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William D. Maixner
University of Nebraska Medical Center

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THE BIOLOGICAL EFFECTS OF THE NITROGEN MUSTARDS ON VARIOUS BODY TISSUES

WILLIAM D. MAIXNER

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UNIVERSITY OF NEBRASKA
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INTRODUCTION

A. The Problem of Neoplastic Diseases

Neoplastic diseases have been recognized as a major health problem throughout the world today. In the United States alone there were over 170,000 deaths per year from this condition. Neoplastic diseases today rank as the second most important cause of death, after heart disease. Fortunately, the outlook for the control of these is more promising today than it has been in the past. The medical profession is better equipped to fight them with improved techniques in surgery and in the use of X-rays and radium, and both physicians and the public are better informed on the need for early diagnosis and treatment. Medical groups and the various workers in the field of sciences are taking a lead in the fight against neoplastic diseases (1).

In spite of all the research and knowledge relative to the therapeutic steps used in the fight against these diseases, none has been satisfactory at their best. As long as the neoplastic disease remains localized it is amenable to surgical or irradiation treatment or both in many instances. However, when

metastasis has occurred before treatment of the disease, then the prognosis becomes decidedly unsatisfactory. For this reason a continuous search is being carried forward in hopes that some day a method of controlling these diseases will be found. Because the nitrogen mustards have an important tissue specificity when administered intravenously, workers in this field of therapeutics are hopeful that some form of the mustards will answer their search for the ideal drug and will be a part of the solution in neoplastic diseases.

B. History Relative to the Nitrogen Mustards.

Since World War I the vesicant action of Nitrogen Mustards has been known. At that time it was believed due to the formation of HCl intracellularly. Such reasoning has been proven erroneous as later investigation indicates that vesication is due to the poisoning of cellular enzymes. In the experimentation during and following World War I scattered reports on the effects of nitrogen mustards on hemopoetic tissue, the gastro-intestinal tract, the electrolyte and fluid balance were also known (2,3,4). In the United States, soon after World War I, several investigators presented their observations on the action of the "mustards" on neoplastic diseases in animals (3,6,7). The medical profession took no advantage of the results of this research until its value was suggested by the Allied catastrophe at Bari during the invasion of Italy in 1943. At this time a more thorough study on the actions of the "mustards" and their potentialities was made (8, 9).

In addition, studies on nitrogen mustards and sulfur mustards were renewed by the advent of World War II by the Allied Powers. But, because much of the

work was classified as "confidential", the presenting of experimental findings in open literature was not possible. It should be stated that the effects of nitrogen mustards on leucopoetic tissue and on growth of tumors received some attention in the interim of the two wars but as a whole, biological research remained relatively quiet until World War II (2,5,9).

The investigation of nitrogen mustards in the treatment of neoplastic diseases has been limited mainly to tris (Beta Chloroethyl) amine hydrochloride and methyl-bis (Beta Chloroethyl) amine hydrochloride. It was begun at the Office of Scientific Research and Development and the Yale University. Authority to carry out the work was subsequently given by the Committee on Atypical Growth of the National Research Council to the University of Chicago Medical School, the University of Utah Medical School, and the Memorial Hospital, New York. Since then other institutions have participated in the experimentation (8,10).

With the knowledge that nitrogen mustards were contact vesicants, that they exerted a cytotoxic action on various tissues after absorption, that cellular susceptibility to these compounds was related to

the degree of proliferative activity, late studies were made on the action of the nitrogen mustards in relation to the fundamental cell processes (2,10,11).

II. The Chemistry and Chemical Reactions Responsible for Action of Nitrogen Mustards

At the beginning of World War II, the warring powers developed an interest in a group of analogues of mustard gas, in which the sulfur ($S-(C_2H_4Cl)_2$) was replaced by nitrogen ($R-N-(C_2H_4Cl)_2$). These were given the name of "nitrogen mustards". These compounds were potentially more versatile than mustard gas, in that a greater variety of radicles (R in the above formula) could be attached to the nitrogen to alter the physical properties of the nitrogen mustard, without changing the structure of the bis-(Beta Chloroethyl) amine moiety. A large number of nitrogen mustards were prepared, and it was shown that these compounds were very similar to mustard gas in their action. The study of systemic effects of the mustard compounds, however, was intensified by the fact that nitrogen mustards were more readily absorbed especially from the skin of experimental animals to produce fatal systemic intoxication. Mustard gas was not considered for clinical purposes because it is volatile, quite insoluble, unstable in water, dangerous to handle and difficult to administer. On the other hand, the

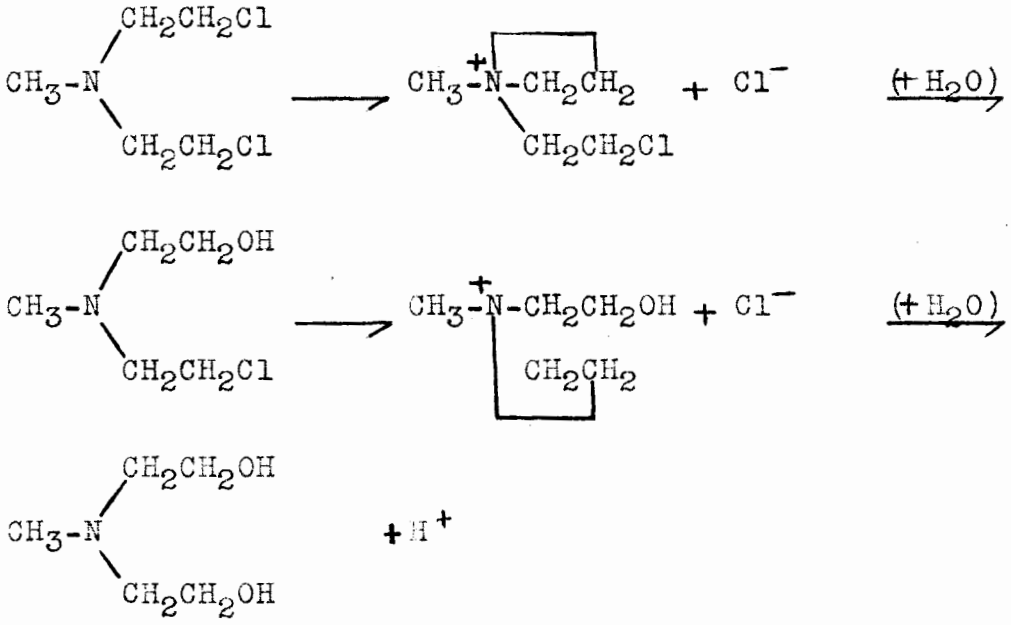
nitrogen mustards form non-volatile, stable, water-soluble hydrochlorides which can be handled with ease and safety, and these were selected for clinical trial. Although many derivatives of nitrogen mustards had been made, the tris-(Beta-Chloroethyl) amine-hydrochloride and the methyl-bis (Beta-Chloroethyl) amine-hydrochloride were used therapeutically, since most of the essential laboratory data on these derivatives were at hand because of their importance as chemical warfare agents (8,11).

The nitrogen mustards owe their physiologico-chemical activity to a basic chemical reaction known as intramolecular cyclization in a polar solvent to form a cyclic onium cation with liberation of a Cl anion. The reaction may be expressed by the following equation:

$$R\text{-}Z\text{-}CH_2CH_2Cl \longrightarrow R\overset{+}{Z}\text{-}CH_2\overset{\frown}{CH_2} + Cl^-$$

The Z represents nitrogen or sulphur atoms. The onium cation (ethylenimonium in the case of Beta Chloro-ethyl-Amines and ethylenesulfonium in the Beta Chloro-ethyl Sulfides) reacts with anions and various uncharged nucleophilic molecules. The following equation of the reactivity of the nitrogen and sulfur mustards will bring about a better, clearer under-

standing of their action:



The nitrogen and sulfur mustards differ in two basically different chemical behaviors. (a) In the use of Beta Chloroethyl amine, the cyclization cannot occur when a proton becomes coordinated with the nitrogen atom. Therefore, solutions of this compound are stable in strong acid, whereas the hydrogen ion concentration does not affect the formation of the sulfonium ring. This distinction is not important at the pH of body fluids. (b) The reactivity of the Ethylenesulfonium ring is so great that it never accumulates in solution in a sufficient amount to permit its isolation. On the other hand, the ethylenimonium

compounds are much less reactive, accumulate and have been isolated. However, the reactivity of this latter compound is sufficient to produce toxic reactions similar to sulfur mustards. For this reason the two compounds can be discussed together if one so desires with respect to basic relations between chemical structure and chemico-physiologic actions.

Methyl-bis (Beta-Chloroethyl) amine can be considered as an example of the Beta-Chloroethyl vesicants. In dilute aqueous alkaline solution the series of reactions as indicated earlier occurs. The majority of the nitrogen mustards are bis-(Beta-Chloroethyl) amines. The third valence of nitrogen is occupied by one of a variety of alkyl groups. The rate of cyclization and the activity of the ethylenimonium cation is influenced by substituent groups on the molecule. This also leads to a large number of nitrogen mustards of different physico-chemical and pharmacological properties (2,8,12,13).

Ethylenimonium and sulfonium cations react with water in pure aqueous solutions at physiologic hydronium concentration. But, if other substances are present, they can react competitively. Sometimes the

reactivity with water is negligible in the presence of other substances. This is important since it is known that the onium compounds are able to alkylate the functional groups of compounds of biological importance. Among these are the alpha-amino, imidazole, sulfhydryl, sulfide, phenolic, epsilon-amino, and imino groups of amino acids and peptides; inorganic phosphate, glycerophosphate, and hexose-phosphate; the amino groups of adenosine and thiamine; the pyridine-N of nicotinic acid amide and pyrodoxine. The carboxyl and amino groups of numerous proteins as hemoglobin, insulin, gelatin, crystalline egg albumin, tobacco mosaic virus, ovalbumin, silk fibroin, protamine, and various purified crystalline enzymes are also involved in reaction.

The above paragraph does not imply that systemic toxic action of the mustards is due to the reaction with any single compound listed and nor is it intended to imply so. Rather, it can be assumed that the basic mechanism of the action of nitrogen mustards involves a similar reaction with some vital cellular constituent (2,12,13,14,15,16,17).

III. Biological Effects

A. Findings in Vivo or in Vitro or Both

Cytotoxic Action: Experimentation has shown that certain tissues of the body are more affected by the nitrogen mustards than others. However, it should be made clear that when the nitrogen mustards are present in sufficient strength or concentration, their action will kill any type of cell whether it is a normal or an abnormal cell (13,14,18). The outstanding systemic action of these compounds is cell death. The mechanism by which cell death results is not completely explained at this time. But, recent experimental work suggests that there is a relationship between the action of mustards on enzyme systems and their cytotoxic effects. It has also been shown that cellular susceptibility to the cytotoxic action of mustards is directly related to the proliferative activity of the cells (2,5,11,12, 14-17,19,20).

In lethal and sublethal doses the most sensitive tissues are the blood forming organs (bone marrow and lymphoid tissue) and the intestinal mucosa. In therapeutic doses the intestinal effect may be minimal or insignificant (2,13,15,18,21). The changes seen in

these tissues will be discussed later and further discussion pertinent to these will not be added here in order to prevent repetition.

Acute actions to Lethal Doses: The toxicity, clinical and pathological effects of the nitrogen mustards were appraised in mice, rats, rabbits, dogs, and pigeons by administering the agents as a gas, or as a liquid, cutaneously, orally, subcutaneously, intraperitoneally, and intravenously. Studies on human beings were also made on subjects exposed to the nitrogen mustards. On contact these compounds have a necrotizing action on the skin, cornea, or mucous membranes. Inhalation results in injury to the respiratory mucousa. Ingestion of the compounds results in lesions of the mouth and upper gastro-intestinal tract. Regardless of the method of administration the pattern of systemic effects is similar in all cases (2,12,13,19,22).

Supra lethal doses are followed by prominent signs of central nervous excitement leading to convulsions and acute death. Parasympathomimetic effects, that is, salivation, miosis, etc., are followed by parasympatholytic action. Death occurs usually within 24 hours. Sub-convulsive doses are followed by a

progressive muscular paralysis and death usually occurs from paralysis of the respiratory muscles in 3 to 6 days. If the animal survives the injury, recovery is usually complete. Lymphocytopenia and granulocytosis occur within the first 12 to 24 hours after exposure. This is followed by a progressive severe leucopenia for 3 to 4 days. The erythrocyte count is affected very little. These events are closely paralleled by abrupt atrophy of lymph nodes, spleen, and thymus, due to lymphocyto-chexis, by aplastic degeneration of the bone marrow in 2 to 4 days, and by degenerative changes in the mucosa of the small intestine. In these respects the clinical pathological effects resemble those induced by X-ray irradiation (2,8,13,18,22,23).

Inactivation of Enzymes Theory: For some time it has been believed that many poisons act by attacking one or more of the essential intracellular enzymes in that way producing a "biochemical lesion". The actual damage is the result of the metabolic disturbances. Similarly, it was reasoned to expect changes in cells and tissues by the nitrogen mustards either in vivo or in vitro.

Both nitrogen mustards and the sulfur mustards produce local and general effects. Small amounts applied to the skin are followed first by a delay of about two hours, after which edema and erythema appear; this is followed later by vesication. Larger amounts produce necrosis of the skin instead of the vesication. The compounds, being readily absorbed through the skin into the circulation, produce general systemic effects, especially a degenerative effect on the white blood cells and a consequent leucopenia, and damage to the gastro intestinal tract. Death occurs if large amounts are absorbed. Because of these facts, it was believed that the general systemic effects and the local skin effects are produced by different mechanisms, and at first they were considered separately (12,15,24).

As a result of the above assumption the problem was approached from two different aspects: (1) a study of the changes in the metabolism of the skin as a result of application of the compounds; (2) a systematic study of the action these compounds on isolated enzymes of various tissues in vitro. As the work progressed it became evident that both lines of experimentation led to the same conclusion, that is, the very specific enzyme poisoning by the mustards which was ob-

served in vitro would produce exactly those changes in metabolism which are found and correlated with skin damage and the damage in other tissues (15).

Young rats were used in testing the effect of the mustards by rubbing the mustard over the undersurface of the young rats and then keeping the rats for varying times up to three hours before killing. Untreated rats of the same litter acted as controls. There was no immediate metabolic change noted, but after about two hours signs of skin damage began to appear which became more marked after three hours with two well marked changes in metabolism. (a) There was a sharp fall in the Respiratory Quotient (R.Q.) with glucose from 0.9 to 0.56, although the rate of oxygen uptake remained slightly below normal. This indicated that carbohydrate could no longer be utilized. (b) The Anaerobic formation of lactic acid from glucose was much inhibited. This inhibition increased with time and was parallel to the onset of the symptoms until the glycolysis had fallen to the value obtained without added glucose (2,15). Before continuing with this discussion it can be added at this time that early observations showed that in vitro exposure to the

mustard compounds had disappeared by hydrolysis. The effect is therefore a specific one on the initial phorylation step which is concerned with phosphate transfer to or from adenylic compounds (2,12,15).

In the experimentation on skin it was shown (a) that, as in muscle, the glycolysis is of the phosphorylation type; (b) that hexokinase is present in skin and is present a short time after mustard application; (c) that hexokinase is absent from mustard treated skin once the inhibition of glycolysis has developed.

In research on the effect of chemical warfare agents (mustard gas) on crystalline hexokinase it was determined that this enzyme was especially sensitive as an enzyme but it was not so when considered as a protein. In other words, its sensitivity is not due to any special reactivity of the mustards, but rather that in this enzyme the activity depends on the groups which react with the mustards. It is supposed that metabolic disturbances and consequent tissue damage can result only when the combination of the enzyme with the mustards affects its activity. It is believed that this enzyme has a high competition factor for mustards

tative correlation between the degree of glycolysis inhibition and the severity of the skin damage. In addition, there was also a correspondence in time relations, that is, the inhibition by the mustards occurs after a delay period, and it is only then that visible damage develops.

Because the mustards do not inhibit the glycolysis of hexosediphosphate suggested that the initial phosphorylation of glucose was inhibited. In other words the poisoning or inactivation of hexokinase was the cause of depressed glycolysis. The enzyme catalyzes the reaction GLUCOSE + ADENOSINETRIPHOSPHATE = GLUCOSE-6-PHOSPHATE + ADENOSINEDIPHOSPHATE. This was proven directly by the use of an aqueous extract of ordinary muscle acetone powder which contains all the enzymes of the glycolysis system except hexokinase. The hexokinase was prepared from yeast. The mixing of the two preparations produces a complete glycolysis system changing glucose to lactic acid. Just as in the skin, this system was inhibited by the addition of mustards. If the muscle extract alone was treated with the mustards and later the hexokinase added, no inhibition of glycolysis was produced except after the

sulfur mustards by minced tumor tissue resulted in a moderate reduction of oxygen consumption and a sharp depression of the aerobic and anaerobic glycolysis of glucose (25,26). Similar results were found with minced brain and chick embryo tissue. Inhibition of respiration and anaerobic fermentation of yeast cells, inhibition of respiration and glycolysis in bone marrow, spleen, and thymus, as well as depression of glycogen synthesis in the liver and intestinal absorption of glucose were also noted (2,12,19,24). Nitrogen mustards have been shown to inhibit respiration in isolated slices of lymph nodes, bone marrow, spleen, brain, liver and kidney. Utilization of pyruvate by kidney slices and synthesis of urea by liver slices were found to be sensitive to nitrogen mustards in vitro as well as in animals gassed by these compounds. It was demonstrated that lactic acid formation from hexosediphosphate or from the substrate present in the skin were not inhibited (2,12).

There is evidence that a direct connection exists between this inhibition of glycolysis and the production of skin damage. Skin damage never was observed without inhibition and there appears to be a good quanti-

when compared to other enzymes. By "competition factor" of a substance is meant the ratio of the velocity constant of its reaction with mustards to that of the hydrolysis of the mustards (12,15).

Because the diverse cells and tissues mentioned earlier in the discussion showed similar findings as to inhibition of respiration and cellular activity, this lead to the theory that possibly other enzymes might be affected. Investigation was made in vitro of many enzymes and enzyme systems. This included proteins, dehydrogenases, hydrolytic enzymes, catalysts involved in the metabolism of glucose, intracellular and extracellular proteolytic enzymes, various oxidases, acetyl-choline esterase, ribonuclease, hyeluronidase, carboxylase, and vitamins (2,12,14,16, 17,24). The majority of the enzymes were found to be only moderately sensitive to inactivation by the "mustards". The markedly sensitive were hexokinase, creatinine, pyruvate phosphokinase, inorganic pyrophosphatase, adenylic acid deaminase, chick pepsin, kidney pepsinase, peptidase, choline oxidase, and acetylcholine esterase. The most sensitive were hexokinase and phosphokinases as a group (2,12,14,24). Thus with these findings in vitro there is much doubt

that hexosekinase in vivo is the specific mechanism of the toxic action of the mustards.

At this time a final statement cannot be made on the "enzyme inactivation theory". It is evident that in vitro some enzymes are more sensitive to the mustards. Further experimentation and investigation in vivo will be required to determine whether this inactivation leads to the cell pathology. This is especially true when experimentation on mammalian cornea (2,12) and yeasts (27) produce changes in mitotic activity in the presence of mustard concentrations below that which affect respiration and glycolysis (28, 29). In addition it has been shown that the mustards react with protein constituents, amino acids, and peptides (these substances are found in the blood and other body fluids). They react with alpha-amino and alpha-carboxyl groups common to most amino acids and the characteristic side chains are present in many cellular tissues and cells. Since many of these groups are found in intact protein molecules, it seems that the mustards can react with proteins in vivo (15,16,24). Then, too, it has been found that the mustards can combine with a number of vitamins which are essential to the economy of the living cell. The vitamins referred

to are nicotinic acid or its amide, pyridoxine, and thiamine (17).

Nucleotoxic Action: It should be remembered that toxic amounts of the mustards results in diverse systemic effects but threshold doses and therapeutic doses bring out pathologic changes in cells and tissues which have a high rate of proliferation and growth. Minimally effective doses inhibit the mitotic activity of a variety of cells. This has been demonstrated by work on unicellular, invertebrate, amphibian, mammalian and plant organisms (2,10,14,18, 20,30). Exposure of yeast resulted in reduction of growth rate. Salamander larvae so exposed reacted by an immediate cessation of growth explained by an inhibition of mitosis in the proliferative regions of all tissues of the embryo (2). Similar findings were found in experimentation on embryos of Triturus torosus and amblystoma punctatum (20,31). It should be noted that cells in which mitotic activity was completed at the time of exposure continued functional differentiation in the normal manner (2,15,20,31). Threshold amounts of the mustards applied to the eye or administered by the way of the parenteral routes

depleted the corneal epithelium of mitotic figures for several days. There was no evidence of cytoplasmic or nuclear damage (2,12,15)

The inhibition of mitosis is confined to the resting phase so this does not necessarily suggest a primary nucleotoxic action. Since cells already in mitosis, when exposed, do complete their division, the consequence is an inhibited tissue entirely void of mitosis or mitotic figures. However, higher or toxic doses brings forth some suggestion of a more direct toxic action on the nuclear mechanism by the appearance of much nuclear fragmentation and chaotic chromatin dispersal which may be considered pathological and incomplete mitosis (2,14,15,20,31).

It is supposed that while the mustards may fail to precipitate cytoplasmic proteins, they might be able to penetrate the nucleus and produce characteristic changes. In this connection it may be noted (a) that the effect of the mustards on cells as with X-rays produce nuclear change, and (b) that mustards also behave like X-rays in producing mutations. The most significant demonstration of this specific nucleotoxic action has been shown in experiments on

Drosophil Melanogaster (2,14,32) and on Neurospora (8,33). An example of this is shown by profound disturbances produced by mustards on the structure and function of chromosomes in Drosophila Melanogaster. Exposure of both male and female to sublethal doses reduced or suppressed fertility through disturbances of miosis and mitosis in the gametogenesis of both sexes. By exposing the adult male only to lower dosages fertility was not reduced greatly, but the genetic analysis of X chromosomes revealed a high percentage of sex linked lethals which was much in excess of the natural rate of mutation as well as a significant number of translocations and inversions (2). At present the mechanism by which these changes in chromosomes are brought about is unknown and needs further investigation to determine if this is by chemical reaction with the chromosomes' component compounds or if this is the result of structural instabilities induced by the inactivation of associated nuclear enzymes.

Even in the presence of such nuclear changes, it is not believed to be the sole mechanism associated with the cytotoxic action of the mustards because other cytoplasmic parts of the cells show some effect of the

mustards as structural changes in mitochondria of embryonic membrane bone, and swelling of cytoplasmic fat globules of sclerotic chick tissues in culture (2,32). Then, too, avian erythrocytes have been affected by immersion into dilute mustard solutions so that their swelling action was inhibited in the presence of applied detergents. Since this type of erythrocyte has no nuclear mechanism, the interpretation suggests a primary change in cytoplasmic stroma (2). Thus, much work remains in order to explain what part is played by the nucleotoxic action of mustards in causing the various tissue reactions.

On the Gastro-Intestinal Tract: Toxic and lethal doses of the mustards produce severe gastro-intestinal changes. Metabolic changes run parallel with the histological changes seen in the gastro-intestinal tract. There is a definite diminution in glycolysis. It seems, however, that the fact that the rats on which the experimentation was performed ceased to eat, may account in part for this finding. The glycolysis in this tissue depends very much on whether the animal had been recently fed. Hexokinase is not inhibited in this tissue in systemic poisoning. At the same time

the fall in metabolism of the gastric mucosa and the fall in glycolysis were similar in badly affected rats, and there was a rise in both in those which spontaneously recovered (15).

After toxic or non-toxic doses nausea and vomiting occur in a few hours which may be due to reflex response from the gastro-intestinal mucosa or possibly medullary stimulation. With toxic doses there are produced necrotizing desquamating lesions of the mucosa; diarrhea begins within 24 hours and becomes progressively worse. The vomitus and feces may both show blood which escapes from necrotic areas. Fluid and electrolyte loss from the gastro-intestinal system is marked, partly accounting for death if the outcome is fatal. The small intestine suffers to a greater degree than any part of the tract (2,13,22).

In therapeutic doses the only gastro intestinal reaction is nausea and vomiting in varying degrees which begins usually within one to eight hours after injection. This is not necessarily severe nor is it of long duration. Anorexia may be present one to three days after which the appetite returns with a feeling of well-being (10,21).

On the Hemopoietic System: The metabolism in bone marrow showed a decided fall in anaerobic glycolysis within 4 hours after injection of mustards running parallel with or slightly preceding the cell damage observed histologically. It was not disclosed, however, that this was due to hexokinase alone but the poisoning of hexokinase seemed to be predominant. In marrow, the whole metabolism with respiration fall after mustard injection and the effect is not specific on one reaction only (15).

Marrow studies showed that the nucleated cell count dropped from 100,000 per cubic millimeter to 6,400 per cu. mm. on the twenty-first day. This fall was followed by an increase to 65,000 per cu. mm. in the fourth week. Depression of blast forms and promyelocytes is noted in the first week but increased in number during the second week. Myelocytes and metamyelocytes drop in number during the second week but increase during the third and fourth weeks. The erythroid series was depressed for two weeks followed by regeneration of proerythroblasts and basophilic erythroblasts in the third week and of the orthochromatic erythroblasts in the sixth week.

The peripheral blood showed a depression of all elements. In therapeutic doses the depression of the blood increased with the number of injections per course of treatment. The hemoglobin and red cell count decreased 0.7 gm. per cent and 140,000 red cells after two injections to a total decrease of 1.6 gm. per cent and 460,000 after six injections. Associated was an increasingly severe decline of 35%, 65%, and 67% in the leucocyte count after 2,4, and 6 injections respectively. Differential blood counts revealed that there is a transient leukocytosis due to a granulocytosis. Both the lymphocytes and eosinophiles drop immediately on injection. The total leukocyte count falls within 24 to 48 hours. The platelet count usually shows a severe lowering in number. Recovery of the bone marrow, however, is rapid and peripheral blood returns to normal in surprisingly short periods of time. The leucocyte count returns to normal following each course of treatments (2,13,21).

Of all the blood forming elements the most susceptible tissue to the mustards is the lymphatic tissue. An abrupt atrophy of lymph nodes, spleen, and thymus occurs due to lymphocytorhexis (14,22).

The fact that the lymphocyte count drops immediately has already been mentioned. To this can be added that the lymphocyte count in the body lymphatics decreases rapidly. Lymphatic tissue injury in the spleen consists of fragmentation of the lymphoid cells with phagocytosis of chromation particles, and cellular depletion of the sinuses. In the thymus, cytolysis of the lymphoid cells of the thymic corpuscle and interstial tissue occurs. The abrupt injury to lymphatic tissue is paralleled by the disappearance of the lymphocytes from the peripheral blood (13). The action of the mustards is directly a lymphocytotoxic effect and is not due to adrenal cortex stimulation (the "alarm reaction"), because adrenalectomized animals showed this same lymphocytic fragmentation when treated with the mustards (34).

Infections during a course of treatment or following injection of the mustards, or following exposure to mustards are extremely rare. This fact lead to the investigation of the effect of the mustards on antibody production. In this investigation goats were immunized against ricin. It was found that the antibody levels were decreased or held lower in the

presence of the mustards. After the effect of the mustards had disappeared, the antibody titre rose comparably to that of the normal controls. The deleterious effect of the mustards on the formation of circulating antibodies can be reasonably attributed to their toxic actions on leucopoietic tissues. In addition, the fact that processes of immunity are susceptible to the actions of leucotoxic agents is comparable with current theories of the importance of lymphoid tissues in the formation of antibodies. However, it has not as yet been demonstrated that lymphoid tissues are the only leucopoietic elements involved in the production of antibodies (35). The part played by adrenal hormones resulting in dissolution of lymphocytes and the release of circulating antibodies has been demonstrated (36,37). To support the belief that the effect of the mustards on antibody production is mediated through direct action on lymphocytes rather than through adrenal cortex tissue, one group of animals were injected with corticosterone and one group with mustards. Those receiving injections of cortical hormones elicited an elevated antibody level along with a lymphopenia and lymphoid

atrophy which, some believe, are related to enhancement of the circulating antibodies (37). Injection of the mustard resulted in a prolonged lymphopenia but no enhancement of antibody production. Thus, it is believed that antibody suppression is by direct leukotoxic action (35). Similar findings were obtained in antibody titre in rabbits treated with mustards and typhoid vaccine (38).

Effect on Embryonic Tissue: Experimentation on both Triturus Torosus and Amblystoma Punctatum revealed (as has been mentioned earlier) that proliferating regions were selectively affected by the mustards to the exclusion of non-proliferating regions and even within the single cell proliferation was inhibited while other features of the cell gave the appearance of normal function.

By experimentation on these embryos it was found that sensory ganglia and cartilage-forming mesenchyme were not indiscriminately destroyed. In both cases those organs more advanced in development, that is, nearer in point of time to the formation of definitive tissue, showed less effect than organs in the earlier formative stages. Mucosa cells and skin cells were affected, but the cell as a whole was not necessarily

destroyed. Rather there was an abnormal enlargement and they carried on complicating differentiation processes at the same pace as those occuring in normal animals. The result was a change in tissue appearance similar to changes occuring in normal animals. In this respect the mustards act as a specific inhibitor rather than a cell poison (20).

The inhibition appeared to be connected specifically with mitotic activity in the various organs. In the neural tube and eye, in which mitotic activity patterns can be sharply defined and localized, the pattern of tissue destruction parallels the mitotic pattern. In other organs as the cranial ganglia and cartilage, liver, stomach, and epidermis, mitotic increase in the number of cells was stopped, but the organs continued to differentiate. The complete inhibition of mitosis in the limb bud and the spinal ganglia resulted in the complete destruction of these organs, because the entire primordium was undergoing mitosis at the time of exposure (20,31).

The effects on mitotic cells took two main forms: (a) In some the cells broke down completely into chromatic fragments; (b) in many others, exposure to mustards cause a great increase in cell size which

was followed by abnormal mitotic behavior. These effects are answered by study of the effects of mustards on embryonic nervous tissue. The embryonic neural tissue gave three different reactions to the mustards: (1) Resistance to exposure, as indicated by absence of break down or enlargement and by normal progression of differentiation; (2) gradual break-down within 5 days, in which the cells of the affected region disintegrate into chromatic fragments; and (3) initial enlargement of the cells and nuclei increasing up to ten days, followed by a break down of the cell in which disarrangement of the mitotic mechanism played a role.

The cells of the embryonic vertebrate nervous system are separated into those which divide and those which differentiate, the two activities never being found in the same cell. Reaction No. 1 above can be explained by the fact that complete absence of any visible effect on cell size or cell differentiation or cell integrity was characteristic of cells which had abandoned mitotic activity. The other reactive types No. 2 and No. 3 above were located in regions of the neural tube which had subsequently different fates. The cells of reaction No. 3 occurred

in a region of the tube which was destined to be persistently mitotic even in later stages of development. It had the property of continuous proliferation. Reaction No. 2 occurred in a region which in a normal animal lost its mitotic activity within 10 days of the exposure date, that is, these cells were cells that are still undergoing generalized embryonic cleavage and do not possess the growth characteristics of other types of proliferating cells. Thus, the size of these cleavage cells is gradually decreased as compared to proliferating cells which grow to the same size as the parent cell. Exposure to the mustards may delay or inhibit mitosis of proliferating cells, while apparently not interfering with the growth mechanism, resulting in the abnormally large cells seen in reaction No. 3. On the other hand exposure of cleaving cells did not result in the enlargement of the cells as indicated by reaction No. 2 since the growth mechanism characteristic of proliferating cells was apparently not in operation here (20,31).

Effect on Neoplasms: From the preceding discussion, it has been shown that the mustards are injurious to many types of tissue, and exert the greatest effect on

rapidly growing tissue. For this reason, it was suggested that the mustards might be a solution to malignant diseases. Early studies concerning the effects on tumour tissue has been stated. In the discussion that follows, therapeutic doses of nitrogen mustards have been used.

Hodgkin's disease is one which has been given a moderately fair trial of treatment with the mustards. All investigators report the same findings in regard to this disease. Unfortunately, in the treatment of this disease and other malignant or neoplastic disease the majority of the cases treated were in advanced or terminal stages and many were considered resistant to roentgen irradiation. Thus, the full results of the treatment cannot be evaluated. In nearly every case of Hodgkin's disease some benefit was obtained from mustard therapy. The clinical results were sometimes dramatic. Those who were resistant to irradiation have been restored after a course of nitrogen mustards. In some cases the response to the mustards was better than from any previous course of radiation treatment. The result of treatment was rapid partial or complete disappearance of Hodgkin's tumor masses; most patients experienced improvement in appetite,

weight, strength, and sense of well-being; fever, if present, disappeared within 24 hours. Symptom free remissions varying from two weeks to at least seven months have been observed. The lymph nodes, liver, and spleen decreased in size, but in advanced or active forms of the disease, regressions are less frequent and shorter in duration. Large masses of matted lymph nodes, invasion of the disease beyond the lymph nodes, and bone lesions may not show favorable response, even though general symptomatic improvement including relief from pain due to bone involvement may occur. The anemia of Hodgkin's disease sometimes improved. Skin lesions due to Hodgkin's disease may or may not be affected and pruritus is sometimes only slightly and inconstantly relieved (9,10,18,21,39,40).

Just as in Hodgkin's disease many patients afflicted with lymphosarcoma were in the terminal stages. Many had received previous X-ray therapy and a great number were irradiation resistant. The clinical results observed were qualitatively similar to those described for Hodgkin's disease but were more frequently unsuccessful. Failures were encountered without obvious explanation for the lack of satisfactory response. It was impossible to predict be-

forehand which patients would or would not respond satisfactorily. Many patients showed dramatic results. Satisfactory responses were obtained even in radiation resistant patients. In addition to definite reduction or complete clinical disappearance of lymphosarcoma masses and the signs and symptoms attributable thereto, the therapeutically induced remissions frequently were associated with an improvement in appetite, strength and weight and with reduction of fever plus a sense of well being. The highly aggressive, rapidly growing type of lymphosarcoma usually was not affected by either maximum doses of mustards or X-ray therapy. On the other hand the lymphosarcoma characterized by normal or elevated white blood cell count, consisting chiefly of lymphocytes, and with enlarged lymph nodes may respond well to the mustards as evidenced by a prolonged depression of the white blood cell count and regression of enlarged nodes. Significant remissions were produced varying from 3-18 months in duration (8,9,10,18,21, 39,40).

Effect on Leukemias: The chronic lymphatic leukemia appeared to respond better than any other

forms of leukemia. But, even in this disease the results are none better than those obtained by X-ray. In all chronic forms of leukemia, the size of the lymph nodes, spleen, and liver decreased markedly. Fifty to eighty per cent of the chronic lymphatic leukemias will respond in some form or another. Where concomitant clinical and symptomatic benefits did not occur, nitrogen mustards often caused a reduction in the leukocyte count, a more normal differential formula, improvement in the appearance of the bone marrow and more persistence in the effect of blood transfusions. Sometimes the signs and symptoms of hypermetabolism were considerably relieved. Most cases responded to the initial course of the drug and remissions occurred varying from two to twenty-one months.

Chronic myelogenous leukemia did not give this response to mustard therapy. Except for a transitory symptomatic improvement and a reduction in the leukocytes of the peripheral blood, results have been as a whole unsatisfactory. It was noted, however, that in these same patients so treated, their response to X-ray was largely unsatisfactory, too. The patients that did respond to the mustards had remissions of six to twelve months duration.

All forms of subacute and acute leukemia have been treated. At the present time, the clinical results in most cases are not particularly encouraging. In some patients clinical and hematologic remissions were obtained, but, as a rule, the ultimate fatal outcome was only briefly postponed if at all. The decrease in thrombocytes caused by the mustards was a complicating factor in the use of this agent in several patients with platelet values which were already quite low. Improvement in the white blood cell count, differential formula or the bone marrow picture was not always paralleled by clinical or subjective gain (9,10,11,18,21,39).

Effect on Other Diseases: A number of other diseases have been placed through the test of mustard therapy. Giant Follical Lymphoma (Brill-Symmer's Disease) exhibited a moderate to marked reduction in the size of lymph nodes and spleen. As a rule, this type of lymphoma responds well but the mustards are not a cure (9,10,21,39).

Polycythemia Rubra has been treated because of the effect of the mustards on hemopoietic tissue. Definite remissions are produced by the use of the mustards; definite symptomatic and hematologic re-

missions were produced. The remissions were comparable with those of radioactive phosphorous and the duration of the remissions was six to eighteen months (10,9,18, 21).

Melanosarcomas respond poorly to this therapy. Only one case treatment resulted in edema around the mass which delineated it from uninvolved tissue and permitted relatively simple enucliation at resection (9,10,39).

Both metastatic mammary carcinoma and carcinoma of the cervix have shown no decided change by treatment with the nitrogen mustards (10,39).

Sympathoblastomas appear to respond well to this therapy. The drug produced a rapid decrease in size of the tumor mass and although remissions may be short, the general condition of the patients improved. The total duration of their disease after diagnosis was ten to sixteen months which compares favorably with roentgen therapy. It was of interest that these undifferentiated tumors of nervous origin respond to the drug and suggested that its physiologic effect may depend on reaction common to all rapidly proliferating cells (10,18).

Multiple Myeloma responds unsatisfactorily except for the relief of pain. No other improvement in the patient is noted. Peripheral blood reacted as described earlier. Studies of sternal aspirations showed a decrease in total nucleated cell count but the tumor cells were not reduced in number nor were morphologic changes seen in myeloma cells. There was no regression of nests of plasma cells seen on biopsy (10,18).

Mycosis fungoides seemed to respond well. The mustards appeared to be extremely effective in controlling the pruritus and caused involution of the tumors and infiltrated plaques. The lesions disappeared rapidly leaving a non-indurated skin (9,40,41). However, the mustards did not cure.

IV. Comments

Because the margin of safety in the use of the mustards is narrow, it necessitated the discussion of the toxicity from the stand point of lethal dosage, sublethal dosage, and therapeutic dosage. It should be realized that the sulfur mustard had not been used and should not be used for therapeutic purposes because of its undesirable physical properties and extreme chemical reactivity which make the mustard sulfur both difficult to administer and very toxic. But, nitrogen mustards in the form of their hydrochloride salts are water-soluble, less reactive, crystalline compounds, which can be readily dissolved in sterile saline for intravenous use. Consequently, the clinical investigations in humans has been confined entirely to the nitrogen mustards.

It was appreciated early that sulfur and nitrogen mustards were not only contact vesicants but, following absorption by any route, could exert cytotoxic actions on a variety of tissues. Furthermore, cellular susceptibility to these compounds seems to be related in a general way to the degree of proliferative activity.

Since it was important to determine the mechanism

by which the mustards act and since these substances apparently had some degree of tissue specificity, the study on the fundamental process of the cytotoxic action of the mustards was pursued. It was determined that many cellular enzymes and biological substances were affected by the mustards, and one enzyme, hexokinase, was highly susceptible to the action of these drugs. These studies also revealed a type of action on cells which can be likened to that of no other chemical agent, but resembles in many ways that of X-rays.

The therapeutic dosage for mustards as well as possible combination of this therapy with radiation or other agents, -- for initial, continuation, or interim prophylactic treatment remain to be determined. While indications and contraindications have not been established definitely, the investigators believe that the nitrogen mustards are deserving of further trial in Hodgkin's disease lymphosarcoma, and the leukemias.

At the present time the dosage of the nitrogen mustards agreed upon is as follows: The standard single dose of 0.1 mgm per kilogram of body weight is injected daily or every second day until three to

six doses are administered, but the single dose never exceeds 8 mgm. This is considered to represent the initial treatment or course required to induce remissions in suitable cases. In the very ill patient the single dose was reduced to 0.05 mgm. per kilogram. The number of single doses used is determined by the patient's response and the hemopoietic status. Similarly, subsequent courses of treatment vary with each patient and this depends on the clinical response, the hemopoietic status, and the duration and completeness of remissions.

The nitrogen mustards must be administered only by the intravenous route and great caution must be observed to prevent extravasation. The solution is freshly made by adding 0.9 per cent sterile aqueous sodium chloride solution to sterile glass bottles each containing exactly 10 mg. of the dry salt. Injection is to be accomplished within five minutes after preparation of the solution, because of the rapid hydrolysis which may occur, with consequent loss of efficacy. Probably the best way to administer the drug is through an intravenous infusion set up. No ambulatory therapy is to be attempted.

The immediate local or systemic effects of mustard drug administration such as pain on injection, thrombophlebitis of injected veins, nausea and vomiting, anorexia and headache are relatively inconsequential and can be avoided or mitigated by careful technic. Preliminary sedation with a barbiturate and the withholding of food (overnight fast) prior to treatment tends to decrease these systemic effects. More serious late toxic effects are concerned with the blood-forming organs and include leukopenia, granulocytopenia, thrombocytopenia, and anemia, but these can be largely avoided by adherence to safe dosage schedules and careful follow up by blood studies. These toxic effects are in some respects merely extensions of the therapeutic effects. Although the chemicals seem to have a selective action on primitive cells and abnormal hemopoiesis, in sufficiently large doses the compounds affect all elements of the bone marrow producing the picture described above. The objective in treatment is to keep the dosage within the relatively narrow range of safety, so that maximal salutary clinical results can be obtained with a minimal untoward effect on the unformed elements of the blood.

The evaluation of the clinical status of this group of compounds will require many more years of careful study. At the present time there is no basis for assuming that the therapeutic efficacy of the mustards is any greater than that of X-ray. The mustards like X-ray do not cure. It seems advisable that X-ray is indicated as the first treatment especially in localized diseases as therapy can be applied locally. When the disease is generalized and is attended by severe systemic intoxication with fever, anorexia, and weakness, then nitrogen mustard therapy should be attempted and if gratifying remissions are obtained, they should be continued as long as the hematologic status permits.

It is possible that the potential value of nitrogen mustards in the treatment of neoplastic diseases will only be fully realized when the opportunity to explore the relationship between chemical constitution and pharmacodynamic action has been exhausted. So far only two compounds have been investigated, -- tris (beta-chloroethyl) amine and bis (beta-chloroethyl) amine which have been evaluated more for their action as toxic chemical warfare agents rather than of compounds of therapeutic interest. Hundreds of

congeners of these compounds can be synthesized and remain to be evaluated. Thus a series of compounds which can reproduce in many ways the cellular effects of X-ray is available for chemical and biological investigation. It is hoped that just as in the evolution of other chemotherapeutic drugs, a parent compound may be found in the beta-chloroethyl amines by chemical alteration which will be sufficiently specific in toxic action for certain types of proliferative cells and thereby possess therapeutic value.

V. Summary

(1) The problem of neoplastic diseases has been discussed for which there is no satisfactory therapeutic approach.

(2) The evolution and history of nitrogen mustard therapy was brought forward by the advent of World War II and by experimentation by the various groups named.

(3) The formation of the intramolecular cyclization (ethylenimonium in the case of (Beta-chloroethyl) amine) with its great power and variety of reaction accounts for physiologico-chemical activity of these compounds.

(4) Nitrogen mustards are cytotoxic to certain tissues, namely rapidly proliferating tissue, hemopoietic tissue, and gastro-intestinal mucosa (esp. in lethal and sublethal doses). Lethal doses also have in addition a parasympathomimetic effect.

(5) The "biochemical lesion" or biological lesions produced by the drugs appears to be through inactivation of many biologically important substances. Hexokinase appears to be the most easily affected biological substance.

(6) These drugs apparently possess a nucleotoxic action not fully understood but resembles X-ray irradiation.

(7) Lethal doses produce severe pathologic lesions of the gastro-intestinal tract. In therapeutic doses these are not found and the effects on the gastro-intestinal tract can be disregarded.

(8) Hemopoietic tissue reacts by a marked fall in leukocytic elements and a slight anemia but this tissue has a rapid recovery power to normal readings within a few weeks. Lymphatic tissue is most affected by mustards. Antibody production is also much depressed.

(9) Proliferating embryonic tissue is highly susceptible to the action of the mustards.

(10) Hodgkin's disease responds to mustard therapy most favorably of all neoplastic diseases. Chronic lymphatic leukemia responds better than any other type of leukemia. The mustards at present are worthless in acute leukemia. Lymphosarcoma and polycythemia rubra respond moderately well. Other neoplastic diseases have been subject to mustard therapy with varied responses but as a whole not satisfactorily.

(11) The toxic reactions produced by therapeutic doses of mustards can be disregarded. The mustards do not cure and at present are none better than X-ray therapy. Only two mustards have been investigated. There are hundreds of similar compounds, one or two of which may have a greater specificity for pathologic or neoplastic tissue.

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