50} dose of HN-2 and merophan (o-di-2-chloroethylamino-DL-phenylalanine) resulted in the following findings: cysteine offered protection against both nitrogen mustards; thiosulfate was effective only against HN-2, a SN_2 reactor (mustards that cyclize rapidly) but not against merophan, a SN_1 type of mustard (cyclizes slowly); in combination, they gave only slight protection against HN-2 and little protection against merophan. The authors suggested that systeine efficacy against both mustards was due to its entrance into cells to increase the thiol levels, while the presence of thiosulfate interfered with its entrance into cells (Connors *et al.* 1964).

- Sodium Citrate

The injection of 2750 mg/Kg thiocit (sodium citrate) i.p. 10 minutes before or after injection of 6.75 mg/Kg sulfur mustard provided protection for all rats subject to the agent. Moreover, intravenous injection of thiocit was more protective against sulfur mustard than even intravenous injection of thiosulfate; however, oral administration of thiocit was not any more protective than thiosulfate (Callaway and Pearce 1958).

- Other Drugs

A comparative study of the effectiveness of several drugs on the degree of protection offered to rats acutely poisoned by either sulfur or nitrogen mustards (sc injection of $3LD_{50}$) revealed that besides sodium thiosulfate (i.p.) , dexamethasone (im) promethazine (im), heparin (im), Vitamin E (im), and atropine (im), all injected 30 minutes after the mustards, gave good protection. However, the most effective protection (increased survival time and reduced lethality) against sulfur mustard was achieved by dexamethasone and Vitamin E, and against nitrogen mustard by sodium thiosulfate and Vitamin E while atropine was the least effective of all the drugs tested. Simultaneous administration of thiosulfate with any of the other drugs further increased the protective activity, particularly in acute poisoning with nitrogen mustard; however, much better protection was offered when three of the drugs, sodium thiosulfate, dexamethasone and promethazine, were simultaneously injected, protecting 90% of the rats compared to 50% when two drugs were combined (Vojvodic *et al.* 1985).

A steady loss in body weight was observed in all rats injected with the mustard, whether or not they had received a protective drug and this loss became maximal four days after injection of the mustard, but treatment caused a steady body weight increase from that point to the termination of the experiment seven days later (Vojvodic *et al.* 1985).

Inhalation of dexamethasone (Auxiloson) immediately in large doses has been suggested in order to prevent lung edema after mustard contamination; inhalation of dexamethasone every 10 minutes by taking five deep breaths of the drug for one day is the preferred therapy (Wegner 1975a; Wegner 1975b).

Recent Iranian Mustard Victims - Regimen

The burn injuries of several Iranians were initially treated in several Vienna hospitals as second degree burns, and as such were covered with antibiotic ointment and the patients given calcium and anti-histamines to reduce itching. On assuming that these burns had been incurred by mustard gas, further treatment was pursued with Dimaval, a BAL derivative (Mandl and Freilinger 1984). After confirmation that the burns had been induced by mustard gas by laboratories both in Vienna and Ghent, a collaborative effort resulted in the following schedule of therapy: skin decontamination by washing with 5% chloramine solution; 10-20g/day animal charcoal together with magnesium sulfate as a laxative and to accelerate urinary excretion, patients had to drink 2-3 liters/day or receive these drugs by infusion; four infusions of 10-20 ml 0.5% cysteine solution to decontaminate the blood or hemoperfusion over charcoal in difficult cases; and Vitamin K was given prophylactically for three days. This treatment was successful in six out of 10 patients, three of whom had been considered as hopeless cases (Heyndrickx and Heyndrickx 1984; Mandl and Freilinger 1984; Pauser *et al.* 1984).

A recent pamphlet obtainable at the Third International Symposium on Protection Against Chemical Warfare Agents, June 11-16, 1989, gives the latest treatment for poisoning with sulfur and nitrogen mustards; it advises immediate i.v. infusion of up to 500 mg sodium thiosulfate/Kg

bw (Kohler 1989). Injection of 500 mg sodium thiosulfate/kg BW i.v. within the first 20 minutes after contamination with mustard will avoid systemic intoxication and even lethal effects (Wegner 1975a; Wegner 1975b).

MUTAGENICITY OF SULFUR MUSTARD

Sulfur mustard is a cell poison which causes disruption and impairment of a variety of cellular activities which are dependent upon a very specific integral relationship. These cytotoxic effects are manifested in widespread metabolic disturbances whose variable characteristics are observed in enzymatic deficiencies (Dixon and Needham 1946; Cullumbine 1947, Cullumbine 1954), vesicant action (Lynch *et al.* 1918), abnormal mitotic activity and cell division (Darlington and Koller 1947; Dustin 1947), bone marrow disruption and disturbance in hematopoietic activity (Krumbhaar 1919; Krumbhaar and Krumbhaar 1919; Pappenheimer and Vance 1920) and systemic poisoning (Lynch *et al.* 1918; Warthin and Weller 1919; Anslow *et al.* 1947; Graef *et al.* 1948; Walpole 1958; Hassett 1963). Indeed, mustard gas readily combines with various components of the cell such as amino acids, amines and proteins (Price 1958; Wheeler, 1962; Lawley 1966). In 1947, Berenblum and Schoental reported that crude extracts of rabbit skin shaken with mustard gas formed precipitates which contained not only sulfur but also phosphorous, and so appeared to indicate that the interacting material was not a protein but a nucleoprotein. This was confirmed when calf thymus nucleoprotein formed similar precipitates with mustard, while pure proteins such as serum globulin, albumin, fibrinogen and gelatin did not. Indeed, Butler *et al.* (1950) reported that mustard caused depolarization of nucleoprotein and that the loss in structural viscosity of the nucleoprotein incurred by the action of mustard and X-ray irradiation were quite similar.

In 1946 Auerbach and Robson reported that mustard gas vapor was as effective as X-rays in producing chromosomal breaks and rearrangements. When *Drosophila melanogaster*, male fruit flies, were exposed to mustard gas, 95 sex-linked lethals out of 1300 treated sex chromosomes were obtained which represented a mutation rate of 7.3% compared to three sex-linked lethals observed in a similar number of untreated sex chromosomes, representing a mutation rate of 0.2%. Later tests reported much larger mutation rates of sex-linked lethals induced with mustard such as 24% (Auerbach 1947; Auerbach *et al.* 1947a, Auerbach *et al.* 1947b). In addition to the potent tissue penetrability, vesicant action and cytotoxic activities, mustard also appeared to be a mutagen. Dustin (1947) has alluded to those chemical substances such as mustard that affect chromosomes in similar fashion, as do the ionizing radiation of X-rays, as being radiomimetic.

When pollen grains of *Allium cepa* were exposed to the vapors of sulfur mustard, the mitotic and meiotic activities were impaired with chromosome breakage observed similar to that following the ionizing radiation of X-rays (Darlington and Koller, 1947). Moreover, when embryos of *Amblystoma punctatum*, at the tail-bud stage, were exposed to 0.001% bis(2-chloroethyl)methyl amine for two days, all mitotic activity was abolished (Bodenstein, 1946). Thus, evidence appeared to indicate that both X-ray irradiation and radiomimetic poisons

acted on the level of the nucleus, specifically on the chromosomes (Darlington and Koller 1947); Loveless and Revell 1949).

Elmore *et al.* (1948) suggested that the cytotoxic effects of sulfur mustard might be a reflection of the interaction of mustard with nucleic acids through the cross linking of mustard with certain groups of the polynucleotide chains. Bodenstein and Kondritzer (1948) reported that developing embryos of *Amblystoma punctatum*, showed a steady increase in the nucleic acids, RNA and DNA, as the embryos passed through the various developmental stages. With increasing age, they continued to show a definite increase in RNA, while the DNA did not rise but remained at the same level as that earlier observed, if the embryos had been exposed to 0.001% bis(2-chloroethyl)methyl amine for 45 minutes.

Male mice of an inbred wild-type strain were injected i.p. with 0.08 mg bis(2-chloroethyl)methyl amine and although most of the treated males died, one did survive and was mated. Of the 24 offspring, 16 were tested for the presence of recessive gene mutations by outcrossing each, and then backcrossing the offspring or mating them among themselves. Only one mutant was obtained, which was due to a single recessive gene giving normal ratios with 100% penetrance. Phenotypically, they displayed folding ears, absence of hair on the tail or behind the ears, small kinks at the tip of the tail and a short thin coat with hairs pointing toward the midline of the back; both sexes were fertile (Auerbach and Falconer, 1949). The investigators contended that spontaneous visible mutations in mice were quite rare even in inbred strains and that the mutation observed was induced by the action of the nitrogen mustard.

Asexual spores of wild-type 1A of *Neurospora crassa* were exposed to two drops of mustard gas for 30 minutes and mated sexually with wild type E5297a, and single ascopores were isolated for germination on a complete medium (Horowitz *et al.* 1946). In the treated series, 760 spores germinated, of which 29 or 3.8% were mutants, compared with the untreated controls where one doubtful mutant or 0.13% out of 769 germinated spores was obtained. The mutants in the mustard-treated group consisted of 17 visible mutants (morphological and pigmentation) and 12 biochemical mutants, one of which was a new type never encountered previously in that laboratory, termed "albino". Indeed, nutritionally deficient mutant strains of *N. crassa* were induced to revert to the non-deficient state, reverse mutation, by sulfur mustard and monochloro-mustards (Auerbach and Moser 1950; Stevens and Mylroie 1950).

The pioneer work of Avery MacLeod and McCarthy demonstrated that the transforming principle which was responsible for the conversion or transformation of a harmless strain of Pneumococcus into a virulent one, was the nucleic acid, DNA, the genetic material of heredity. Evidence that the cytotoxic activity of mustard was exerted through an action on DNA was shown when the transforming principle from *Pneumococcus* was completely inactivated by a 2-hour exposure to sulfur mustard in concentrations as low as 6×10^{-5}M (Herriott, 1948) and that of *Haemophilus influenza* by six hours exposure in nitrogen mustards such as 10^{-5}M bis(2-chloroethyl)ethyl amine or 10^{-4} bis(2-chloroethyl)methyl amine (Zamenhof *et al.* 1956).

Crathorn and Roberts (1966) have shown that doses of mustard gas, at the mean lethal dose, inhibited the incorporation of thymidine into DNA but had no influence on the incorporation of uridine into RNA. Indeed, at a level of 0.5-1 mole ^{35}S-labeled mustard gas/mole P had no effect on the incorporation of amino acids into the polypeptides directed by poly U in the Nirenberg-Matthaei cell-free system other than alkylation of the terminal 5'-phosphate group of poly U (Abell *et al.* 1965). Thus, the primary action of a difunctional alkylating agent, such as sulfur mustard, was the inactivation of DNA through the formation of cross-linkages between DNA and mustard; thus preventing the separation of the strands of DNA for replication, while RNA synthesis did not appear to be disturbed (Price 1958; Kohn *et al.* 1965; Lawley and Brookes 1965; Lawley 1966, Lawley *et al.* 1969).

Mustard gas induced mutations in specific regions of DNA such as those r-RNA regions coding for the bb (bobbed) locus on RNA forming genes in Drosophila (Fahmy and Fahmy, 1971; Fahmy and Fahmy, 1972). Indeed, linear relationships were observed between the frequency of X-linked recessive lethals in *Drosophila spermatozoa* and molar doses of several different mustards (Fahmy and Fahmy 1960), and for mustard gas induced chromosome breaks and rearrangements in *Drosophila* (Nasrat 1954, Nasrat *et al.* 1954).

Using murine leukemia L5178Y/As$^-$ cell suspensions, mustard gas induced chromosome mutations for asparagine dependence and reversed cells to asparagine independence with doses of 100 mg/kg (Capizzi *et al.* 1973; Capizzi *et al.* 1974).

Mice bearing implants of 10^7 asn requiring L5178Y murine leukemia cells were exposed to 0.1 mg sulfur mustard vapor administered in a closed chamber for six hours a day for five days a week; however, the frequency of spontaneous reversion to asn independence was not statistically significant (Rozmiarek *et al.* 1973). In another experiment, the dominant lethal mutation procedure which measures germ-cell mutations was performed on adult male virgin rats that were exposed to sulfur mustard from one to 52 weeks, using two concentrations, 0.1 mg/m^3 and 0.001 mg/m^3. A significant difference in dominant lethality was observed only at the higher concentration; the dominant lethal mutation rate was cumulative, reaching a maximum at 12 weeks of exposure. It was estimated that a total dose of 0.63 mg sulfur mustard/Kg might have entered the lungs after 12 weeks of exposure at 0.1 mg/kg (Rozmiarek *et al.* 1973).

The mutagenic potential of sulfur mustard was evaluated in the standard plate incorporation version and preincubation modification of the Ames *Salmonella*/microsomal assay with tester strains TA97, TA98, TA100 and TA102, with and without S9 activation using concentrations of 1, 10, 50, 100 and 500 μg/plate. Sulfur mustard induced point mutations in strain TA102 and frameshift mutations in TA97 but no mutagenicity was observed against strains TA98 and TA100; sulfur mustard was approximately four times more potent for the frameshift mutants (TA97) than for the substitution mutant (TA102). The mutagenic response due to sulfur mustard was dose-dependent over the range of 1-50 μg plate and was independent of metabolic activation by Aroclor induced rat liver microsomes (S9). Extensive sulfur mustard induced cell killing was seen with the excision repair deficient strains (TA100, TA98 and TA97) but not with the wild type for excision repair strain TA102 (Stewart *et al.* 1987; Stewart *et al.* 1989).

Recently, an investigation has been concluded that sought to determine the dominant lethal effect in both male and female rats orally ingesting sulfur mustard. Male and female Sprague-Dawley rats 6-7 weeks of age were gavaged with one of several concentrations of sulfur mustard diluted with sesame oil to yield dosages of 0, 0.08, 0.20 or 0.50 mg/Kg, for five days/week for 10 weeks, and the dominant lethal effects determined at the end of the gavaging period. To evaluate the female dominant lethal effect, females treated with mustard were mated to mustard-treated and non-treated males during a 3-week post-treatment mating period, and the fetuses examined 14 days after copulation. Male dominant lethal effects were evaluated by mating of treated male rats with non-treated females. No significant female dominant lethality was observed at any of the sulfur mustard doses. However, significant male dominant lethal effects were seen in mustard-treated male rats that had been mated to untreated female rats two and three weeks following the 10-week exposure interval. Increased incidences of early fetal resorptions, preimplantation losses and decreases in total live embryo implants were observed at the 0.50 mg/Kg dose of mustard and frequently at the lower doses. Although no effects were observed on male reproductive organ weights, or on sperm mortality, an increase in the percentage of abnormal sperm was found in those male rats treated with the 0.50 mg/Kg dose of mustard. The investigators suggested that the dominant lethal effects observed were consistent with an action during the postmeiotic stages of spermatogenesis, involving the sensitive spermatids (Sasser *et al.* 1989b).

A doctoral dissertation by W. Hellmann (1970b) and a summary by Lohs (1975) citing the former investigator's work reported that dominant, sex-linked, lethal mutations were observed in the offspring of 134 former poison gas factory workers. This reproductive effect was seen as an increase in sex ratio, namely, presence of more female births with presumably a very high mortality of male fetuses, in offspring of fathers that had worked in a poison gas factory which produced both sulfur and nitrogen mustards during World War II. Abnormal spermatogenesis and damaged sperm were also found in these former poison gas factory employees. While nitrogen mustard is a very effective mutagen, it is difficult to find sulfur mustard equally culpable under these conditions. There was a lack of information regarding the exposures the men were subjected to while employed in the factory (Chem Stock Dispo Draft Environ Input Statement 1988). Epidemiological studies of germ cell mutations in human populations have been carried out on the children of retired Japanese workers who had been employed formerly at the Okuno-jima poison gas factory (Fujita *et al.* 1983; Neel *et al.* 1985; Yamakido *et al.* 1985a; Fujita, 1987). Examination of the blood proteins of these children by electrophoresis, and determination of enzyme activities using variant proteins as indicators of the genetic effects of sulfur mustard on the germ cells of the parents, did not reveal the presence of any variant proteins, which were a consequence of mutations of parental germ cells; no statistically significant difference in mutation rate from that of the spontaneous mutation rate was observed.

Fisherman trawling off the Danish island of Bornholm in the Baltic Sea have picked up in their nets containers and shells filled with mustard gas which had been dumped into the sea at the end of World War II. These shells are corroded and break up easily to contaminate fishermen handling them. So far, 11 cases of acute intoxication have been observed in which the fishermen have displayed inflammation of the skin, axilla and genitofemoral areas with

blisters on hands and feet and eye irritancy and temporary blindness. Examination of their lymphocytes have revealed a statistically significant increase in sister chromated exchange indicating mutagenitity (Wulf *et al.* 1985).

Considering the experimental evidence for the mutagenic action of the mustards in *Drosophila mulanogaster* (Auerbach and Robson 1947; Auerbach *et al.* 1947a; Auerbach *et al.* 1947b) *Aspergellus nidulans* (Horkenhall, 1948), *Neurospora crassa* (Horowitz *et al.* 1946; Stevens and Mylroie, 1950), dormant barley and wheat seeds (Mac Key, 1954), corn, Zea Mays (Gibson *et al.* 1950), Amblystoma embryos (Bodenstein and Kondritzer, 1948) and on nucleoproteins (Berenblum and Schoental, 1947; Bulter *et al.* 1950), r-RNA regions in Drosophila (Fahmy and Fahmy 1971), murine leukemia cell suspensions and dominant lethal mutations in rats (Rozimarek *et al.* 1973), the inescapable conclusion was that the mutagenic action of mustards was on the hereditary maternal, DNA (Papirmeister 1961; Wheeler, 1962; Lawley, 1966; Hueper, 1971; Fox and Scott, 1980). Consequently, molecules such as the cytotoxic mustards were, essentially, "chemical bullets" of great reactivity that formed addition compounds with DNA to cause chemical alterations, mistakes or mutations (Wheeler, 1962; Hueper 1971; Haddow 1973; Fox and Scott, 1980).

ALKYLATION

The special property which gives sulfur mustard its tremendous chemical reactivity is the presence of two sulfur atoms, each of which has an unsaturated valence. Under certain conditions, a cyclic ethylene sulfonium ion may form as a consequence of the ionization of one of its chlorine atoms. However, the reaction which is favored is the ionization of the chlorine atom with formation of a carbonium ion which then will react with a variety of nucleophilic centers in the cell, such as the guanine moieties of DNA (Loveless and Revell, 1949; Lawley, 1966; Van Duuren *et al.* 1974; Fox and Scott, 1980). This activity is enhanced by the presence of the 2-side chains in sulfur mustard, ($Cl\text{-}CH_2\text{-}CH_2\text{-}$), so that the molecule acquires the capacity to insert alkyl groups into other molecules or form addition products, that is, alkylation capability (Peters, 1947; Loveless, 1951; Auerbach, 1958; Wheeler, 1962; Lawley, 1966; Alexander 1969, Hueper 1971). Evidently, sulfur mustard reactions proceed much more rapidly with nucleophiles, than do nitrogen mustards (Fox and Scott, 1980). Indeed, nucleic acids, such as DNA, contain nucleophilic sites in both strands of their polynucleotides that readily react with chloroalkyl groups to form ester linkages with their guanine residues (Haddow, 1959; Lawley, 1966; Van Duuren *et al.* 1959, 1974) resulting in cross-linkages within the same strand or between the two complementary strands (Elmore *et al.* 1948; Goldacre *et al.* 1949; Lawley 1966; Fox and Scott, 1980).

Among alkylating agents, difunctional ones such as the mustard, bis (2 chloroethyl) sulfide, which has two chloroethyl groups, appears to exert a more potent cytotoxic activity than do monofunctional ones such as the hemi-sulfur mustard, 2-chloroethyl 2-hydroxyethyl sulfide, which has merely one chloroethyl group (Loveless, 1959; Brookes and Lawley 1961; Brookes and Lawley 1963; Lawley and Brookes, 1965). Indeed, monofunctional mustards such as the hemi-sulfur, n-butyl-2-chloroethyl sulfide and several monochloro-nitrogen mustards are effective

in producing lethal mutations in *Drosophila melanogaster* (Auerbach and Moser, 1950) and many biochemical mutations and reversions in *Neurospora crassa* (Jensen *et al.* 1950; Stevens and Mylroie, 1950; Steven and Mylroie, 1952; Stevens and Mylroie 1953).

In 1959, Loveless reported that inactivation of bacteriophage T2 required the presence of two alkylating groups. Brookes and Lawley (1961) found that alkylation of nucleic acids occurred at the N-7 position of guanine and that hydrolysis of the alkylated products led to the formation of 7-alkylguanines by monofunctional agents and both 7-alkylguanines and diguanin-7-yl derivatives by the action of difunctional agents. These investigations, therefore, suggested that difunctional alkylating agents such as mustard gas formed cross-linkages between the two strands of DNA to prevent strand separation and subsequent replication of DNA without any effect on RNA or protein synthesis or growth (Brookes and Lawley 1961; Papirmeister, 1961; Brookes and Lawley 1963; Lawley *et al.* 1969).

Although both resistant and sensitive strains of *E. coli* do not show any difference in the extent of their initial alkylation by mustard gas, resistant strains continue to grow after alkylation when incubated in growth media, since they are able to excise the cross linkages from their DNA (Papirmeister and Davison, 1965; Lawley and Brookes, 1965; Kohn *et al.* 1965; Venitt, 1968; Papirmeister *et al.* 1969). The resistant strains showed a preferential excision of the diguaninyl alkylation products from their DNA, but only partial excision of the monofunctionally-alkylated guanine derivatives (Lawley and Brookes 1965; Roberts *et al.* 1971). This difference between the resistant and sensitive strains in bacteria demonstrated that the significant lesion in DNA which prevents its replication is the difunctional alkylation. Thus, the cytotoxic action of mustard gas was due to the formation of interstrand cross-linkages in DNA and the excision of those cross-linked portions of the DNA strands restored the ability of DNA to act as a template for the synthesis of more DNA (Wheeler, 1962; Papirmeister and Davison 1965; Hueper 1971; Haddow 1973; Fox and Scott 1980). Thus, lesions caused by an alkylating agent such as sulfur mustard could be prevented, mitigated or reversed by a variety of excision and repair mechanisms; this ability may account for the differential response of certain tissues to carcinogens. Such mechanisms appear to exist in *Escherichia coli* (Kohn *et al.* 1965; Lawley and Brookes, 1965; Venitt, 1968), yeast cells (Kircher *et al.* 1979), He La cells (Crathorn and Roberts, 1966; Roberts *et al.* 1968; Reid and Walker, 1969; Roberts *et al.* 1971), mouse lymphoma cells (Crathorn and Roberts, 1966) and Yoshida lymphosarcoma cells (Scott *et al.* 1975; Scott, 1977). Moreover, the sensitivity of cells to the action of alkylating agents such as mustard gas varies at different stages of the cell cycle so that mouse fibroblasts show the greatest resistance to mustard gas in the G_2 stage (Walker and Helleiner, 1963), while Hamster cells exhibit resistance in the S period (Mauro and Elkind, 1968). Perhaps, the sensitization, protection and repair mechanisms involve enzymatically mediated elimination from the cell of some of the entering mustard (Kohn *et al.* 1965; Lawley and Brookes, 1965) through reaction with free thiol groups to reduce the extent of alkylation of DNA (Bacq and Alexander, 1964; Connors, 1966; Alexander, 1969).

CARCINOGENICITY OF MUSTARD

Animals

Many chemical substances are mutagenic, however, they are not necessarily carcinogenic (Wheeler, 1962; Haddow, 1973; Fox and Scott, 1980). Now, although it has been demonstrated that the nitrogen mustards were among the most potent mutagens (Auerbach and Robson, 1946; Horowitz *et al.* 1946; Hockenhill, 1948; Stevens and Mylroie, 1950), the question of whether sulfur mustard is a potential non-human carcinogen has not been settled (Walpole, 1958; Haddow, 1959).

Using an inbred strain of mice, strain A, which had a known incidence of spontaneous development of pulmonary tumors, Heston (1950) injected i.v. 0.25 ml of a 1:10 dilution of a saturated solution of mustard gas (0.06-0.07%) for a total of four injections at two-day intervals. Ten months later, 93% of the treated mice had developed pulmonary tumors, averaging 2.6 tumors per mouse, while 68% of the untreated mice had developed similar tumors with an average of 0.93 tumors per mouse (Heston, 1950). In another, experiment, strain A mice were injected i.v. with 0.1 mg of bis (2-chloroethyl) methyl amine for a total of four injections at two-day intervals. Indeed, ten months later 100% of the treated mice had developed multiple tumors, averaging 9.6 tumors per mouse, while 62.5% of the untreated mice had developed tumors with an average of 0.81 tumors per mouse, which was the usual incidence for that strain of mouse (Heston, 1950). This experiment confirmed the findings of an earlier preliminary investigation using bis (2-chloroethyl) methyl amine (Heston, 1949) and led (Heston, 1950) to conclude that both nitrogen and sulfur mustards showed a positive correlation between their mutagenic and carcinogenic activities.

In another investigation, strain A mice housed in cages, were placed in large desiccators which contained a piece of filter paper on which had been placed 0.01 ml of mustard gas. Vaporization occurred with the help of a small electric fan which also circulated the mustard gas vapors so that the mice received a 15 minute exposure to mustard gas daily. Ten months later, 49% of the treated mice had developed pulmonary tumors, averaging 0.66 tumors per mouse, while 27% of the untreated mice had developed tumors with an average of 0.31 tumors per mouse (Heston, 1953a). The difference in tumor incidence in treated and non-treated mice as well as the number of tumors per mouse were statistically significant with no dimorphic differences being observed. Moreover, Heston (1953b) was also able to induce tumor development in several different strains of mice besides strain A, such as C3H and C3Hf, using either sulfur or nitrogen mustards which were injected subcutaneously. Tumors developed, especially, at the site of the injection, but in other regions as well, which were remote from the injection site and included pulmonary and mammary tumors, hepatomas, and sarcomas 15 months after the last injection (Heston 1953b).

Two groups of mice, 20 mice per group, received weekly s.c. injections of either 1.0 mg bis (2-chloroethyl) methyl amine or 1.0 mg tris (2-chloroethyl) amine /kg bw for 50 and 10 weeks respectively; 14 mice survived for more than 250 days and of these 10 had tumors which

included eight lung tumors, two lymphosarcomas, a uterine fibroma, and at the site of injection a spindle-cell sarcoma (Boyland and Horning, 1949). Swiss mice and albino rats injected i.v., s.c., or i.p. for 40 weeks with either bis (2-chloroethyl) methyl amine or tris (2-chloroethyl) amine and sacrificed one year later revealed a high incidence of tumors (Griffin *et al.* 1951). Tumors were first observed 6-7 months after the injections began, and s.c. injections appeared to be the most effective route of administration, if one dose of the agent was given; however, all routes were effective if the agents were administered in multiple weekly injections (Griffin *et al.* 1951). No other details were given in the abstract. Haddow (1959) reported that aromatic nitrogen mustards such as, N-phenyl nitrogen mustard was also carcinogenic in the mouse, rat and hamster when the agent was administered not only s.c. but also orally. Mustard gas injected s.c. was also carcinogenic in the rat (Haddow, 1959). Moreover, a variety of alphatic and aromatic derivatives of nitrogen mustards such as uracil mustard, L-phenylamine mustard, and chlorambucil enhanced the development of pulmonary tumors in strain A mice (Shimkin, 1954; Shimkin *et al.* 1966).

Rats, mice, guinea pigs, rabbits and dogs were maintained in closed chambers and exposed to mustard gas vapors at a concentration of either one or 100 $\mu g/m^3$, for six hours/day and five days/week, at Edgewood Arsenal. The duration of exposure to the agent varied from one to 52 weeks depending on the different species, but was insufficient to derive any significant data for the guinea pigs, rabbits and dogs. Chronic exposure to the higher concentration of mustard gas vapor increased the frequency of skin tumors significantly in rats, but not in mice; the majority of tumors being squamous cell and basal cell carcinomas (McNamara *et al.* 1975).

In an earlier investigation, Fell and Allsop (1948a) reported that addition of low concentrations of mustard gas, 0.05 mg/ml, to tissue cultures containing small pieces of choroid and sclera obtained from 12 day chick embryos resulted in the appearance of multinucleate, hypertrophic and other abnormal cells, resembling cells usually associated with malignant tumors. Consequently, these investigators applied small doses of mustard gas, 2.5 $\mu g/ml$ to 12.5 $\mu g/ml$ to the skin of mice by means of a pipet, five times per week, for varying intervals, up to 278 days but with no evidence of any tumor formation (Fell and Allsop, 1948b). Although abnormal mitoses, multinucleate cells and cystic hair follicles were observed in the epidermis, the dermis contained newly formed collagen fibers and the treated areas of the skin appeared quite normal (Fell and Allsop, 1948b).

Recently, a treatment-related lesion associated with sulfur mustard gavage has been reported (Sasser *et al.* 1989b; Sasser *et al.* 1989c). Seventy-two Sprague-Dawley rats of each sex, six to seven weeks of age were distributed into six groups of 12 animals/group/sex and were gavaged with either 0, 0.0033, 0.011, 0.033, 0.3 mg/kg of sulfur mustard in sesame oil five days/week for 13 weeks. A significant reduction in body weight in both sexes was observed only in the group receiving the highest dose and only two out of 144 animals died during the 90 day study. Hematological and chemical determinations revealed no consistent alterations as a consequence of any of the doses used. The primary toxic effect observed was epithelial hyperplasia of the forestomach in both sexes receiving the 0.3 mg/kg dose and in males receiving the 0.1 mg/kg dose. These lesions were minimal and were characterized by increased

mitotic activity of the basilar epithelial cells with cellular disorganization and thickening of the epithelial layer. There was no evidence that the forestomach lesions observed were precancerous.

Man

A comparative examination of the mortality records of 1267 British war pensioners, gassed with mustard gas in 1917-1918 during World War I, was made for the interval, January 1, 1930 to December 1, 1952 (Case and Lea 1955). The study included two control groups consisting of men who had never been exposed to mustard gas and comprised a group of 1421 war pensioners afflicted with chronic bronchitis and a group of 1114 war pensioners who were amputees. There were 29 deaths from lung and pleural cancer in the mustard gas exposure group, which was double the death rate expected for a normal population, and the same number of deaths from cancer in the chronic bronchitis group; for both groups, the incidence was 29 observed to 14 expected. However, there were only 13 deaths from lung cancer in the amputee control group; the incidence being 13 observed to 16 expected. It is particularly important to note that almost all of the pensioners in the mustard gas exposed group suffered from chronic bronchitis with a mortality estimate for that disease of 217 observed to 21 expected. The conclusion reached by the investigators was that the mustard gas had not acted as a direct carcinogen, but rather had increased the risk of lung cancer, indirectly, by way of a variety of pulmonary disorders, most particularly, chronic bronchitis.

In another study (Beebe 1960), the mortality records of World War I American veterans were examined who had been hospitalized after mustard gassing in 1918. All of the veterans had been between 24-30 years of age in 1918, and comprised three groups: 2718 men hospitalized after mustard gas exposure because of evidence of mustard gas injury to the respiratory tract, eyes, and skin; 1855 men hospitalized for pneumonia during the influenza epidemic of 1918 who had no prior evidence of exposure to mustard gas; and 2578 men hospitalized because of wounds to the extremities who had no prior contact with mustard gas. The interval between 1919 to 1955 was divided into calendar periods of approximately 10 years, and revealed a difference in mortality only during the 1930-1939 decade, when the mustard gas exposed group had the highest mortality, predominantly, from pneumonia and tuberculosis. The number of deaths from lung cancer were 36 (1.3%), 14 (0.8%), and 126 (11%) in the mustard gas, pneumonia, and leg-wounded groups, respectively. However, chronic bronchitis was also prevalent: the incidence being 65% in the mustard gas group, 35% in the pneumonia group, and 20% in the leg-wounded group. When the incidence in mortality from lung, trachea, and bronchii were compared with the expected values based on U.S. mortality rates, the following ratio of observed to expected were obtained: 39/26.6 (1.47); 15/18 (0.81); and 30/26.2 (1.15) in the mustard gas, pneumonia and leg-wounded groups, respectively. The mustard gas group appeared to differ, but not always significantly from the other groups in having higher mortalities from tuberculosis and pneumonia. Beebe's own statistical analyses of his data indicated, and he himself stated, that the causal relation between mustard gas exposure and lung cancer in these circumstances was weak or equivocal. An additional examination of the same records perused by Beebe in 1960, but extended for an additional 10 years, from 1956-1966,

included 2718 men who had been gassed with mustard gas; however, the original conclusions were not altered (Norman 1975).

Of 511 people who had worked in a British factory producing mustard gas during 1939-1945 (World War II), 84% were traced up to the year 1974 and the mortality rate for cancer determined for the 29-year interval. Although the number of deaths for all neoplastic diseases amounted to 37 men and eight women for a total of 45, these findings were only slightly higher than the expected mortality from the National Death Rate and not significant statistically. However, seven deaths from carcinoma of the larynx was much more than the expected 0.75, and so the conclusion was that workers exposed to mustard gas over a long period of time have an increased risk of developing cancer of the larynx (Manning *et al.* 1981). Although mortalities from lung cancer, pancreatic cancer, pneumonia, and accidents were higher among those who had formerly been exposed to mustard gas, the findings were not statistically significant. Manning *et al.* (1981) reflected on the small scale of their study, the deficiencies in identifying the data recorded, the incompleteness of the records available, the inability to trace some of the former workers, and the lack of information about the personal habits of these people in regard to use of alcohol and smoking. Nevertheless, they concluded that the findings of their study provided evidence that exposure to mustard gas led to a significant risk of laryngeal cancer (Manning *et al.* 1981).

The first case of occupationally induced cancer due to mustard gas which provoked an extensive and still ongoing series of investigations of former workers at the poison gas factory was reported in 1952, when a 30-year old man died of bronchial cancer. He had been engaged in the production of mustard gas for 16 months during 1941 (Yamada *et al.* 1953; Wada and Miranishi 1954).

The poison gas factory was located in Okuno-jima, a small island in the Inland Sea of Japan and produced several poisonous gasses such as mustard gas, Lewisite, diphenylcyanarsine, hydrocyanic acid, chloracetophenone, and phosgene from 1929 to 1945, with intensive production between 1937 to 1944. The workers there were subjected to a working environment in which there were few health or safety precautions (Wada *et al.* 1963; Wada *et al.* 1968). The quantity of mustard gas produced on a monthly basis was almost three times the total quantity of all the other gases combined (Inada *et al.* 1978), resulting in a concentration of the gas in the work environment ranging from 0.05 to 0.07 mg/L (Nakamura 1956). The protective clothing available to some of the workers did not prevent the gas from penetrating the clothing and caused acute symptoms of mustard gas exposure such as dermatitis, blistering and skin lesions on the trunk and upper portions of the lower extremities, in addition to conjunctivitis, rhinitis, and bronchitis (Inada *et al.* 1978). More than half of the former workers previously engaged in the production of poison gas, particularly mustard gas, suffered from chronic bronchitis (Shigenobu 1980) and also irreversible airway obstruction (Nishimoto *et al.* 1970).

Additional cases of carcinoma of the larynx and three of bronchial carcinoma were reported in workers 17 to 24 years after occupational exposure to mustard gas; the length of exposure had been from five to 13 years. Thus, a total of 32 deaths from cancer, 12 from respiratory cancer,

during the period from 1946 to 1957 had been recorded (Yamada *et al.* 1957; Yamada 1959). Another report found that 27.9% (48) of the deaths of former workers (172) at this poison gas factory were due to cancer and that 16.3% (28) had occurred in the respiratory system, particularly, in the upper portion of the respiratory tract where the inhaled mustard gas exposure was intense (Yamada 1963). According to Miyaji (1962), the primary sites of growth of pulmonary neoplasia were found in the hilar region and middle zone of the lung rather than in the peripheral regions and their growths were predominantly adenocarcinomas. An additional study of deaths that had occurred between 1952 to 1967 in men who had worked at the poison gas factory during 1929 to 1945, extended the number of deaths from neoplasms of the respiratory tract to 33 (Wada *et al.* 1968). Thus, there have been 33 deaths of former mustard gas workers since 1952, compared to 0.9 such deaths expected for males from 1952 to 1967,on the basis of the Japanese national mortality rates for males of the same age distribution as the gas workers (Wada *et al.* 1968). The association between mustard gas workers and the eventual development of respiratory neoplasms was significant.

Of 104 male former poison gas factory workers who had developed malignant tumors of the respiratory system in 1952, 93 had died by 1979 (Shigenobu 1980). The means, respectively, for age of death, work period, and latent period for development of cancer of the lungs in these workers was 61 years, 5.6 years, and 32 years, and for development of cancer of the upper respiratory tract 57 years, 7.4. years, and 25 years (Shigenobu 1980). There appeared to be no significant difference in the rate of cancer of the respiratory system between those workers who smoked or did not smoke (Shigenobu 1980). Observations of respiratory neoplasms in former poison gas workers from 1952 through 1981 revealed an increase in mortality of 102 cases up to that time: 79 cases of cancer of the lungs, 17 cases of cancer of the larynx, and six cases of cancer of the pharynx (Nishimoto *et al.* 1983; Yamakido *et al.* 1985b). Tokuoka *et al.* (1986) carried out autopsies on former poison gas factory workers, 52 of whom had died of respiratory tract cancer including 37 who had died of lung cancer. Histological examination of 19 lung cancer cases of former mustard gas workers confirmed the earlier findings of carcinogenicity (Yamada 1963; Wada *et al.* 1968) and indicated an increase in frequency of moderate to severe bronchial lesions in former poison gas workers who had been diagnosed as not having lung cancer (Tokuoka *et al.* 1986). The majority of these former mustard gas workers were heavy cigarette smokers and a correlation was found between smoking, mustard gas exposure and pre- and early cancerous changes in the lungs (Tokuoka *et al.* 1986).

Gastric cancer in former workers of the Okuno-jima poison gas factory has also been reported as a consequence of mustard gas involvement (Shimura *et al.* 1978) as well as a case of early gastric cancer with wide spread metastases of the lymph nodes in a man who had chronic bronchitis and had worked with mustard gas for six years (Hirano *et al.* 1984).

Lesions of the respiratory tract and skin were observed in former workers of the Okuno-jima poison gas factory as delayed effects of mustard gas exposure (Yamada 1959; Yamada 1963; Wada *et al.* 1963; Inada *et al.* 1977; Inada *et al.* 1978). Inada and colleagues (1977, 1978) reported the presence of numerous pigmented and depigmented spots on the trunk and upper extremities and hyperkeratotic papular eruptions on the skin of five patients. All of

these patients were former workers at the poison gas factory on Okuno-jima and had been engaged in the production of mustard gas. Examination of the skin lesions revealed the presence of Bowen's disease or intraepidermal squamous cell carcinoma which suggested that multiple Bowen's disease had been caused by exposure to mustard gas (Inada *et al.* 1977; Inada *et al.* 1978). The average length of employment in the factory on mustard gas production was 9.3 years and the interval from first contact with mustard gas to diagnosis of Bowen's disease was 39.2 years.

A close association of mortality from cancer and exposure to mustard gas in former German chemical warfare workers has been reported by Weiss (1959) and U. Hellmann (1970) with an average exposure of 4.6 years (Hellmann 1970) and an average interval from first contact with the agent and death from cancer of 18.5 years (Hellmann 1970).

The production, testing, and destruction of mustard gas and nitrogen mustard were performed from 1935 to 1945 in a German factory employing 878 workers of whom only about half were directly engaged in handling the two chemical agents. During the interval between 1951 to 1972, 85 deaths occurred in the group of workers who had been exposed to nitrogen and sulfur mustard; work records available for these people suggested that 32 of these deaths were due to cancer. When the incidence on mortality rates were compared with the mortality rates for Lower Saxony, the rates obtained exceeded the expected values, however, only the data on the mortality for bronchial carcinoma were significant: 11 obtained to five expected. The findings, however, were confounded by the possibility of exposure to other dangerous chemicals such as phosgene, chloropicrine, bromoacetone, and several organic arsenicals produced at the factory (Weiss and Weiss 1975).

Between 1945 and 1951, during the disbandment of a poison gas plant, Heerresmunitionsanstalt St. Georgen, in Germany, which had produced both sulfur and nitrogen mustards, about 400 people became contaminated through inhalation and handling of these agents due to inadequate precautions. Many of those people are only now, many years later, displaying multiple skin tumors such as basal cell carcinoma, Bowen's disease, Bowen's carcinoma and skin spinocellulare, even in unexposed portions of the skin (Klehr 1984).

The disparities in findings between the carcinogenicity of mustard gas in World War I, and that observed in former workers in poison gas factories who had been exposed to mustard gas repeatedly over many years in the working place, would most certainly result in different effects from those contaminated by an acute poisoning incident on a battlefield (Norman 1975; Manning *et al.* 1981).

A recent report provides strong evidence for a dependent relationship between mustard induced cancer and former poison gas workers for both men and women, who had been engaged in the production of mustard gas in Cheshire England during World War II. The work records of these former employees were examined and their mortality records followed up to the end of 1984; these individuals comprised a group of 3354 people. Comparison of national death rates from cancer of the upper respiratory tract showed very significant excesses in cancer of the

larynx (11 observed vs 4.04 expected); cancer of the pharynx (15 observed vs 2.73 expected); and cancer of additional buccal and upper respiratory sites combined: i.e., lip, tongue, salivary glands, mouth and nose (12 observed vs 4.29 expected). A more moderate excess mortality but still very significant, was observed for lung cancer (20 observed vs 138.39 expected). Statistically, significant excesses in mortality from acute non-malignant respiratory diseases were found: pneumonia and influenza (131 observed vs 9.87 expected) and chronic non-malignant respiratory disease and chronic bronchitis, (185 observed vs 116.31 expected) were also found. Significant excesses in mortality for cancer of the esophagus (20 observed vs 10.72 expected) and stomach (70 observed vs 49.57 expected) were also obtained but displayed no consistency with regard to first time of exposure or duration of exposure and may have been confounded by chance, or other factors or perhaps a combination of the preceding. These results, therefore, give strong evidence that mustard gas exposure causes cancer of the upper respiratory tract predominantly, and also cancer of the lungs and non-malignant respiratory disease; and that the duration of employment is very important (Easton *et al.* 1988).

Indeed, epidemiology, animal experimentation, and evidence obtained from accidental exposure to mustard gas supports the conclusion that mustard gas is a respiratory tract carcinogen in humans (IARC 1975; IARC 1982; Cowles 1983).

Therapeutic Uses

In 1929, Berenblum reported that the induction of warts in mice through the repeated application of tar to their skin could be prevented by the addition of 0.1% mustard gas to the tar; mustard gas apparently prevented the skin of the mouse from responding to the carcinogenicity of the tar. This antineoplastic effect observed by Berenblum (1929) was the rationale for utilizing sulfur mustards and nitrogen mustards in the treatment of a variety of different tumors and neoplastic diseases (Berenblum 1931; Karnofsky 1948a; Karnofsky 1948b; Karnofsky 1948c; Burchenal and Riley 1949; Burchenal *et al.* 1951). Mustard gas has also been used in the treatment of Psoriasis in the form of an ointment which was applied once or twice daily from two weeks to seven months (Illig, 1977). Mustard gas, in the treatment of this disease, was used not only as an ointment but also by inhalation and was considered by the investigator as a comparatively weak carcinogen in humans (Illig, 1977) in contrast to what other investigators have concluded. When the bodies of patients were covered with radioactively-labeled ^{35}S-mustard vaseline ointment (50g) from one to two days, about 1-7% of the radioactivity was eliminated within one week, 0.5%/20 liters of radioactivity was found in the breath while the epidermis contained very little radioactivity; the ambient air contained 1.5-13.7 μCi/20 liters of air (Illig, 1977).

In general, lymphomas and neoplasms of the hematopoietic tissues appeared to be the most susceptible to mustard therapy (Karnofsky 1948a; Karnofsky 1948b; Karnofsky 1948c; Burchenal *et al.* 1951). Both nitrogen and sulfur mustards were once used extensively as therapeutic agents when radiation was ineffective, particularly in leukemia (Karnofsky 1948a; Karnofsky 1948b; Karnofsky 1948c; Burchenal *et al.* 1948; Burchenal and Riley, 1949; Landing and Eisenberg, 1949). Indeed, these activities, particularly the inhibition of tumor growth, all pointed to the

possibility that the mustards might be carcinogenic (Goldacre *et al.* 1949; Walpole 1958; Haddow 1973).

TERATOGENICITY

During organogenesis, a single subcutaneous injection of 0.5 mg/kg or 1.0 mg/kg bis (2-chloroethyl) methyl amine in Sprague Dawley rats, given sometime between 12 and 15 days of gestation, resulted in abnormal development of fetuses. Reduction in fetal size and body weight, receding lower jaws, cleft palate, deformed limbs, absence and fusion of digits, cranial defects and short tails were observed, without any deleterious effect on the mother (Haskin 1948). In the mouse, a single intra-abdominal injection of one μg bis (2-chloroethyl) methyl amine/g b wt, on any day between day 10 to 12 of gestation, resulted in a wide range of developmental anomalies and many dead embryos (Danforth and Center 1954). Embryos are relatively resistant to the teratogenic influence of nitrogen mustards up to day seven or eight of gestation, since the critical period of gestation during which fetal development may be interrupted appears to be day 10 to 16 of gestation (Murphy and Karnofsky 1956). Thus, a single dose of 0.3 to 0.5 mg bis (2-chloroethyl) methyl amine/kg injected sc in pregnant Wistar rats between days nine to 16 of gestation altered normal fetal development (Murphy and Karnofsky 1956). Indeed, a single i.p. injection in pregnant Wistar rats on day 12 of gestation of any of several different polyfunctional alkylating agents, such as bis (2-chloroethyl) methyl amine, triethylene meramine (TEM), and triethylenethiophosphoramide (ThioTEPA), chlorambucil, and Myleran caused fetal resorption with teratogenic effects in surviving fetuses (Murphy *et al.* 1958). These same agents injected into the yolk sac of 4-day old chick embryos caused stunting, reduction in growth, retardation in development of the extremities, missing toes, and shortening of the lower beak (Murphy *et al.* 1958).

Conclusions regarding the teratogenicity of mustard gas needs to be severely reassessed, particularly, in light of the many perplexing questions that may distort the presence of conclusive evidence: the use of many different routes of administration, the very high concentrations utilized and absence of information regarding maternal toxicity (i.e. body weight loss) incurred by the suspect teratogen. In a recent publication (Goldman and Dacre 1989), we have questioned the use of parenteral routes for inducing teratogenicity and the absence of information of maternal toxicity in such studies. Maternal toxicity should be considered as an important diagnostic tool in the assessment of a suspected chemical as a possible teratogen. Indeed, the associated findings of maternal toxicity with fetal toxicity should render a decision of teratogenicity, as not proven.

Exposure of pregnant mice to sulfur mustard vapors administered in a closed chamber, for six hours a day for five days a week, at a concentration of 0.1 mg/m^3 was not effective in inducing fetal toxicity or teratogenicity (Rozmiarek *et al.* 1973). Male rats exposed to either 0.001 mg/m^3 or 0.1 mg/m^3 mustard gas vapor in closed chambers for different lengths of time varying from one to 52 weeks, when mated to female controls displayed no reduction in fertility and there was no evidence of any developmental abnormalities among the fetuses at the end of gestation (McNamara *et al.* 1975).

A recently completed teratology study by Battelle Pacific Northwest Laboratory reported that neither rats that had received 0.5-2.0 mg/Kg sulfur mustard between days six and 15 of gestation or rabbits that had received 0.4-0.8 mg/Kg mustard gas between days six and 19 of gestation, by gastric intubation and sacrificed on days 20 and 30 respectively, displayed any evidence of fetal abnormalities when maternal toxicity was not present. Since fetal defects were observed only at dose levels that caused maternal toxicity, the investigators suggested that mustard gas was not teratogenic in rats or rabbits (Rommereim and Hackett, 1986; Hackett *et al.* 1987).

SUMMARY

Chemical attacks, in which mustard may have been used on Iranian soldiers and civilians during the present Gulf War in 1984 and 1985 and an Iraqi chemical attack on the Iranian occupied village of Halbja in 1988 resulting in many civilian casualties have been reported. Heavy use of chemical warfare in Afghanistan by the Soviet military is a recent innovation in military tactics which has been highly successful and may ensure further use in future military conflicts and terrorist attacks as a profitable adjunct to conventional military arms.

Mustard is a poisonous chemical agent which exerts a local action on the eyes, skin and respiratory tissue with subsequent systemic action on the nervous, cardiac, and digestive systems in man and laboratory animals causing lacrimation, malaise, anorexia, salivation, respiratory distress, vomiting, hyperexcitability and cardiac distress.

Under extreme circumstances, dependent upon the dose and length and of exposure to the agent, necrosis of the skin and mucous membranes of the respiratory system, bronchitis, bronchopneumonia, intestinal lesions, hemoconcentration, leucopenia, convulsions with systemic distress and death occur. Severe mustard poisoning in humans is associated with systemic injury which is manifested as headache, epigastric distresses, anorexia, diarrhea and cachexia and is usually observed at mustard doses of 1000 mg/min/m^3 with damage to hematopoietic tissues and progressive leucopenia.

Sulfur mustard is a cell poison which causes disruption and impairment of a variety of cellular activities which are dependent upon a very specific integral relationship. These cytotoxic effects are manifested in widespread metabolic disturbances whose variable characteristics are observed in enzymatic deficiencies, vesicant action, abnormal mitotic activity and cell division, bone marrow disruption, disturbances in hematopoietic activity and systemic poisoning. Indeed, mustard gas readily combines with various components of the cell such as amino acids, amines and proteins.

While evidence of an association between lung cancer and mustard gas encountered on the battlefields of World War I is at best suggestive if not problematical (Case and Lea 1955; Beebe 1960; Norman 1975), the epidemiological data accumulated from the poison factories of Japan (Yamada *et al.* 1953; Wada *et al.* 1968; Inada *et al.* 1978; Shigenobu 1980; Nishimoto *et al.* 1983; Hirano *et al.* 1984; Tokuoka *et al.* 1986); Germany (Weirs 1958; U. Hellmann 1970;

Weiss and Weiss 1975; Klehr 1984) and England (Manning 1981; Easton *et al.* 1988) are substantial (IARC 1975). Unfortunately, attempts to seek confirmatory and substantial evidence in laboratory animals such as mice (Boyland and Horning 1949; Heston 1950; Heston 1953; McNamara *et al.* 1975) and rats (Griffin *et al.* 1951; McNamara *et al.* 1975; Sasser *et al.* 1988a; Sasser *et al.* 1988b) have not been consistent.

Sulfur mustard has been shown to be mutagenic in a variety of different species using many different laboratory techniques from fruit flies, microorganisms and mammalian cell cultures (Fox and Scott 1980) and evidence is slowly accumulating from human data (Hellmann 1970; Lohs 1975; Wulf 1985).

Evidence for the teratogenicity of mustard has been negative in assessments of fetotoxicity and adverse effects of mustard on the reproductive potential of both human and animal studies. Indeed investigations of women adversely affected by mustard are minimal since most of the studies have been performed on former men employees of poison gas factories and have been negative or questionable. We have recently emphasized the need to assess the affect of a suspected teratogen on maternal toxicity in laboratory animals before any conclusions can be made. Indeed, maternal toxicity should be considered as an important diagnostic tool in assessing whether a chemical is teratogenic. The significance of parenteral routes for inducing teratogenicity is also a problematic one (Goldman and Dacre 1989).

The special properties which give sulfur mustard its tremendous chemical reactivity are due to the presence of a sulfur atom with its unsaturated valence which results in the formation of a cyclic ethylene sulfonium ion. However, the reaction which is favored is the ionization of the chlorine atom with the formation of a carbonium ion which then reacts with a nucleophilic site such as the guanine moieties in DNA. This activity is enhanced by the presence of the 2-side chains in sulfur mustard ($Cl\text{-}CH_2\text{-}CH_2\text{-}$), so that the molecules acquire the capacity to insert groups into other molecules or form addition compounds, i.e. sulfur mustard is a strong alkylating agent. Evidently, sulfur mustard reactions proceed much more rapidly with nucleophiles than do nitrogen mustards.

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