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ADDITIONAL STUDIES PERTAINING TO THE MECHANISM OF CUTANEOUS INJURY BY MUSTARD GAS. AN EXPERIMENTAL STUDY USING MUSTARD PREPARED WITH RADIOACTIVE SULFUR

to
October 1, 1945
by
A. R. Moritz and F. C. Henriques, Jr.

Report OSRD No. 3620-A
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ADDITIONAL STUDIES PERTAINING TO THE MECHANISM OF CUTANEOUS INJURY BY MUSTARD GAS. AN EXPERIMENTAL STUDY USING MUSTARD PREPARED WITH RADIOACTIVE SULFUR.

Service Directive: CWS-2

Endorsement (1) Dr. John A. Zapp, Jr., Technical Aide, Division 9, to Dr. Walter R. Kirner, Chief, Division 9.

Forwarding report and noting:

> "This report is supplemental to OSRD 3620 and is based upon additional data obtained between November 1943 and December 1944. The pages and sections have been numbered to correspond to the pages and section of OSRD 3620 to which the data are supplemental. This report together with OSRD 3620 constitutes a complete and final report of the studies on the mechanism of cutaneous injury by mustard gas done under Contract NDCrc-169 with Harvard University."

(2) from Dr. Walter R. Kirner, Chief, Division 9 to Dr. Irvin Stewart, Executive Secretary of the National Defense Research Committee.

Forwarding report and concurring.

This is a progress report under Contract 9-93, NDCrc-169 with The President and Fellows of Harvard University.

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Supplement to Formal Progress Report
OSRD No. 3620

Additional Studies Pertaining to the Mechanism of Cutaneous Injury by Mustard Gas. An Experimental Study Using Mustard Prepared with Radioactive Sulfur.

Final F ormal Progress Report

Contract NDCrc 169 Division 9 N.D.R.C.

November 1943 to December 1944

by

F.R. Dutra, F.C. Henriques, Jr.,* A.R. Moritz*
and L.A. Patterson
with the assistance of
R.S. Myers, M.D. (Surgical Consultant), K. Lynch, and C. Margnetti

This report is based upon experiments reported in the Informal Monthly Progress Reports (Nov. 1943 to Dec. 1944) on Physiological Mechanisms of Chemical Warfare Agents, Section 9-5, Division 9 N.D.R.C. of the Office of Scientific Research and Development.

*Official Investigators

INTRODUCTION

ii) Scope and Period of these Investigations:

The experiments herewith reported comprise additional information pertaining to the mechanism of mustard vesication primarily through parallel histological and chemical studies of skin exposed to mustard and related compounds prepared with radioactive sulfur.

Since this report comprises only a small portion of the total studies done on this problem under Contract NDCrc 169, it has been prepared with the view of complete integration into O.S.R.D. Report No. 3620. This is to be especially noted in the numbering of the pages, section headings and general form of presentation. Furthermore, the data given herein can be only fully utilized by appending this report to O.S.R.D. Report No. 3620.

These two reports give all of the reliable data pertaining to the mustard investigations that were obtained under Contract NDCrc 169 and that were published as Informal Monthly Progress Reports from November 1940 to December 1944. No attempt has been made to present these data in chronological order.

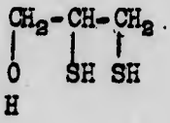
iii) The Radioactive Sulfur:

The radioactive sulfur of 88 day half life used in the investigations undertaken after November 1944 was prepared at the Radioactivity Center of the Massachusetts Institute of Technology under the direction of Prof. R.D. Evans.

The activity of this sample was 50 millicuries in 2 grams of barium sulfate.

v) Glossary:

(k) *:
Denotes substance contains radioactive sulfur.

(l) B.A.L.:
British Anti Lewisite 

(m) CH:
Semi-H or half hydrolysis produce of mustard.

(n) DH-TG:
Sulfonium salt of semi-H and thiodiglycol.

(o) CH-TG:
Sulfonium salt of semi-H and thiodiglycol.

(p) DH-2TG:
Sulfonium salt resulting from the combination of one mustard molecule with two thiodiglycol molecules.

SUMMARY

I. Preparation of Radioactive Mustard and Related Vesicants.

(2) Through techniques similar to these used in the preparation of radioactive mustard, milligram quantities of benzyl mustard, sesquimustard, mustard sulfone and divinyl sulfone respectively containing radioactive sulfur can be prepared in high yields and excellent purity.

IVd. Comparison of the Penetration and Fate of Mustard for Man, Pig and Rabbit.

(3a) At the termination of a one hour application, pig skin tissue has an appreciable reservoir of extractable radioactive sulfur. The composition of these extractables is about 50% unreacted mustard, 25% thiodiglycol, 2.5% sulfonium salt (Di-TG) and 22.5% unknown.

VI. The Pathology of Cutaneous Injury following Exposure to Mustard.

VIc. Tissue Differences in Respect to the Length of the Latent Period between Exposure to Mustard and Microscopic Evidence of Irreversible Injury.

(1) Differences in susceptibility of various tissues (rabbit) to injury by direct exposure to liquid mustard are apparent rather than real. When an epithelial (liver) and a connective (peritoneum) tissue receive the same direct exposure to liquid mustard the reaction of the former is sufficiently rapid that the full extent of irreversible injury can be recognized microscopically in less than 24 hours, whereas the morphologic evidence of irreversible injury in the latter is so slow in developing that 7 to 10 days are required before the dead can be distinguished from living cells.

(2) This difference in the rate at which morphologic evidence of irreversible injury develops in epithelium as compared with connective tissue is probably significant in respect to the difficulties that are encountered in attempting to evaluate the depth of a cutaneous mustard injury during the first few days after exposure. The extent of the epidermal damage can be recognized microscopically in less than 24 hours, whereas the extent to which connective tissue of the dermis has been destroyed may not be recognizable until a week has elapsed.

(3) The depth to which tissue is ultimately destroyed after a standard exposure to liquid mustard appears to be approximately the same whether the application is made to skin, liver, or parietal peritoneum. Inherent differences in the capacity of these tissues for repair and regeneration are such that the damaged cells of one may be disposed of and replaced even before the extent to which another tissue has been injured can be recognized.

(4) On the basis of equal exposure times local fixation of mustard was least in the case of intact skin and it was found in progressively increasing amounts in tongue, liver, peritoneum, and scraped skin. Since the amount that penetrated at each site as

inferred from the amount found to be fixed was in excess of that required to produce a maximum cutaneous injury no conclusions could be drawn regarding differences in tissue susceptibility to injury.

VId. Skin Grafting Experiments to Determine the Viability of Epidermis after Exposure to Mustard.

(1) A series of epidermal transplants on pigs in which the transplants were protected by Harvey's "buried skin" technique provided evidence that the death of the epidermis represents primary injury and is not a secondary effect of dermal injury.

(2) When exposed epidermis is transplanted to normal dermis the epithelium shows no alterative change for several days and has the appearance of coagulated fixed tissue. It is slowly sequestrated in the form of a necrotic crust.

(3) When normal epidermis is transplanted to dermis from which the exposed epithelium has been excised there is a progressive degeneration of the transplant with early reactive changes and usually survival of a thin lamella of basal cells. To date it has not been determined whether or not the surviving basal cells in such a transplant lead to earlier healing of the lesion.

VIe. A Comparison of the Histo-pathologic Characteristics of Thermal and Mustard Injuries of the Skin.

(1) A comparative histo-pathologic study of cutaneous injuries produced by heat and by mustard included lesions which ranged from 1 to 28 days of age. All observations were made on pigs and in all instances the superficial area of injury was oval and measured 6 mm. in width by 12 mm. in length.

(2) In each type of lesion there was a central superficial zone of primary necrosis, an intermediate peripheral zone comprised in part of delayed primary and in part of progressive secondary necrosis, and an outer peripheral zone in which injury was reversible and from which healing took place.

(3) Histologic evidence of cell death in the superficial zone of primary necrosis developed more rapidly in thermal than it did in mustard lesions. In the former the full extent of irreversible epidermal damage was usually apparent within a few hours whereas several days were often required in the case of mustard lesions. Final demarcation of the zone of progressive secondary necrosis in the subcutaneous connective tissue required approximately the same length of time (7 days) in both types of lesions.

(4) In both kinds of lesions repair of the epidermal defect by new epithelium preceded the repair of the dermal defect by connective tissue. Epithelial regeneration occurred as soon as the outermost zone of connective tissue damage became static. It took place on the surface provided by the still viable, original connective tissue. Proliferation of new connective tissue to replace the corium and to fill in the depression occurred only after the surface had been epithelialized.

(5) Regardless of the mechanism by which a defect was produced approximately 3 weeks were required for complete epithelial repair if the entire thickness of the epidermis had been destroyed. Mustard lesions healed as rapidly as did thermal lesions of comparable severity.

(6) The time required for complete restitution of the dermis in lesions having identical surface areas varied in direct proportion to the depth to which the subcutaneous tissue had been destroyed. There was a similar correlation between the depth of an injury and its tendency to undergo cicatricial contraction. Mild superficial injuries whether produced by heat or mustard healed early with complete anatomic restitution and little or no contraction. Deep injuries required many weeks for complete restitution of the dermis and cicatricial contraction increased according to the depth of the lesion. There were no significant differences between comparable thermal and mustard injuries in regard to the time of complete anatomic restitution or the degree of cicatricial contraction.

VIf. A Pathological Study of the Inhibition of Vesication by B.A.L.

(1) Inhibition of vesication by B.A.L. is not limited to injuries by mustard. It is equally effective in inhibiting vesicle formation in human skin after exposure to extreme cold (solid CO_2).

(2) Pathological studies of vesicated mustard lesions compared with lesions in which vesication was prevented by treatment with B.A.L. (human skin) indicate that treatment with B.A.L. prevents vesication but does not reduce the severity of the injury.

(3) The mechanism by which vesication is prevented may be one or any combination of the following: Early coagulation of the epidermal-dermal junction by B.A.L. may cause sufficient hardening of the injured tissue to prevent the disruption that is essential to vesicle formation.

Early coagulation of the injured epidermal cells by B.A.L. may prevent the escape of those substances that are ordinarily responsible for an outpouring of serous fluid.

Early damage of the dermal capillaries by B.A.L. may interfere so seriously with circulation as to prevent the serous effusion that ordinarily results in vesicle formation.

(4) Pathological studies of treated (B.A.L.) and untreated mustard lesions on pig skin disclose:

Early coagulation necrosis of epidermis following B.A.L. treatment.

Partial inhibition of subepithelial exudation in the treated lesions.

Earlier and more severe vascular injury in the treated lesions.

A considerably greater accumulation of fibrin in the interstitial spaces of the dermis of the treated lesions.

VII. Significance and Properties of Fixed-Mustard in Skin Sites of Man and Pig.

VIIa-5. The Effect of B.A.L. on Fixed Mustard in Human Skin.

(1) BAL treatment reduces the amount of fixed mustard present in human skin tissue by about one-half. Thus BAL apparently promotes the disintegration of tissue substances containing fixed mustard.

VIIa-6. The Relative Injury Propensities of Various Vesicants Related to Mustard.

(1) Mustard, benzyl mustard, sesqui mustard, mustard sulfone, and divinyl sulfone produce in the skin tissue types of injury which can be correlated quantitatively with the amount of the fixed agent in the tissue.

(2) Mustard and benzyl mustard fixed in equivalent molar amounts in the tissue produce the same degree of injury; sesqui mustard, on the same basis, is somewhat more potent.

(3) Mustard sulfone and divinyl sulfone when fixed in equivalent molar amounts in the tissue are identical in their injury producing ability. They are only about one-seventh as potent as the mustard type vesicants on the basis of the amount of fixed material required to produce a given type of injury.

(4) The above observations indicate: that only one beta chloroethyl group of mustard is responsible for injury; that the second beta chloroethyl group in sesqui mustard is reacting to some extent with the tissue constituents; that mustard sulfone reacts through the divinyl sulfone intermediate in the tissue.

VIIb-4. Enzyme Hydrolysis of Malphigian Layer of Pig Skin Containing Fixed Mustard.

(1) Pancreatin at a pH of 11 at 37°C in 10 days will convert 83% of the fixed material of the malphigian layer into permeable molecules.

(2) Under the same conditions in 16 days Pancreatin will render soluble 99% of the fixed mustard with the formation of 28% thiodiglycol.

VIIc. The General Mechanism of Mustard Injury.

(2) Although fixed mustard is the initiator of injury, it is not "intrinsically" toxic. Then the removal of fixed mustard after the lesion is developed would probably not shorten the healing time.

VIII. Miscellaneous Control Experiments with Animals.

VIIIa-4. A Method of Increasing the Rate and Severity of Cutaneous Injury by Mustard.

(1) With short-time exposures certain combinations of liquid mustard and ethyl cellosolve are as effective as and others are more effective than pure mustard in the production of injury to the skin of pigs. In mixtures in which the ratio of mustard to ethyl cellosolve is less than 1:9 or more than 1:1 the injuries were of the same order of severity as those produced with pure mustard. In mixtures in which the ratio was 2:8 and 3:7 the injuries occurred earlier and with greater severity than was the case when the skin was exposed to pure mustard.

IX. Certain Chemical Experiments.

1Xc. Investigations Pertaining to the Sulfonium Salts, DH-TG.

(1) When 0.0008 M. mustard* was reacted with blood plasma for thirty minutes at 37°C, 2.4% of the added DH* went to DH*-TG*, 3.1% of the extractables formed was DH*-TG*, and 4.5% of the mustard which reacted went to DH*-TG*.

(2) The complete hydrolysis of 0.0006 M. and 0.0008 M. mustard* in water resulted in about 4.5% of the DH* going to DH*-TG*. The use of 0.01 M. mustard* showed that 10.6% of the DH went to sulfonium salts of various types.

(3) Solutions of DH-TG have been shown to exist as an equilibrium mixture of DH-TG and DH + TG. In water the equilibrium mixture consists of practically all DH-TG, while in acetone and alcohol it is mostly all DH + TG.

TABLE OF CONTENTS

Ib. The Synthesis of Benzyl Mustard, Sesqui-mustard, Mustard Sulfone and Divinyl Sulfone

by P.C. Henriques, Jr., C. Margnetti, and L.A. Patterson

Preparation of Radioactive Benzyl-H $\phi CH_2-S-CH_2-CH_2-Cl$:
The following reaction schemes were used:

(1) H_2S + ethylene oxide = $HOCH_2CH_2SH$. Exactly 1/2 millimole of radioactive H_2S and ethylene oxide were condensed into a thick-walled 1 mm. capillary tube by means of liquid nitrogen and high vacuum technique. The capillary was sealed off such that upon warming all but a trivial amount of the constituents were in the liquid phase (estimated pressure 20 atmospheres). The capillary was placed on a steam bath overnight. Yield of mono-thio ethylene glycol was better than 98%. The capillary was now opened and the contents were centrifuged into a carius tube .

(2) $HOCH_2-CH_2-S^- + \phi CH_2Cl = \phi CH_2SCH_2CH_2OH + Cl^-$

One cc.of absolute methyl alcohol was now added, the contents chilled in ice, and 0.51 millimoles of OH^- as INNaOH were added. Then exactly 0.5 millimoles of ϕCH_2Cl were added, the tube was sealed and then shaken for two days. Sodium chloride precipitated out.

The tube was then centrifuged, cooled and 3 cc. of conc. HCl added, followed by 3 cc. of 20°-40° petroleum ether.

(3) $\phi CH_2SCH_2-CH_2OH + HCL = Benzyl-H + H_2O$

The tube was resealed and shaken for 48 hours at 75°-80° C. The Benzyl-H was then extracted 3 times with 2 cc. of petroleum ether. The petroleum ether was then removed under reduced pressure. Overall yield 96%. Numerous preliminary preparations with ordinary H_2S resulted in yields of 95 ± 2%, and analysis of these preparations for both sulfur and chlorine indicated a purity of at least 97%.

Preparation of Radioactive Sesqui Mustard $Cl\,CH_2-CH_2-S-CH_2-CH_2-S-CH_2-CH_2-Cl$
To an 8 mm. tube sealed at one end and containing 50 mg. of anhydrous sodium carbonate and 0.3 ml. of water was added 34.6 mg. of monothioglycol. After the tube was shaken to form the sodium salt of the mercaptan, 23.6 mg. of 1,2-dichloroethane was added. The tube was sealed and shaken overnite in a water bath at 75-80°C. The tube was opened and placed in a sublimation apparatus. The distillation was carried out at a pressure of 1 mm. with water circulating through the cold finger. After the water was removed at room temperature, the sesqui glycol was collected by gradually raising the bath temperature to 150°C. The sublimation tube was cleaned, 0.3 ml. of dry ether added, and the sesqui glycol removed from the cold finger by refluxing. The tube containing the ether solution of sesqui glycol was cooled in an ice bath while first 1.7 ml. of dry ether, then 1 ml. of thionyl chloride in 0.1 ml. portions were added. The reaction mixture was allowed to stand in the ice bath for two hours, then the thionyl chloride and the ether were removed under reduced pressure. The tube was attached to the sublimation apparatus and the residual sesqui H distilled at a pressure of 1 mm. as the bath temperature was gradually raised to 150°C. The sesqui

H was obtained in 90% yields. It melted at 53°C (literature, 53-54°C) and had a purity of 97% on the basis of chlorine and sulfur analyses.

Preparation of radioactive mustard sulfone $ClCH_2-CH_2-\overset{\overset{O}{\|}}{S}-CH_2-CH_2-Cl$: 14.1 mg. of radioactive mustard (DH) and .2 cc. of conc nitric acid centrifuged into a reaction tube. When the acid became clear the nitric acid was pumped off at room temperature leaving white crystals of the sulfoxide.

Then 12 mg. of potassium chromate and 0.01 cc. of sulfuric acid in 0.1 cc. of water were centrifuged into the reaction tube. The tube was sealed and then steamed for four hours. The reaction tube was then opened and the sulfone extracted with four 1 cc. portions of chloroform into a micro-vacuum sublimation apparatus. The majority of the chloroform was pumped off at -60°C; the temperature was then slowly increased to 175°C whereupon the sulfone sublimed onto the cold spot. Yield 15.1 mg. 90% theor.) M.P. = 55°C. The melting point is indicative of the purity of the sulfone.

Preparation of Radioactive Divinyl Sulfone $CH_2=CH-S-CH=CH_2$: To a 10 ml. Erlenmeyer flask containing 60 mg. of radioactive mustard sulfone dissolved in 4 ml. of dry benzene was added 1.2 ml. of dry benzene solution containing 63 mg. of triethyl amine. The reaction mixture was allowed to stand 24 hours at room temperature, then filtered into a 15-20 ml. ground glass flask containing a long neck constricted above the bulb. The benzene solution was evaporated to a volume of about 0.5 ml. by means of a current of air drawn in through a capillary. A plug of glass wool was inserted in the constriction of the flask; a still was attached containing a cold finger having an inverted cup-shaped end and having near the middle a concentric glass ring to collect the product in when the still is inverted. The remainder of the benzene was removed at a pressure of 10 mm. at room temperature while water was run through the cold finger. Dry ice and acetone were placed in the cold finger and the distillation of the residual divinyl sulfone carried out at a pressure of 3 mm. while the bath temperature was raised slowly to 100°C. A white solid product collected on the cold finger. The still was inverted and allowed to come to room temperature. The liquid product was removed from the cold finger by attaching a ground glass test tube, adding 2 ml. of ether, and refluxing a few minutes. The ether solution was transferred to a weighed ground glass centrifuge tube. The ether (about 4 ml.) was evaporated to about 0.3 ml. using an ice bath and at a pressure of 150 mm. The remainder of the ether was removed by drawing air with a vacuum pump into the tube through a capillary for a couple of minutes first at room temperature, then at 35-40°C. A colorless liquid product was obtained in 95-98% yields, N_D^{25} 1.4773.

A preparation as small as 3.5 mg. of divinyl sulfone was carried out in a 70% yield with a purity of 97% for the product.

The purity of the product was unchanged after a storage of three weeks in the ice box. A 10% cellosolve solution was perfectly stable during two weeks of storage in an ice box.

$C_4H_6O_2S$	Calculated	S, 27.1
	Found	S, 27.1

IIIj-3a. Chemical Analysis of the Skin Sites. Determination of the Amount of Sulfonium Salts Present in the Tissue Extract (see IIIj-3) of Some of the Short Time Post Application Period Tissues.

The most likely solfonium compound to be formed from the hydrolysis of dilute mustard solutions present at skin tissue sites is probably the DH-TG compound. The CH-TG compound may be formed to some extent also. The DH-2TG compound is probably not formed to any extent because of the low concentration of thiodiglycol present.

To formulate a satisfactory method for the analysis of DH*-TG* in tissue sites containing extractable mustard compounds it was necessary to study the properties of DH-TG and to try several different procedures. A procedure was tried in which an attempt to separate the three main components of the tissue extractables, DH*, TG*, and DH*-TG*, from each other so that the relative amounts of the three substances would not change during the analytical procedure. DH* was separated from the TG* and DH*-TG* by shaking the extractables with a water-pentane mixture. Attempts to separate TG* from DH*-TG* in the aqueous phase by the use of organic solvents was unsuccessful. Isoamyl alcohol by repeated extractions would remove 100% of the TG* but would also remove 70% of the DH*-TG*.

The essential step to be carried out in any analytical scheme for DH*-TG* is the addition of either DH-TG plus picrylsulfonic acid or DH-TG picrylsulfonate plus picrylsulfonic acid as carriers to give the DH*-TG* picrylsulfonate mixed with the DH-TG picrylsulfonate. The derivative is then isolated and the radioactivity determined. Under no circumstances should the DH*-TG* be precipitated when the procedure involves the standing of DH* or TG* in contact with DH-TG or its picrylsulfonate for any length of time in an aqueous solution. The DH-TG picrylsulfonate has been found to undergo a rapid exchange with TG* in water, 65% of the TG* exchanging in 60 minutes. The same thing would probably happen in the case of DH*. The exchange of DH* with DH-TG picrylsulfonate in alcohol was slow, only 1% over a three hour period. The analysis for DH*-TG* in an acetone solution containing DH* and TG* was tried but was not successful as 6% of the DH* and TG* present either exchanged with the DH-TG picrylsulfonate added as carrier or reacted to form DH*-TG*. Most of the exchange undoubtedly took place when the acetone solution was evaporated to isolate the DH-TG picrylsulfonate, as the water present favored rapid exchange as the acetone evaporated.

A method was worked out which gave only a small amount of exchange of DH* and TG* with DH-TG picrylsulfonate. Cold acetone was used to extract the tissue containing mustard* extractables. The acetone extract was partly evaporated after analyzing aliquots of it for total extractables. The residual acetone was diluted with ice water and shaken with pet. ether. This procedure removed the DH* so it could not participate in an exchange reaction with the DH-TG added in the next step. The DH-TG carrier was added to the cold aqueous solution containing DH*-TG* and TG*, then quickly converted to its picrylsulfonate by adding picrylsulfonic acid. The solution was shaken with nitromethane. This procedure gives only a short time for the exchange reaction between TG* and DH-TG to take place.

The nitromethane extracted the DH-TG picrylsulfonate out of the water layer so that an appreciable exchange could no longer take place. DH*-TG added to acetone in the presence of pig skin tissue was recovered in yields of 98% when carried through this procedure.

Procedure for the Analysis of Pig Skin Extractables for Radioactive DH-TG Sulfonium Salt. The frozen tissue shavings from two application sites were placed in a large test tube containing 15 cc of cold acetone (cooled to 0°C) and shaken for two or three minutes. The acetone extract was separated from the tissue by filtering through a coarse fritted filter packed with asbestos. The filter was washed twice with 4 cc portions of cold acetone. The acetone filtrate was diluted to 25 cc with acetone. Two 1 cc aliquots were removed and analyzed for total radioactive extractables after adding to each sufficient DH, TG, and DH-TG as carriers to give 7 mg. of benzidine sulfate. The residual acetone extract was evaporated rapidly with a water pump to a volume of about 8 cc in a 500 cc round-bottomed flask. The residual liquid was poured into a large test tube containing 10 cc of pet. ether (b.p., 30-60°C) and shaken. The flask was quickly rinsed out twice with 10 cc portions of cold water, adding the rinsings to the test tube containing the pet. ether and shaking. The pet. ether layer was removed and discarded. The aqueous layer (kept at near 0°C) was extracted twice with 10 cc portions of pet. ether. Then 5 cc of ice cold aqueous DH-TG solution[a] was added, the mixture was quickly shaken and 1 cc of water containing 40 mg. of picrylsulfonic added. After the mixture was shaken, it was extracted with 20 cc of nitromethane. The nitromethane layer was removed following centrifugation and then evaporated at a pressure of 2 mm. in a 500 cc round bottomed flask which was swirled continuously and warmed with the hands to hasten evaporation. The residual liquid (about 0.1 cc) was treated with about 5 cc of cold absolute alcohol (cooled in dry ice-acetone bath). A colorless oily solid separated and adhered to the bottom of the flask. The alcohol was decanted from the precipitated DH-TG picrylsulfonate which was then washed twice with 5 cc portions of chloroform, once more with 5 cc cold alcohol, and finally with chloroform. The residue was taken up in acetone and transferred to a large weighed ground glass tube. The acetone was removed with a water pump and the product dried with a vacuum pump. The residue was weighed, dissolved in a definite volume of acetone and two aliquots taken which contained the equivalent of 7 mg. of benzidine sulfate. The aliquots were analyzed for the amount of radioactivity per milligram of benzidine sulfate. This value multiplied by 94 x 1.1 (60 mg. of DH-TG picrylsulfonate carrier added to 23/25 of the acetone extract and it yields 94 mg. of benzidine sulfate, gives the amount of DH*-TG* in the original acetone extract. However, 1.3 of DH*-TG* was formed under the conditions of the experiment so this amount subtracted from the DH*-TG* obtained in the experiment gave the actual amount of that substance present. The results are given in Table 1.

The above procedure was tested in the following manner:

---

[a] The DH-TG solution was prepared according to the method of Smith (Informal Report, June 10, 1944). To a warm solution of 72 mg. of DH-TG picrylsulfonate in 2.6 cc of water and 0.8 cc of acetone was added 2.1 cc of water containing 21 mg. of the dichloro cyclic dimer of TL 146. The reaction mixture was diluted to 3 cc, cooled, centrifuged, and the supernatent DH-TG solution removed.

IVa-2a. Nature of the Chloroform Pig Skin Tissue Extractables. The Sulfonium Salts.

by L.A. Patterson and F.C. Henriques, Jr.

Introduction: The sulfonium salts have been given considerable attention as possible agents concerned in the systemic toxic effects of mustard. Rydon (B-2979; Porton Report No. 2483) has postulated the existence of sulfonium salts formed by the action of mustard on mustard-protein molecules, e.g. $(Pr\text{-}CH_2CH_2SCH_2CH_2OH)^+$ $(CH_2CH_2SCH_2CH_2Cl)Cl^-$, in which Pr is a protein molecule. These sulfonium salts are supposed to be carried about in the blood liberating mustard in various parts of the body. While there is no evidence for the existence of such compounds, the formation of sulfonium salts derived from the action of mustard or semi-mustard on thiodiglycol has been shown when mustard is allowed to hydrolyze in water (Bergman, Informal Reports, May 23, 1942, June 20, July 10, 1943). These latter substances may be responsible for much of the systemic effects of applied mustard. Bergman (Informal Report, April 10, 1943) showed that DH-2TG at 37°C and a pH 7.3 decomposes slowly in aqueous solution to yield products that react with thiosulfate or cysteine. Toxic products may result from the decomposition of the sulfonium compound under physiological conditions. Smith (Informal Reports, June 10, August 10, September 10, 1944) has studied the toxic effects of aqueous DH-TG solution administered cutaneously and intravenously to various animals. The toxicity of DH-TG parallels that of mustard in many cases and in some cases it was more or less toxic.

In view of the physiological importance attached to mustard-thiodiglycol sulfonium compounds and in view of their ready formation when mustard is hydrolyzed, it is important to know if any such compounds are actually formed under the conditions in which mustard penetrates the skin in such small amounts.

Purpose: To determine the percent of chloroform extractables that were DH-TG sulfonium salt.

Procedure: See III (a-1, d-3, e-2, f-1, g, n-2, j-3a). Four skin sites of a pig received applications of mustard (1 mg. per sq.cm.) for 1 hour at an environmental temperature of 92°F. The post application period was of 0 hr. duration.

A radioactivity of about 1500 counts per gamma-H enabled the determination of the amount of DH*-TG* in the acetone extract of the tissue brie from two sites, by the procedure given in section III-j-3a.

Results: The results of two experiments are given in Table VIIa.

Table VIIa
Analysis of Pig Skin Extractables for DH*-TG*

Run No.	Extractables (as gamma DH*) in Acetone	DH*-TG* (as gamma DH*) in Acetone	Correction for DH*-TG* (a) in gamma	Net gamma DH*-TG* in Acetone	% of DH*-TG* in Extractables
1	50 γ	2.5 γ	1.3 γ	1.2 γ	2.4%
2	37 γ	2.3 γ	1.3 γ	1.0 γ	2.7%

(a) See IIIj-3a (Part 1) for the method of determining the 1.3 gamma of DH*-TG* formed under the conditions of the experiment.

IVa-3. Discussion of Data from IVa-1, IVa-2 and IVa-2a.

(5a) No more than about 2.5% of the unidentified extractables can be attributed to sulfonium salt formation. Thus the possibility of the compound being responsible for the systemic action of mustard is unlikely.

PART VI
THE PATHOLOGY OF CUTANEOUS INJURY FOLLOWING EXPOSURE TO MUSTARD

VI-c. Tissue Differences in Respect to the Length of the Latent Period between Exposure to Mustard and Microscopic Evidence of Irreversible Injury

by F.R. Dutra, A.R. Moritz, and F.C. Henriques, Jr.

Purposes: To compare the rate of reaction and the extent of injury in different tissues after direct exposure to liquid mustard.

To compare different tissues in respect to local fixation of DH*.

To compare the amount of DH* fixed in normal skin with the amount fixed in skin from which the stratum corneum had been scraped previous to exposure.

i) Experiments on Rabbits:

Procedure: See III (a-2, d-2, e-2, f-3, g, h-1, i). Different tissue surfaces (skin, liver, parietal peritoneum) were exposed to 1.1 mg. per sq. cm. of mustard for a 10 min. period subsequent to immediate decontamination. The rabbits were sacrificed at varying times after exposure and observations of the different stages in the pathogenesis of the lesions from the initial reaction to repair were made.

Results: Table XVa shows the number of animals used, the sites of exposure, the recovery time, and the microscopic characteristics of the lesions.

The outstanding difference was in the rate with which the extent of the injury became apparent. In the epidermis and liver the total extent of irreversible damage was apparent within 12 hours. Necrosis was present at this time, and all subsequent changes in these tissues were directed toward removal of debris and regeneration of tissue.

The degree of injury of the dermis, and of the connective and muscle tissues covered by the exposed peritoneum was not apparent. At first the only evidence of injury was provided by the presence of edema and occasional exudative cells. Focal degenerative changes became focal areas of necrosis and these eventually coalesced. The inner half of the striated muscle of the abdominal wall showed foci of necrosis within 48 hours but the full extent of the injury was not apparent until 7 days.

The demarcation of the irreversibly altered from the viable tissue was recognizable only after proliferation of fibroblasts was well advanced, and was not complete in the subperitoneal tissue until the 10th day. At this time, the entire periphery of the lesion was surrounded by a zone of loose connective tissue. On the 14th day this had further thickened and the central mass of necrotic debris was smaller.

TABLE XVa
Rabbit Experiments

Tissue	Post-application Time	Specimen Number	Microscopic Features
Liver	12 hrs.	571-1	Coagulation necrosis, fairly sharply demarcated. Diffuse leucocyte infiltration.
		571-2	Coagulation necrosis, sharply demarcated, in subserous portion as well as deeper in liver (focal). Diffuse and focal leucocyte infiltration.
	24 hrs.	483-5	Well demarcated zone of necrosis. Diffuse infiltration of leucocytes.
		486-1	Ditto
		487-1	Ditto
	48 hrs.	493-1	Well demarcated zone of necrosis. Diffuse infiltration of leucocytes. Cytolysis.
	4 days	570-1	Lysis of cells nearly complete. Much granular debris. Peripheral fibroblast proliferation.
		570-2	Ditto
	7 days	500-1	Loosely arranged mass of cellular connective tissue. Debris absent.
		500-2	Loosely arranged mass of cellular connective tissue containing lymphocytes and foreign body giant cells. Debris absent.
	10 days	511-1	Thin layer of relatively dense connective tissue.
		511-2	Ditto
		511-3	Ditto
	14 days	501-1	Thin layer of relatively dense connective tissue.
		501-2	Ditto
Skin	12 hrs.	571-5	Necrosis of epiderm. Edema and leucocyte infiltration of corium, Vascular dilatation.
		571-6	Ditto
	24 hrs.	530-2	Necrosis of epiderm. Edema and leucocyte infiltration of superficial dermis.

(Table XVa cont.)

Tissue	Post-application Time	Specimen Number	Microscopic Features
Skin	48 hrs.	493-4	Epithelium absent. Necrosis of hair follicles. Edema of entire corium. Leucocyte infiltration of corium and subcutaneous tissue.
	4 days	570-5	Superficial corium (including zone of necrotic hair follicles) is necrotic crust over edematous remaining corium in which are proliferating fibroblasts.
		570-6	Ditto
	7 days	500-5	Epithelial regeneration beneath sloughing crust of necrotic corium. Fibrosis beneath regenerating epithelium.
		500-6	Ditto
	14 days	501-5	Regeneration of epidermis complete. Fibrosis of corium. No regeneration of hair follicles.
Peritoneum	12 hrs.	571-3	Moderate amount of edema, infiltration of few leucocytes. No demarcation.
		571-4	Ditto
	24 hrs.	483-4	Marked edema. Leucocyte infiltration. Occasional muscle fiber necrotic. Poor demarcation of lesion.
		486-2	Ditto
		487-2	Ditto
		488-2	Ditto
	48 hrs.	493-3	Entire thickness of inner layer of muscle necrotic. Few fibers undergoing lysis. Edema. Leucocytes. Fibrin on peritoneal surface.
	4 days	570-3	Necrotic zone same. Few fibers undergoing lysis, others contain small amounts calcium salts. Early proliferation of fibroblasts at periphery.
		570-4	Ditto
	7 days	500-3	Demarcation poor. Lysis more advanced. Leucocytes present. Moderate amount loose connective tissue.
		500-4	Ditto
	10 days	500-3	Demarcation complete. Fibrosis more marked in periphery. Many necrotic muscle cells, some containing calcium salts. Mononuclear giant cells.
	14 days	501-3	Diffuse fibrosis. Few necrotic muscle cells. Mono- and multi-nuclear giant cells. Lymphocytes. Fibrin on peritoneal surface organized.
		501-4	Ditto

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In the liver, the lysis and disappearance of necrotic cells was much more rapid than in the peritoneum. Complete organization occurred early (7 days) and there was some regeneration of parenchyma by mitosis of adjacent cells.

The sequence of events after exposure of the skin differs from that observed in the other sites in regard to disposal of dead tissue. Marked edema was evident within 12 hours after exposure, and necrosis of hair follicles was noted at the end of 48 hours. Epithelialization proceeded from the periphery of the lesion inward, beneath the crust of necrotic debris, and the destroyed tissue was eliminated from the body in this crust. It was not until epithelialization was complete that sharp demarcation of viable from necrotic corium could be identified. There was no evidence of regeneration of hair follicles in the material studied. In no case did the necrosis extend to the subcutaneous striated muscle.

ii) Experiments on Pigs:

Procedure: See III (a-1, d-2, e-2, f-3, h-1, i). In addition to treating the liver, skin, and peritoneum of pigs with liquid mustard in a manner identical with the above, the dorsum of the tongue was also exposed. Furthermore, applications were made to skin from which the cornified layer had been scraped. This scraping was done with a sharp knife immediately before the application of mustard and removed virtually all the stratum corneum with little disturbance of subjacent tissues. The post-application time for all pigs was 24 hours.

Results: It is obvious that the number of observations on each tissue is too small for a satisfactory statistical comparison. If the available data be representative (see Table XVb) it appears that after a 10 minute exposure the fixation of DH in normal skin and normal tongue is of the same order of magnitude (3.2 and 3.7 gammas per cm^2) whereas its fixation following direct exposure of liver, peritoneum, and scraped skin is of a higher order of magnitude (5.4, 6.7, and 7.5 gammas per cm^2). Since in all instances the amount fixed is in excess of that required to produce a maximal injury (of the skin) the experiments are not definitive in establishing a quantitative relationship between fixation and injury. If fixation of DH can be regarded as an index of penetration as is true in the case of skin it appears that intact skin is more resistant to penetration than any of the other tissues which were tested. Pre-exposure scraping of the skin sufficient to remove all of most of the stratum corneum increases its penetrability to such an extent that the amount of fixed mustard recovered from it is comparable to the amounts recovered after direct exposure of liver or peritoneum. Whether this increased penetrability results from the removal of some of the mechanical barrier (stratum corneum) or from the induction of a physiologic disturbance in the dermis is not clear.

Discussion: There appears to be a fundamental difference between epithelium and connective or muscle tissue in the rate of local reaction after exposure to mustard. At the end of 12 hours, the entire thickness of exposed epidermis was visibly necrotic, whereas the connective tissue and smooth muscle cells of the dermis showed little change. The liver parenchyma beneath the portion of capsule exposed to mustard was recognizably necrotic and sharply demarcated from adjacent viable parenchymal tissue at the end of 12 hours. In contrast to the liver the muscle and connective

TABLE XVb
DH (gammas per cm^2) Fixed in Various Tissues
(Pigs-all 10 Minute Exposures)

		0-1.9	2-3.9	4-5.9	6-7.9	8-9.9	Mean Amt. DH in gamma fixed per cm^2
Intact Skin	**Site No.**						
307	60		2.25				
+307	120		2.40				
+307	10		2.75				
502	13		2.75				
+307	360		3.10				
+307	10		3.30				
+307	20		3.30				
+307	20		3.30				
+307	60		3.50				
502	12		3.55				
+307	120		3.90				
+307	260		3.90				3.2 gammas (total)
Tongue							
502	9		2.80				
506	14		2.90				
506	15		3.80				
502	8			5.12			3.7 gammas (total)
Liver							
506	9		2.10				
506	10		2.90				
502	3			5.70			
502	1			5.75			
502	2				6.60		
502	4					9.50	5.4 gammas (total)
Peritoneum							
502	7			4.75			
502	5			5.55			
506	13				6.40		
502	6					8.15	
506	12					8.50	6.7 gammas (total)
Scraped Skin							
502	19		2.90				
502	15			5.64			
502	18				6.30		
502	14					9.65	7.5 gammas (total)

+Data from pig A-307 (see Table XII, p. 38) for post-application periods greater than 10 min.

tissues beneath the exposed parietal peritoneum showed remarkably little change after 12 hours, although there was extensive edema and infiltration of inflammatory cells.

Although the reason for this difference in response to DH* is not clear, it is in accord with the general rule that epithelial cells undergo earlier morphological alteration by noxious agents than do stromal cells. Presumably the greater vascularization of the liver is chiefly responsible for the relatively rapid removal of its debris.

The experiments on pigs confirmed the differences in manifestation of injury to DH* that had been observed in rabbits 24 hours after exposure. A peculiar feature of the tongue lesions was that they shared the characteristics of both the skin lesions and those of the parietal peritoneum. Unlike the skin, the epithelium of the tongue has no appreciable subjacent layers of connective tissue or fat. There is a thin lamina of connective tissue separating stratified epithelium from striated muscle. Hence, although the epithelium of the tongue was obviously necrotic (and partially desquamated) 24 hours after exposure, there were likewise changes in the underlying muscle analagous to the changes beneath exposed peritoneum. In no case did the skin lesions extend to the subcutaneous striated muscle.

Removal of the stratum corneum before applying the mustard allowed appreciably more DH to be fixed than in sites left unscraped, although the lesions were not appreciably different.

The increased quantity of fixed DH in scraped skin may represent increased penetration, in that the stratum corneum may act as a barrier to the passage of DH. Another possible explanation for the increase in fixed DH is that the hyperemia induced by scraping the surface before applying the DH may favor penetration.

VI-d. Skin Grafting Experiments to Determine Viability of Epidermis after Exposure to Mustard.

by F.R. Dutra, A.R. Moritz and R.S. Myers (Surgical Consultant)

Purpose: The results of certain unpublished preliminary experiments (Herriott) have suggested that the death of epidermis following exposure of the skin to mustard may be secondary to injury of the dermis rather than to its direct effect on the epithelial cells. The experiments herewith reported were undertaken to investigate this question.

Procedure: See III (a-1, d-2, e-2, f-3, g, h-1, i, j). On a young pig an area of skin was shaved and cleaned. Four sites separated from one another by approximately 1.5 cm. situated along the long axis of the prepared area were selected and to two of them (A and B) liquid mustard was applied for 20 minutes beneath small glass cups. At the conclusion of the exposures the skin was decontaminated.

Fifteen minutes later, using aseptic surgical technique, the full thickness of the epidermis was removed from sites B, C, and D with a sharp knife. Site A was left undisturbed as a control. A small disc of the exposed epidermis from site B was transplanted to the unexposed dermis at site C. A disc of unexposed epidermis from site D was transplanted to the

exposed dermis at site B. A disc of unexposed epidermis from site C was transplanted to exposed dermis at site D. In each instance the dermis had been denuded over a larger area than was to be covered by the graft so that a gap of several mm. separated the margin of the transplant from the edge of the surrounding original epidermis.

The sites were in a line and close together. By means of two curved incisions it was possible to isolate all four of them on an oval island of skin. A sheet of non-irritating animal membrane+ was laid over this island and anchored with sutures so as to keep the grafts in place and protect the surface. The edges of the skin adjacent to the island were then undercut and brought together over it.++

Thus, the buried island included the following:

A - Undisturbed exposed epidermis.
B - Exposed corium upon which unexposed epidermis had been placed.
C - Unexposed corium upon which exposed epidermis had been placed.
D - Unexposed corium on which unexposed epidermis had been placed (control).

In a few instances the control site A was not buried beneath the adjacent skin. The thickness of the grafts was determined by microscopic examination and was found to consist of epidermis and a narrow (25-50 micra) lamella of attached dermis.

Two such experiments were conducted on each pig and were spaced several days apart. Seven experiments in all were completed with post-application times ranging from 3 to 12 days.

Results: The information obtained from these experiments is presented in Table XVc.

Normal graft to exposed site:

There was survival of some epithelial cells of the normal grafts in all cases where the recovery period was 7 days or less. The epithelium of the 12 day graft was necrotic. The surviving epithelium consisted of cells of the basal layer which were present in isolated groups. Although basal cell survival was seen in six of the seven uninfected grafts of normal epithelium to exposed dermis the apparently viable epithelial cells did not proliferate or contribute to the healing of any of them. In the one experiment wherein sufficient time elapsed to allow evaluation (12 days) it was found that all of the transplanted epidermis was dead.

+Cargile Membrane, Johnson and Johnson

++A modification of Harvey's 'Buried Skin' technique--Harvey, S.C., Contract OEMcmr-83; Annual Report, July 1, 1943.

TABLE XVc

Animal No.	Side	Recovery Time	Fate of Epithelium of Graft		
			Exposed epidermis on Unexposed corium	Unexposed epidermis on Exposed corium	Unexposed epidermis on Unexposed corium
566	Left	--	--	--	--
564	Left	--	--	--	--
533	Right	3 days	Necrosis	Survival of basal cells	Survival of some basal cells
566	Center	4 days	Necrosis	Survival of few basal cells	Full thickness survival
534	Right	5 days	Necrosis	Survival of few basal cells	Survival of basal cells
564	Right	6 days	Necrosis	Survival of basal cells	Full thickness survival
533	Left	6 days	Necrosis	Survival of basal cells	Full thickness survival
534	Left	7 days	Necrosis	Survival of basal cells	Necrosis
566	Right	12 days	Necrosis	Necrosis	Full thickness survival

In most instances the normal corium was readily capable of survival when transplanted to an exposed site and its death in the 12 day survival is believed fortuitous, in light of its apparently complete reception on the other exposed sites. The epithelial structures (hair follicles and sweat glands) within the corium of the exposed receiving sites were necrotic, and the fusion of graft corium with receiving corium was often so complete as to leave no visible demarcation. Since the epithelial structures of the receiving site had been destroyed by H, there was no regeneration of epithelium from them to prevent close proximity of the graft's dermis with that of the receiving site.

Exposed graft to normal site:

In all instances, the epithelium was dead. The corium of the grafts was, in some instances, overgrown by epithelium regenerating from the edges and from hair follicles of the receiving sites, and was being incorporated into these sites. There were capillaries within these grafts which appearedunaltered and which contained blood. In other cases, the corium appeared viable and included blood-containing capillaries but the regenerating epithelium of the receiving site was undermining its edges. A third condition observed was a combination of the above two, so that the regenerating epithelium was partially over the surface of the graft's dermis (between the dermis and the necrotic epithelium of the graft) and other processes of regenerating epithelium were extending some distance beneath the graft's dermis. Another condition, encountered fairly frequently, was regeneration of the epithelium of the receiving site beneath the graft, with necrosis of both epidermis and dermis of the latter.

In several instances, grafts covered the upper ends of transected hair follicles, and tongues of epithelium from the follicles were observed spreading peripherally beneath the dermis of the graft. This was observed both in instances where the corium of the graft was viable and where the entire graft was being sloughed.

Unexposed graft to unexposed corium:

All but one of these had surviving epithelium present. Four of them had full thickness survival; that is, the basal and complete malpighian layers were alive. Several showed marginal proliferation of epithelium onto adjacent denuded surface of the receiving site.

Dermis:

The changes in the dermal tissues of the buried sites which had been exposed to H contrasted with the H-treated controls which had not been buried. In the latter there was a superficial zone of hyaline material covering a zone of collagenous tissue, wherein the fibers were coarser, more separated, and better defined than usual. The capillaries in this zone were dilated and engorged. The connective tissue cells of the dermis were few or absent, but in some cases there were a few infiltrating leucocytes immediately beneath the hyaline zone. The hair follicles and sweat glands were necrotic. The unburied control with 12 day recovery showed a thick necrotic

hyaline plaque overlying a zone of connective tissue which was infiltrated with leucocytes. Beneath the latter zone, there were early reparative changes as evidenced by fibroblastic proliferation.

The dermis of buried controls and of the receiving sites which had been exposed to H showed little superficial eosinophilic material, and this was never as dense as in the unburied controls. The capillaries were engorged and the increased definition of collagen fibers was noted, but leucocyte infiltration was absent or consisted only of a few cells scattered throughout the corium.

The unburied lesion at the end of 12 days showed early demarcation within the outermost portion of subcutaneous fat, with sequestration of the overlying tissue. The buried receiving site of the 12 day experiment which had been exposed to H appeared viable and although the hair folicles had been severely injured, they had numerous surviving basal cells.

Isolated "Grafts":

Bits of epidermis from exposed and unexposed grafts which were enclosed in membranous bags and placed within the wound cavity for four days showed necrotic changes of epidermis in each instance. The dermis of each showed no evidence of necrosis or of exudation.

Discussion: These experiments indicate that the epidermis exposed to liquid H is killed directly and that its death is not secondary to any effect that the H produces in the dermis.

Furthermore, it appears that a considerable part of the damage sustained by the dermis after exposure of intact skin to H may be due to the destruction of its protective epithelium. In effect, a new protective layer has been substituted for the damaged epidermis in these experiments, and the major changes usually ascribed to primary injury by H were not seen in the dermal tissues.

The beneficial effect of covering a mustard lesion was clearly shown by comparing unburied mustard lesions with these that had been buried with the island of skin. Whereas deep dermal injury occurred in the case of the former, the amount of dermal damage seen in the latter was insignificant.

VIe-a. Comparison of the Histo-pathologic Characteristics of Thermal and Mustard Injuries of the Skin.

by F.R. Dutra, A.R. Moritz and F.C. Henriques, Jr.

Purpose:

1. To determine if there be any difference in the development or repair of cutaneous lesions resulting from mustard and those resulting from heat.

Method:

A series of lesions produced in the skins of pigs by liquid mustard and by heat were studied microscopically after recovery periods ranging from 1 to 28 days.

Mustard lesions were produced as described by III (a, d-1, e-2, f-3, g, h-1, i). The application times were 5, 10, and 20 minutes, each in triplicate for each post-application period. The room temperatures varied between 66 and 74°F. Immediately after exposure the surface was decontaminated with pentane.

The heat lesions were made by contact with an oval copper surface which measured 6 mm. in width and 12 mm. in length. The apparatus was designed in such a manner that the pressure of the hot metallic surface on the skin was identical in all experiments. This surface formed the bottom of a container in which liquids were boiled by passing current through a high-resistance wire. The temperatures depended on the liquids used, and after some preliminary tests it was decided that lesions made at the temperatures of boiling methanol (66°C.) and of boiling ethanol (78°C.) would be most suitable for comparison with H-lesions. The duration of application of heat at 66° and 78° were 1/2, 1, and 2 minutes. Duplicate applications of each duration were made for each recovery period.

Changes in the Epidermis:

Mustard Lesions:

After a short latent period, focal degenerative changes may be recognized but in many instances the full extent of the damage was not appreciated until as long as 48 hours after exposure. The initial changes were nuclear and consist either of pyknosis or swelling. Concurrent cytoplasmic changes consisted of vacuolization and liquefaction or coagulation with increased eosinophilia. Disappearance or pyknosis of nuclei constituted evidence of cell death.

Twenty-four hours after a five minute exposure to H, focal changes were present in some of the deeper epithelial cells. Although these changes were more extensive after 48 hours, the full extent of the irreversible damage was not apparent until 4 days had elapsed in some instances.

The sites where the applications of H were for 10 or 20 minutes showed alteration of the entire epithelium within 24 hours. The change is best characterized as coagulation necrosis with condensation of nucleus and cytoplasm and a marked increase in the staining intensity.

After necrosis was fully established the progress of the lesion was toward elimination of the necrotic epithelium and re-epithelialization of the denuded surface. The epithelium together with its subjacent dead corium was incorporated into a crust which slowly separated from the surviving connective tissue.

As early as the fourth recovery day there was marginal re-epithelialization. This was evidenced by enlargement of basal cells at the periphery of the lesion and the growth of a thin layer of newly formed cells from the periphery toward the center of the lesion. These cells were found between the necrotic crust and the layer of still viable corium. Although after 5 and 10 minute exposures the regeneration of epithelium was apparent in 4 days it was not seen before the 7th survival day when the exposure had been for 20 minutes. Indeed, complete demarcation of necrotic from viable corium was not apparent until the 7th day.

Besides this growth from the periphery, there was also epithelial regeneration from hair follicles beneath the crust. The cells lining the follicles enlarged, and numerous mitotic figures were present. Tongues of newly formed epithelium projected centripitally from them, beneath the necrotic crust, and these tongues finally united with the epithelium growing inward from the edges.

It was found that although re-epithelialization began sooner in the lesions resulting from exposures of 5 minutes and 10 minutes than those from 20 minute exposures, all three required approximately 3 weeks for complete repair. Differentiation into the various epidermal strata was not complete at this time, and although it was more advanced after 28 days it was not complete in all of the specimens.

Heat Lesions:

There was practically no latent period before the extent of injury was evident in lesions resulting from heat. At the end of 24 hours, the entire epithelium was necrotic even with the shortest application (1/2 min.) of the least heat (66°C.) used.

The necrosis was marked by shrinkage of cells and nuclei with increased chromatophilia of each. There was none of the cytoplasmic vacuolization seen in H lesions. A peculiarity of heat burns was the elongation of cells and their nuclei. These cells, which also stained darkly, were long and had elongated and often slightly curved nuclei. This change was present only in cells of the basal or immediately suprajacent layers and persisted up to the fourth day after application.

In some of the burns of lesser intensity (66°C. for 1/2 or 1 minute, 78° for 1/2 minute) miliary sub-epidermal collections of fluid were found 24 hours after the applications. These were small collections of fluid of high protein content (as evidenced by staining reaction). The sequence of their formation was evident in some sections, and consisted of enlargement and finally liquefaction of some of the cells which had elongated following the application of heat.

The subsequent changes in the epithelium resembled those of H lesions closely. The necrotic epithelium was sloughed alone or with debris from the dermis. New epithelium growing from the periphery and from hair follicles (except where entire follicles had been killed) covered the surface.

The regeneration of epithelium was not apparent in any of the heat lesions until 7 days had elapsed. This differed from 5 to 10 minute mustard lesions, in which early regeneration was present on the fourth day. However, in lesions resulting from either heat or H, regeneration was

progressing on the 14th and complete on the 21st day.

Changes in the Corium:

Mustard Lesions:

Application of liquid H to the skin was followed by a characteristic series of changes in the corium. Twenty-four hours after exposure, there was moderate to marked dilation of the superficial capillaries and infiltration of a few polymorphonuclear leucocytes into the tissues around these vessels. At the sites where the exposure time had been 10 or 20 minutes, there were also many extravasated erythrocytes. The infiltration of leucocytes was more marked at the end of 48 hours, and the capillary dilatation was maximal.

The first evidence of necrosis of the corium from liquid H was noted in lesions which had had a 2 day recovery period. The changes consisted of a zone of relatively homogeneous eosinophilic material over a base of apparently viable tissue. This homogeneous structure represented what had appeared as a "crust" grossly. Incorporated into it were the leucocytes of the exudate (which had shrunk and appeared as small basophilic granules in the crust) and the tissue which had included the dilated capillaries.

When the regeneration of epithelium was apparent, the demarcation of necrotic debris from viable corium had already become established, and the crust existed as a sequestrum to be cast from the body. This crust appeared to persist over the proliferating epithelium, and may have helped to protect it from desiccation and external trauma. As its edges separated from the underlying new epithelium, keratinization began to appear in the more superficial epidermal cells, so that separation of the crust and keratinization of the new epithelium progressed simultaneously and each was completed at approximately the same time.

The extent to which new connective tissue formed beneath the ingrowing epithelium varied according to the depth to which the tissue had been destroyed. When the original defect was shallow only a thin layer of new connective tissue formed between the base of the lesion and the new epithelium. When the defect was deep a thick mass of granulation tissue formed under the new epithelium.

Heat Lesions:

The reaction of the corium following application of heat to the skin differed in several respects from the reaction to H. In this material there was recognizable necrosis in the corium as early as 24 hours after exposure to 66°C. for ½ minute. The depth to which the necrosis extended varied with the amount and duration of application of heat. It was considered to be superficial as long as the sweat glands and hair follicles were not entirely destroyed and the coagulum demarcated above the layer of fat cells.

All burns produced at 78°, and those resulting from 66° for 2 minutes, ultimately progressed to necrosis of the entire thickness of the corium. Only the sites exposed to H for 20 minutes showed necrosis of this degree, and this was not apparent until the 7th day after exposure.

Superficial hyperemia, hemorrhage, and exudation were less pronounced in thermal than in mustard lesions. A feature of thermal injuries not seen in mustard lesions was thrombosis of small veins and arteries in the zone of reversible reaction and occasional hyalin arterial necrosis. There was some indication that thermal have less tendency to residual inflammation of the repair tissue than do mustard lesions. Otherwise the repair of thermal injuries does not differ significantly either in manner or rate, from that of mustard lesions.

VIf. A Pathological Study of the Inhibition of Vesication by B.A.L.

by F.R. Dutra, A.R. Moritz and R.S. Myers (Surgical Consultant)

Purpose: To investigate the mechanism of the inhibition of vesication in mustard lesions treated by B.A.L.

Experiments:

Experiments on human subjects: See III (a-3, d-2, e-2, f-3, g, h-1, j-1). Bilateral abdominal applications of liquid mustard (1 mg. per sq. cm.) were made on each of four volunteers. At the conclusion of the exposures, the skin was decontaminated with pentane. One site on each subject served as a control and the other site was treated with B.A.L.

The treatment was commenced thirty minutes after decontamination and consisted of the application of two grams of 10% B.A.L. ointment. This was allowed to remain on the surface and the area was covered with a water-proof dressing for varying periods (see Table XVd). At different intervals after the conclusion of the treatment both sites were excised prepared for histological examination.

TABLE XVd

Subject	Exposure Time in min.	Treatment	Post Application Period	Type Injury	Fixed H per sq. cm. gammas	Ratios of Fixed
H628	10	BAL 48 hrs	48 hrs	No vesication	0.61	0.54
	10	Vaseline 48 hr	48 hrs	II* "	1.13	
H619	10	BAL 12 hr	24 hrs	No "	0.84	0.54
	10	Vaseline 12 hr	24 hrs	II* "	1.56	
H596	15	BAL 48 hrs	72 hrs	No "	1.67	0.63
	15	Vaseline 48 hr	72 hrs	II* "	2.60	
H613	20	BAL 24 hrs	96 hrs	No "	3.15	0.53
	20	Vaseline 24 hr	96 hrs	III* Marginal Vesication	5.90	
H630	1 min)Dry	BAL 48 hrs	72 hrs	No Vesication		
	1 min)Ice	Vaseline 48 hrs	72 hrs	II* "		

*A precise definition of Type I, II & III injuries is given in Section VIIa-1, page 58.

In both the treated and the untreated sites there was complete necrosis of the epidermis. However, the treated epidermis remained relatively compact and deeply acidophilic. Although there was early and complete loss of nuclear staining, the dead epidermal cells remained attached to one another and to the dermis and the liquefaction of the more deeply situated epithelial cells that characterized the untreated mustard lesions failed to occur.

The differences in the dermis of the treated and untreated lesions were most pronounced in and around the superficial blood vessels. The dermal capillaries and arterioles of the treated lesions were greatly dilated and appeared to be occluded by compact masses of partially disintegrated pyknotic erythrocytes. The walls of the affected vessels appeared granular and indistinct and perivascular hemorrhage was prominent. Small aggregations of fibrin were present in the interstitial spaces of the dermis of treated lesions.

Effect of B.A.L. treatment on thermal (cold) burn of human skin. Preliminary experiments disclosed that vesication of human skin regularly occurred between 12 and 24 hours after a contact exposure to dry ice of 50 seconds' duration. A human volunteer received bilateral abdominal exposures to dry ice for 50 seconds, and one site was allowed to go untreated as a control while the other site was treated with B.A.L. for 48 hours in the manner described in the preceding section.

At the end of 24 hours there was complete vesication of the untreated lesion and no vesication of the treated lesion. At the end of 72 hours the vesicle of the untreated lesion had collapsed and the epidermis of the treated lesion was still intact. The epidermis of the latter was opaque, everywhere attached to the dermis, had a yellow-gray cast and was separated from the surrounding normal skin by a narrow zone of erythema.

Microscopic examination of the lesions excised 72 hours after injury disclosed complete coagulation necrosis of the intact epidermis of the treated lesion with vascular changes in the dermis similar to those described in the case of treated mustard lesions. The detached epidermis of the untreated thermal lesion was completely necrotic and lay loosely over the exposed dermis.

Effect of B.A.L. treatment on mustard lesions in the pig. Three pairs of liquid mustard applications (1 mg. per 1 sq. cm.) were made to the skin of a young pig. The first pair of applications were for 10 minutes and the second and third pairs were for 20 minutes. One application of each pair was treated with 10% B.A.L. ointment according to the method of McMaster and Hogeboom (OMSr 434, Div. 9, NDRC: Informal Monthly Progress Report, No.v 10, 1943). The treatment was begun 30 minutes after decontamination and consisted of the application of 1 gram of 10% B.A.L. ointment which was rubbed into the skin for 30 seconds. The excess was allowed to remain on the surface. This procedure was repeated at hourly intervals for 6 applications. The sites were excised 12 hours, 30 hours, and 6 days after exposure.

No significant macroscopic differences were observed between the treated and untreated sites.

The treated and untreated lesions excised 12 hours after exposure showed no significant microscopic differences. There was diffuse nuclear degeneration throughout the epidermis and generalized engorgement of and localized hemorrhages around the dermal capillaries.

In the lesions excised 36 hours after exposure the necrotic epithelium of the treated site was compact and deeply acidophilic in contrast to the somewhat swollen amphophilic epidermis of the untreated site. Beneath the epidermis at the untreated site there was diffuse exudation of leucocytes. In places the damaged epidermis was separated from the dermis by collections of exudate. Exudation was much less pronounced at the treated site and nowhere was the damaged epithelium separated from its dermis. Capillary engorgement and perivascular hemorrhage was more pronounced beneath the treated sites and collections of interstitial fibrin were prominent.

In lesions excised 6 days after exposure there were no significant differences between treated and untreated sites. In both the necrotic epidermis was undermined by a lake of exudate. In both, the superficial portion of the dermis was necrotic and undergoing organization by granulation tissue.

Discussion:

The mechanism whereby vesication is inhibited by B.A.L. is unknown. The fact that vesicle formation at the sites of exposure to solid CO_2 is prevented by BAL indicates that the phenomenon is non-specific. The similarity between mustard vesicles and those produced by extreme cold is that both have a latent period of between 12 and 24 hours. Other vesicating agents (heat, cantherides) which have been tested have latent periods of less than 6 hours and are therefore not amenable to treatment by B.A.L.

The action of BAL may be to fix the injured epidermis to the underlying dermis by coagulation of proteins at the interface. That such coagulation does occur may be inferred from the changed appearance and staining reaction of the B.A.L. treated epidermis.

Another mechanism whereby fixation of protein may act to prohibit vesication is by preventing the liberation of hydrophilic degradation products from the injured tissue.

Still another possible explanation for the vesicle inhibiting effect of B.A.L. treatment is the manner in which B.A.L. affects the dermal blood vessels at the site of mustard or thermal injury. B.A.L. treatment results in early and severe damage to capillaries and arterioles which may interfere with circulation to such a degree as to prevent the exudation of fluid that is necessary for vesicle formation.

The question of why B.A.L. affects damaged tissues but does not affect normal skin deserves further investigation.

VII. Significance and Properties of Fixed-Mustard in Skin Sites of Man and Pig.

VIIa-5. Effect of B.A.L. on Fixed Mustard in Human Skin.

by F.C. Henriques, Jr. and C. Margnetti

Purpose: To determine the effect of B.A.L. on fixed mustard in human skin in which B.A.L. has prevented vesication.

Procedure: See Section VIf (page 56n)

Results: The date are given in Table XVd (page 56n).

Discussion: There can be little doubt that B.A.L. treatment reduces the amount of fixed mustard present in human skin tissues. There are two possibilities: either (1) the B.A.L. ointment dissolves some of the dead epiderme which contains fixed material; or (2) B.A.L. promotes the dinintegration of tissue substances containing fixed mustard and these substances are carried away by the body fluids. Conclusion (1) is held to be extremely unlikely since there is no histological evidence that any of the upper layers of the epidermis slough off during the B.A.L. treatment. Conclusion (2) is believed to be much more probable[a] In view of the stability of mustard alkalated compounds, the actual breaking of the mustard carbon bond by B.A.L. is extremely unlikely[b].

VIIa-6. The Relative Injury Propensities of Various Vesicants Related to Mustard.

by L.A. Patterson, F.R. Dutra, and F.C. Henriques, Jr.

Purpose: To study the relative injury producing abilities of mustard, benzyl-mustard, sesquimustard, mustard sulfone and divinyl sulfone.

Introduction: The correlation of the amount of fixed mustard to the degree of injury produced by mustard has been well established (section VII). This relationship is one of the principle reasons for the belief that the formation of fixed mustard is the direct cause of injury, that is, it is the initiator of the physiological reactions producing injury in the tissue. A study of other vesicants was undertaken to determine if the relationship between the amount of fixed material and the degree of injury still held.

(a) An attractive but highly speculative theory would be that a portion of the fixed mustard tissue constituents are directly responsible for vesication. These substances then have their properties sufficiently altered by reaction with BAL that the body is able to break them down and carry them away.
(b) One possibility, admittedly remote, would be the reaction of mustard attached to a methionine type sulfur (sulfonium type bond) with BAL to form "methionine" and the BAL mustard thioether linkage.

These various vesicants were chosen for these specific reasons, namely: Benzyl mustard because it is a one handed vesicant. Sesquimustard since its beta-chlorethyl groups are both widely separated and attached to different sulfurs. Mustard sulfone because it alkylates by a different mechanism than that of the above three vesicants. Divinyl sulfone since it is supposed to be the intermediate by which mustard sulfone alkylates.

Procedure: The preparation of the five radioactive vesicants is given in section II.

In view of the difficulty of obtaining uniform lesions with undiluted sesquimustard, mustard sulfone and divinyl sulfone, these radioactive compounds were dissolved in ethyl celloselve to give 1,5 and 10 per cent solutions.(a) Five mg. amounts of these vesicant solutions were placed in a shallow circular glass cup (2 sq. cm. in area and containing no filter paper - IIId-3). The remainder of the procedure was standard. See III (a-1, e-2, f-3, g, h-1, i, j-1.)

The procedure used with both mustard and benzyl-mustard is described in III (a-1, d-2, e-2, f-3, g, h-1, i, j-1).

The application periods were varied with each vesicant so that mild, moderate and severe lesions would result in each case. The post-application periods were of 48 hrs. duration.

Experimental Results: The data are given in Tables XXIa and XXIb.

Discussion: The correlation between the degree of injury and the amount of fixed vesicant for mustard, benzyl mustard, sesqui mustard, mustard sulfone, and divinyl sulfone is given in Table XXIa. The values for the amount of fixed material are expressed in 10^{-9} moles per sq. cm. of skin surface. The degree of injury to the tissue section was determined microscopically and the grading of the lesions was in some cases difficult, due to an unequal distribution of the injury in the tissue section.

All five of the agents showed a definite correlation of the degree of injury with the amount of fixed material. The amount of the fixed vesicant required to produce a given degree of injury has been summarized in Table XXIb. In two or three instances the limits for each type of injury could not be sharply defined because of the lack of sufficient data. Benzyl mustard and mustard are nearly identical in their injury producing ability; sesqui mustard is somewhat more potent. These three substances apparently react by the same mechanism with the tissue constituents. Mustard and benzyl mustard react through only one beta chloroethyl group. Sesqui mustard probably is capable of reacting to some extent through both beta chloroethyl groups because

(a) Experiments with ordinary mustard sulfone, divinyl sulfone, and their celloselve solutions, as well as with radioactive H and its celloselve solutions, have shown that celloselve has no effect on the lesions produced and the correlation between amount of fixed mustard and the degree of injury remains the same in the case of mustard. The use of celloselve effects a considerable saving of the radioactive agent.

TABLE XXIa

Correlation of Injury in Pig Skin with the Amount of Fixed Vesicant* Data derived from experiments on six pigs

Vesicant	Type of Injury with the Amount of Fixed Vesicant in 10^{-9} moles per sq. cm. of Skin Surface		
	Severe	Moderate	Mild
Mustard	57.0 20.0	5.7	0.9
	43.0 18.0	4.4	0.7
	41.0 14.0	4.1	0.25
	36.8 13.1	3.3	
	32.5 13.0	3.0	
	24.0 11.0	1.8	
	21.0 9.6	1.5	
		1.3	
Benzyl Mustard	64.0*	4.4*	0.4
	42.0*	3.1*	0.2
	29.0*	2.5*	0.1
	16.0*	1.9*	0.1
		1.9*	
Sesqui Mustard	7.4 3.0	1.9	1.5
	6.6 2.2	1.1	1.2
	4.1 2.1		0.6
	3.7 2.1		0.5
	3.2 2.0		
	3.2		
Mustard Sulfone	62.0 54.2	32.6	7.5 2.2
	58.7 52.1	28.0	3.3 2.1
	57.7	20.0	3.6 1.9
		12.8	2.8 1.3
Divinyl Sulfone	142.0 83.8	25.3	10.3 5.8
	116.5 83.4	24.6	8.7 4.9
	105.0 62.1		7.1 4.4
	93.7 38.5		6.2 4.0

*Data derived from experiments on six pigs

TABLE XXIb

Comparison of Amounts of Each Vesicant in 10^{-9} moles per sq. cm. of Skin Surface Required to Produce a Certain Injury

Vesicant	Type of Injury		
	Severe	Moderate	Mild
Sesqui H	>2.0	1.5-2.0	<1.5-1.0
Benzyl H	>6.0*	1.0-6.0*	<1.0*
Mustard	>6.0*	1.0-6.0	<1.0
H Sulfone	>40.0	10-40	<10.0
Divinyl Sulfone	>40.0	10-40	<10.0

*These values were estimated by careful individual grading of the mustard and benzyl mustard histological sections and a comparison of the amounts fixed.

of the remoteness of the two chloro groups from each other. Such a situation would explain the increased potency of sesqui mustard over the other two agents. The similarity of these three substances bears out more than ever the importance of the role that the fixed material plays in causing injury in the skin tissue.

Mustard sulfone and divinyl sulfone were found to have only about one-seventh the potency of mustard in producing injury for an equivalent amount fixed in the tissue. Since they react by a different mechanism than mustard and do not have the same relative reactivities towards tissue groups, this divergency is not surprising. Apparently the mustard type vesicants are more selective in attacking the vital groups.

The marked similarity of divinyl sulfone and mustard sulfone in their injury producing ability indicates that mustard sulfone reacts through the divinyl sulfone intermediate in the cutaneous layer.

VII-b. Certain Properties of Fixed Mustard.

VIIb-4. Enzyme Hydrolysis of the Malphigian Layer of Pig Skin Containing Fixed Mustard.

by L.A. Patterson, C. Margnetti and K. Lynch

Introduction: Mustard reacts with several groups present in the constituents of skin tissue. One or more of these tissue group combinations with mustard in the form of fixed mustard may be responsible for initiating the physiological reactions which cause tissue injury. With the aid of radioactive sulfur and using the isotope dilution method it should be possible to identify many of the substances in the tissue containing mustard attached to them. Much of the mustard fixed in the tissue is undoubtedly attached to proteins. Hydrolysis of the protein in the tissue in such a manner as to keep the mustard attached to the amino acid residue would enable one to ascertain the nature of the linkages between mustard and protein. Du Vigneaud has hydrolyzed insulin containing fixed benzyl H* or butyl H* with a 1:1 HCl-HCOOH mixture and found 3% of the vesicant residues were attached to the amino group of phenylalanine (Du Vigneaud, Informal Report, August 10, 1943). Although it was shown not to be true in the case of phenylalanine, some amino acid vesicant residues could probably be formed during the hydrolysis procedure (due to the formation of mustard from thiodiglycol by the reaction medium). Alkaline hydrolysis of the tissue would convert nearly all of the fixed mustard to thiodiglycol.

Proteolytic enzymes might split the proteins under mild conditions without the formation of appreciable amounts of thiodiglycol, so we have performed some preliminary work along this line, testing the action of pancreatin on the fixed mustard of the malphigian layer(d) of the pig skin.

EXPERIMENTAL

Preparation of Malphigian Layer Containing Fixed Mustard. One side of a shaven pig was treated with 12 applications of 900 γ of radioactive mustard in circular shallow cups 4.5 sq. cm. in area for 10 minutes at 70°F (this procedure gives only mild injury to the tissue and prevents an overloading of the tissue with fixed mustard). The sites were decontaminated with petroleum ether. The pig was sacrificed 30 minutes after the exposure to mustard had been completed. The skin of the pig was removed and the malphigian layer isolated in the manner described in section IIIm-2. The malphigian layer after being dialyzed against water containing merthiolate to remove all permeable substances was washed with cold acetone and dried under vacuum in the cold.

Enzymatic Hydrolysis of Malphigian Layer. (1) Determination of Permeable Material Formed. The method of tissue hydrolysis was essentially that used for the pancreatin hydrolysis of casein to

(d) The malphigian layer was used because it probably contains the fixed mustard material which is responsible for the damage to the skin tissue. The fixed mustard in the epidermis (dead cells) represents 70% of the total in the skin tissue and probably has no significance.

tryptchane (Org. Syntheses, Col. Vol. II). Two 50 mg. samples of malphigian tissue powder were each placed in a flask with 3 ml. of distilled water, 1 ml. of sodium carbonate-sodium fluoride solution (prepared from 60 g. of anhydrous sodium carbonate, 6 g. of sodium fluoride, and 1 liter of water), and 5 drops of toluene. To one of the flasks was added 50 mg. of Merck's pancreatin. The flasks were allowed to stand with occasional shaking at 37°C, during which time the tissue in the flask containing the pancreatin gradually disappeared. The contents of each flask were washed into a cellophane dialysis bag with 4-5 ml. of water. The contents of the dialysis bags were dialyzed four times with a 24 hr. dialysis period, each time using 30 ml. portions of water containing toluene (temperature 10°C). The permeates were evaporated and the amount of radioactive material determined. The impermeates of the dialysis bags were also analyzed.

(ii) Determination of Amount of Thiodiglycol Formed. Malphigian tissue weighing 775 mg. of 6 ml. of water and 2 ml. of sodium carbonate-sodium fluoride solution was treated with 200 mg. of pancreatin. The mixture containing a few drops of toluene stood at 37°C for four days, then 150 mg. more of pancreatin was added; finally 100 mg. of pancreatin was added at ten days. At the end of the sixteenth day the contents of the flask were filtered through a coarse fritted glass funnel packed with asbestos. The residue was washed first with 1 ml. of water (washing added to filtrate), then several times with water. The latter washings were analyzed for radioactive material, as was the residue in the filter mat. The filtrate (hydrolysate), by adding ordinary thiodiglycol carrier to it and extracting the thiodiglycol with chloroform, was analyzed for the percentage of radioactive thiodiglycol according to the method given in the section IIIj-3.

Experimental Results. The data are given in Table XXIVa.

TABLE XXIVa
Hydrolysis of Malphigian Tissue Containing Fixed Mustard with Pancreatin at pH 11.0

Run A (10 days at 37°C)		
	%S* in Impermeate	%S* in Permeate
With Pancreatin	17	83
Without Pancreatin	37	63

Run B (16 days at 37°C)		
%S* in Tissue Residue	%S* in Hydrolysate	%S* as Thiodiglycol
1	99	28

Discussion: The data in Table XXIVa show that pancreatin at pH of 11 in 10 days will convert 83% of the fixed mustard of the malphician layer into permeable molecules while by buffer action alone 63% was rendered permeable. In 16 days all of the fixed material was made soluble (the percentage of this that was permeable was not determined but it was probably between 90 and 100%), 28% going to thiodiglycol.

Unfortunately the hydrolyses unintentionally were carried out at a pH of 11.(e) The use of the optimum pH of 8-9 for pancreatin or the use of other proteolytic enzymes at a lower pH would undoubtedly lower the percentage of thiodiglycol formed to a much lower value.

These two preliminary experiments indicate that enzymes will hydrolyze proteins containing fixed mustard to amino acid residues with most of the mustard still attached, thus furnishing an approach to the identification of the foregoing mentioned substances.

(e) In using the hydrolytic medium employed in Organic Syntheses for the preparation of tryptophane from casein, we assumed it gave the optimum pH.

VIIc-a. The General Mechanism of Mustard Injury.

(i) In section VIIc it was shown that the experimental data given in section VIIa indicate that mustard injury is initiated by the following reaction, namely:

(1) $H + X \longrightarrow HX$ (fixed mustard) where H is mustard and X denotes impermeable or insoluble constituents present in tissue which remains impermeable or insoluble when reacted with H. The actual fixation process is the alkylation by mustard of certain specific groups of the tissue constituents, X.

To the evidence given in sections VIIa-1 to VIIa-4 should be added that of section VIIa-6, namely: On a molar basis of fixed vesicant, there is no qualitative or quantitative difference between the degree of tissue injury in pig skin produced by either benzyl mustard or mustard.

This data also indicates that the skin injury produced by the mustard is due to alkylation via only <u>one</u> of the beta chlor-ethyl groups. Thus the "intrinsic" vesicantcy of mustard is <u>not</u> effected by the theoretical possibility of the cross bridging of proteins through double alkylation. Since on a molar basis of fixed vesicant sesquimustard is apparently slightly more potent, there is some evidence of double alkylation in this particular case.

(ii) The question was also raised in section VIIc as to whether or not the removal of fixed-H after the appearance of injury shortens the time of healing, i.e., is fixed-H intrinsically toxic? The fact that B.A.L. reduces the amount of fixed mustard in human tissue by about 50% but does not decrease the healing time (section VIIa-5) indicates that fixed-H is probably not "intrinsically" toxic. Thus after the breaking of the chain of some metabolic process by the fixation of H produces cell death, the healing rate of this injured tissue is simply a matter of removal and repair. This is further evidenced by the fact that for comparable degree of injury there is no difference in the healing time of skin damage produced by either mustard or heat (see section VIe).

VIII. Miscellaneous Animal Experiments.

Bad

VIIIa-4. A Method of Increasing the Rate and Severity of Cutaneous Injury by Mustard

by F.R. Dutra and L.A. Patterson

Purpose: To investigate the possibility of increasing the injury-producing capacity of mustard by combining it with certain other substances. Information gained from previously reported penetration studies suggests that it should be possible to increase the rapidity and severity of injury if mustard were combined with an agent which was freely miscible with it and which would penetrate the skin more rapidly than mustard alone.

Experimental: See III(a-1, b, d-3, f-3, g). Because, it was contemplated to test certain substances that are not miscible with pentane (the decontaminant which has been used by us in previously reported experiments) it was thought desirable to use some other agent for decontaminating the skin after exposure to test mixtures. A series of experiments were carried out in which the decontaminating effectiveness of acetone, dechloramine T in absolute alcohol aqueous perphthalic acid, a mixture of green soap and sodium hypochlorite, and sodium hypochlorite in water containing tergitol were compared with pentane. The effectiveness of these decontaminants was judged by comparing threshold mustard burns that had been treated with the decontaminants being tested with threshold mustard burns treated with pentane. It was found that a 5% solution of sodium hypochlorite in water containing a small amount of tergitol (a wetting agent) was as effective a decontaminant for pure mustard as pentane and in addition, was a good decontaminant after exposure to the various mixtures of mustard with other agents which could not be removed from the skin with pentane.

Applications of a variety of mustard-containing mixtures were made to the skin of young pigs in an effort to determine whether or not any of them gave promise of being better than pure liquid mustard. Substances compared with mustard were: acetyl chloride, triethanol amine, phenacyl chloride, dichlorobenzalacetone, dibromobenzalacetone, monobromacetone, monochloracetone, allyl isothiocyanate, dimethyl sulfate, methyl salicylate, benzyl chloride, nitrobenzene, ethyl cellosolve, dichloracetone, formic acid in several concentrations, and several mixtures of formic acid and ethyl cellosolve.

It was found that certain combinations of ethyl cellosolve and mustard produced lesions more rapidly and of greater severity than were produced by comparable exposures to pure mustard, although ethyl cellosolve alone is non-irritating. The experiments were conducted in a constant-temperature room maintained at 50°F. Five mg. of H or an equal volume of the mustard mixture was applied in triplicates by means of shallow circular cups fitted with a filter paper and having an area of 2 sq.cm. Exposed sites were swabbed immediately at the end of exposure time with 4 cotton swabs soaked with the aqueous sodium hypochlorite containing tergitol.

Exposed sites were examined at the end of 24 and 48 hours and the injuries graded as follows:

0 - No reaction

+ - Faint but definite cup-sized area of erythema

++ - Intense erythema - blanched by pressure

+++ - Cup-sized hemorrhagic lesion

++++ - Hemorrhagic necrosis and edema

Discussion: The results of the experiments in Table XXIVb show that a 1% solution of H in ethyl cellosolve is just as effective as pure H alone is in producing lesions on a pig when applied for the same length of time. It was found that mixtures containing up to 10 parts of H to 90 parts of cellosolve were likewise just as effective as pure H. However, when mixtures containing 20 to 30 parts of H were applied the time required to produce an injury comparable with pure H was reduced by a factor of 2. Lesions resulting from applications of such mixtures for the same time intervals as for pure H were found to be far more severe than those produced by the latter. When the ratio of H to cellosolve reaches nearly 1:1 the mixture is again only as effective as pure H. Thus, there is a maximum in the curve when the concentration of H in the cellosolve is plotted against the time required to produce injury. This maximum occurs at about 25 parts by volume of H.

Since cellosolve is known to be a rapid skin penetrant, it is probably it is probably carrying the mustard through the skin at an augmented rate. There is also the possibility that the cellosolve increases the amount of mustard fixed at the local application site, thereby increasing the severity of the injury.

In certain experiments, pure cellosolve was applied by means of a cup to three application sites for a period of 2 minutes; these cellosolve applications were immediately followed by 2 minute triplicate applications of pure mustard. No increase in the severity of the lesions as compared to the controls could be observed. These experiments indicate that cellosolve is not altering the state of the epidermis so as to increase the penetrability of H "per se."

TABLE XXIVb

A Comparison of Injuries Produced on Skin of Pigs by Mustard and Mustard-Cellosolve Mixtures

Pig No.	Agents Applied in Triplicate-% by Volume Mustard	Et. Cellosolve	Application Time in Min.	No. of Lesions in Each Category of Injury 0	+	++	+++	++++
528-R	100	0	2	3				
529-L	100	0	2	3				
529-R	100	0	2	3				
530-L	100	0	2	3				
528-R	50	50	2		2	1		
529-L	30	70	2	1	1		1	
529-R	30	70	2	1	2			
530-L	30	70	2		3			
529-L	20	80	2	1	2			
529-R	20	80	2		3			
530-L	20	80	2		3			
529-R	10	90	2	3				
528-R	1	99	2	3				
528-R	100	0	5	1	2			
529-L	100	0	5	1		2		
529-R	100	0	5		3			
530-L	100	0	5		2	1		
528-R	50	50	5		2		1	
529-L	30	70	5					3
529-R	30	70	5					3
530-L	30	70	5				2	1
529-L	20	80	5				2	1
529-R	20	80	5				1	2
530-L	20	80	5				1	2
529-R	10	90	5			1	2	
528-R	1	99	5	2	1			
529-L	100	0	8				2	1
529-R	100	0	8				3	
530-L	100	0	8					3
		Average Values						
	100	0	2	*				
	30	70	2		*			
	20	80	2		*			
	100	0	5		*			
	30	70	5					*
	20	80	5					*
	100	0	8				*	

Bad

IX. Certain Chemical Experiments.

IXc. Some Investigations Pertaining to Sulfonium Salts.

by L.A. Patterson and F.C. Henriques, Jr.

Introduction: In section IVa-2a it was shown that the maximum amount of the sulfonium salt (DH-TG) present in the pig skin tissue extractables following mustard application is trivial, namely, 2.5%. In the case of human exposures to mustard, the amount of extractable radioactive sulfur is extremely small under all circumstances and thus it is concluded that aside from the 12% of the penetrated mustard that is fixed at the site, the remaining 88% is carried away immediately by the body fluids (see section IVc and V). Therefore it is of interest to determine the quantity of sulfonium salt (DH-TG) that is formed when small concentrations of mustard are allowed to stand in both blood and water.

Experimental:

Formation of DH*-TG* during Hydrolysis of 0.0006 M. Mustard* in Blood Plasma in Vitro. To a vial containing 3 cc of blood plasma at 37°C was added 0.3 cc of alcohol containing 276 γ DH*. The vial stood at 37°C for 30 minutes. A 1 cc aliquot was removed, diluted to 10 cc and analyzed for the total amount of radioactivity by taking two 1 cc aliquots of the diluted solution. Another 1 cc aliquot was analyzed for DH*-TG* by placing it in 15 cc of cold acetone, filtering off the precipitated material, and carrying the acetone filtrate through the procedure described in section IIIj-3a for the analysis of DH*-TG* after removing two 1 cc aliquots for analysis to obtain the amount of acetone extractables. The 30 cc of pet. ether extract was analyzed for DH* by taking a 5 cc aliquot, adding 1½ mg. of DH carrier, shaking it with 3 cc of conc. nitric acid, removing the pet. ether on a steam bath, and taking two 1 cc aliquots of the nitric acid layer. The results are given in Table XXXi.

Formation of DH*-TG* in the Hydrolysis of 0.0006 M. Mustard* in Water. To 3 cc of water at 37°C was added about 300 γ of DH* in 0.3 cc of alcohol. The reaction mixture was allowed to stand 30 minutes at 37°C. A 1 cc aliquot was removed and diluted to 10 cc and analyzed for radioactivity by taking two 1 cc aliquots of the diluted solution and adding enough DH, TG, and DH-TG carriers to give 7 mg. of benzidine sulfate. Another 1 cc aliquot was added to 5 cc of ice cold DH-TG solution in a large test tube. The tube was quickly shaken with 1 cc of water containing 40 mg. of picrylsulfonic acid. The solution was then extracted with 20 cc of nitromethane. The DH-TG picrylsulfonate was isolated and analyzed for radioactivity as previously described. To obtain the actual amount of DH*-TG* formed it was necessary to subtract 1.3 γ from the value obtained in the experiment as that much was formed under the conditions of the experiment. The results are given in Table XXXii.

Determination of Sulfonium Salts* Formed in the Hydrolysis of 0.0009 M. Mustard* by Determining the Amount of Thiodiglycol* Formed. To 5.9 cc of water at 30°C was added 0.1 cc of alcohol containing 769 γ DH*. A 1 cc aliquot was removed, diluted to 10 cc with water and the radioactivity determined on two 1 cc aliquots of the diluted

TABLE XXXI

Formation of DH*-TG* during the Hydrolysis of 0.0008 M. Mustard* in Blood Plasma in Vitro at 37°C. Time of Hydrolysis, 30 minutes.

γDH* added to Plasma	Extractables (in γDH*) in Acetone	γ DH* in Pet. Ether (unreacted)	DH*-TG* (as γDH*) formed (corrected)	% of added DH* as DH*-TG*	% of extractables as DH*-TG*	% of reacted DH* as DH*-TG*
92γ	71γ	43γ	2.2γ	2.4%	3.1%	4.5%

TABLE XXXII

Formation of Sulfonium Salts* in the Hydrolysis of Dilute Solutions of DH* in Water

Molarity of DH*	t°C	Time of Hydrolysis (minutes)	Method used to determine Sulfonium Salts*	% of DH* reacting	% of DH* added going to Sulfonium Salts
0.0006 M.	37	30	(b)	100%	4.4%(d)
0.0008 M.	30	30	(c)	100%	4.7% (e)
0.0008 M.	30	80	(c)	100%	4.4% (e)
0.01 M.	25	70	(c)	97%	10.4% (e)

(b) Analyzed directly for DH*-TG*.
(c) Analyzed indirectly for solfonium salts* by determining the amount of TG* formed.
(d) Determined as DH*-TG*. The correction factor of 1.4γ was used in computing this value.
(e) Determined as total sulfonium salts*. The value may be low due to exchange reaction between DH*-TG* and TG.

solution. At thirty and eighty minutes 2 cc aliquots were removed and analyzed for thiodiglycol as follows. The aliquot was shaken in a separatory funnel containing 200 mg. of thiodiglycol dissolved in 2 cc of chloroform. The chloroform layer was removed and discarded. Three more extractions with 2 cc portions of chloroform were carried out and the extracts discarded (this procedure removes any DH* and CH* present). Two 0.03 cc aliquots of the water layer were removed and the amount of radioactivity per milligram of benzidine sulfate determined. This analysis gives the amount of TG* and sulfonium salts* present. The remainder of the water layer was extracted five times with 10 cc portions of chloroform. The thiodiglycol was isolated from the chloroform extracts, the p-nitrobenzoate prepared and analyzed for radioactivity according to the method given in section IIIj-3. The amount of sulfonium salts* is obtained by subtracting the amount of TG* found from the radioactivity in the water layer after its extraction with chloroform. The results are given in Table XXXII.

Determination of Sulfonium Salts* Formed in the Hydrolysis of 0.01 M. Mustard* by Determining the Amount of Thiodiglycol Formed. The same method was used as that given for the analysis of sulfonium salts* in

the hydrolysis of 0.0008 M. mustard*. A mixture of 7 mg. of DH and 1 mg. of DH* in 0.2 cc of alcohol was added to 4.8 cc water and shaken for 70 minutes at 30°C.

Results: The experimental data are given in Table XXXII.

Discussion: DH* was hydrolyzed in water at molarities of 0.0006, 0.0008, and 0.01 (see Table XXXII). At a 0.0006 M. concentration 4.4% of the DH* went to DH*-TG*. This value checks that obtained for blood plasma (considering only the DH* which reacted, see Table XXXI). At 0.0008 M. the mustard went to 4.5% sulfonium salts (of all types). The method used in this latter determination involved the determination of the amount of TG* formed after removing the DH* and CH*. The residual material was considered to be sulfonium salts (see experimental section). The value obtained by this method might possibly be low if the exchange reaction DH*-TG* + TG $\rightleftharpoons$ DH* - TG + TG* takes place to any appreciable extent when the carrier TG is present under the conditions of the experiment. If it does not, the value of 4.5% for the sulfonium compounds* checks that obtained for the DH*-TG* when the hydrolysate of 0.0006 M. mustard* was analyzed by a different method. It also shows that the DH*-TG* compound is formed almost exclusively at these low concentrations. At a concentration of 0.01 M. mustard* to the amount of 10.4% went to sulfonium salts*. The larger amount of sulfonium salts formed here is to be expected in view of the higher concentration of thiodiglycol present in the hydrolytic medium.

Further Data Pertaining to the Sulfonium Salt. During the development of the analytical procedure (see IIIj-3a) used in the determination of the amount of DH*-TG* formed in either pig skin tissue, blood or water, certain additional data regarding the sulfonium compound was obtained, namely:

The DH*TG sulfonium compound appears to exist in the equilibrium with DH and TG in its solutions.

$$DH - TG \rightleftharpoons DH + TG$$

In acetone and alcohol the equilibrium rests to the right. This fact became apparent when long standing alcohol and acetone solutions of equivalent amounts of mustard and thiodiglycol were analyzed for chloride ion. Only 17% of the theoretically possible DH-TG was formed in alcohol, acetone gave only 3%. Additional evidence for the existence of an equilibrium mixture was obtained when DH*-TG(a) was allowed to stand in 90% alcohol and 90% acetone. The shaking of aliquots removed at various time intervals with benzene and water caused the DH*-TG to enter the water layer which was analyzed for radioactivity. As the equilibrium shifted more and more DH* went into the benzene layer. The non-radioactive thiodiglycol did not enter into the analysis. In water the equilibrium rests far to the left so that there is probably nearly 100% DH-TG present. Evidence for this was obtained by extracting aqueous DH*-TG repeatedly with pet. ether. No appreciable amount of DH* could be extracted.

(a) The preparation of DH*-TG is described in footnote (c) of section IIIj-3a.

TITLE: Additional Studies Pertaining to the Mechanism of Cutaneous Injury by Mustard Gas. An Experimental Study Using Mustard Prepared with Radioactive Sulfur

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ABSTRACT:

Investigations of the mechaism of mustard vesication were made primarily through parallel histological and chemical studies of skin exposed to mustard and related compounds prepared with radioactive sulfur. Details of the synthesis of benzyl mustard, sesqui-mustard, mustard sulfone, and divinyl sulfone are given. These tests were conducted to determine the following; the penetration rate of mustard through pig, rabbit, and human skin; the patholog of cutaneous injury following exposure to mustard; the significance and properties of fixed mustard in skin sites of man and pig; and a method of increasing the rate and severity of cutaneous injury by mustard. Additional investigations were made pertaining to the sulfonium salt, DH-TG.

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