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"THE MECHANISM OF CUTANEOUS INJURY BY MUSTARD GAS. AN EXPERIMENTAL STUDY USING MUSTARD PREPARED WITH RADIOACTIVE SULFUR."

to
November 10, 1943
by
F. C. Henriques and A. R. Moritz, Official Investigators
and
H. S. Breyfogle and L. A. Patterson.

Report OSRD No. 3620
Copy No. 65
Date: May 9, 1944

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Division 9
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Section 5

"THE MECHANISM OF CUTANEOUS INJURY BY MUSTARD GAS.
AN EXPERIMENTAL STUDY USING MUSTARD PREPARED WITH
RADIOACTIVE SULFUR."

Service Directive CWS-2

Endorsement (1) Homer W. Smith, Division Member in Charge to W. R. Mirror, Chief, Division 9 .

Forwarding report and noting:

"This report presents in full the results of over three years study of the penetration and reaction of mustard gas, containing radioactive sulfur as a tracer, into the skin of animals and man.

"Among the more notable facts demonstrated are that:

(a) In man, a small fraction of the penetrating mustard rapidly reacts with the epidermis and corium to produce the cell injury that later leads to vesication, while the remainder is rapidly carried away by the circulation. At no time is there a significant quantity of free mustard in the skin, and hence therapeutic attempts based on destruction of penetrated mustard must necessarily be valueless. This is equally true of light and massive contamination.

(b) In the pig, and to a lesser extent in the rabbit, a considerable reservoir of unreacted mustard is present in the skin for some minutes after exposure, and hence agents designed to destroy free mustard in the skin can to some degree be effective when applied up to 10 minutes after surface contamination. The transfer of therapeutic experiments from these animals to man is therefore unwarranted.

(c) Extensive evidence points to the conclusion that it is the rapidly completed, chemical combination of mustard with one or more cellular proteins (bound mustard) that is responsible for cellular injury.

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Endorsement (Continued)

(4) Since intradermal decontamination offers no hope in man, possibilities for specific therapy are limited to reversal of the chemical reactions between mustard and the essential cellular proteins. The present studies, as well as those of other investigators, indicate that such reversal, under conditions compatible with the life of the cell, presents a very difficult if not impossible problem. Hence the therapy of mustard vesication is in practice limited to rapid and complete surface decontamination.

(5) The rate of penetration increases in direct relationship to the room temperature, probably in consequence of physiological alterations in the skin rather than of local temperature changes at the site of penetration, this increased penetration rate accounting in part for the increased vesicant power of mustard at high environmental temperatures.

"Among other important contributions of this work are pathological studies of a complete, progressive series of mustard burns in man, obtained by biopsy, and a comparison with a similar series from the pig and rabbit, data on the absolute rates of penetration in man, the pig and rabbit, and data on the distribution of fixed mustard in the epidermis and corium and in the nuclear tissue of the epidermis.

"Although the main conclusions of the study are discouraging, so far as the hope of obtaining a specific antidote for mustard is concerned, it is of great value in the vesicant problem, in that it establishes a precise frame of knowledge relative to the limits and requirements basic for decontamination and specific therapy."

(2) from W. 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 9-73, NDCrc-169 with The President and Fellows of Harvard University.

The Mechanism of Cutaneous Injury by Mustard Gas. An Experimental Study Using Mustard Prepared with Radioactive Sulfur

Formal Progress Report

Contract NDCrc 169

Division 9 N.D.R.C.

to

November 10, 1943

by

F.C. Henriques, Jr. and A.R. Moritz (Official Investigators)

and

R.S. Breyfogle and L.A. Patterson

This report is based upon experiments reported in the Informal Monthly Progress Reports on Physiological Mechanisms of Chemical Warfare Agents, Section 9-5 of Division 9, N.D.R.C. (Formerly Section B4-C of Division 8).

INTRODUCTION

The investigation of the mechanism of vesication by mustard through the incorporation of radioactive sulfur into the mustard molecule was suggested by J.B. Conant in November of 1940.

i) Personnel:

Since 1940 the following persons have participated in the work:

Chemical:	
R.S. Halford	January 1941 to August 1941
*F.C. Henriques, Jr.	April 1941-
*G.B. Kistiakowsky	November 1940 to July 1942
C. Margnetti	January 1942 to June 1942--June 1947 -
L.A. Patterson	February 1942-
W.G. Schneider	September 1941 to June 1942
A.K. Solomon	November 1940 to April 1941
J.R. Weisiger	July 1943-
With the assistance of:	
R. Arentzen	August 1942 to March 1943
I. Berman	July 1942 to September 1942
K. Lynch	October 1942-
Pathological:	
H.S. Breyfogle	August 1942 to June 1943
F.R. Dutra	July 1943-
*A.R. Moritz	November 1940-
Surgical:	
R.S. Myers	November 1942-

*Official Investigators:	
G.B. Kistiakowsky	January 1941 to July 1942
A.R. Moritz	August 1942-
F.C. Henriques, Jr.	September 1943-

ii) Scope and Period of these Investigations:

The experiments herewith reported comprise an investigation of the mechanism of vesication through parallel histological and chemical studies of skin exposed to mustard prepared with radioactive sulfur. Thus the report is concerned primarily with:

(1) The rate at which mustard penetrates the skin;
(2) The subsequent fate of the penetrated mustard in the skin;
(3) The significance of these chemical data in relation to the production of injury.

Since the report is intended to integrate the more important experimental findings that have appeared in various monthly Informal Progress Reports, no attempt has been made to present the data in chronological order.

iii) The Radioactive Sulfur (Preparation and Purity).

To date there have been two sources of radioactive sulfur of 88 day half life, namely the cyclotrons at Berkeley, California, and Washington University, St. Louis, Missouri. In both places the preparations were made under the direction of Dr. Martin Kamen of the Radiation Laboratory, Berkeley, California.

Cl^{35} Radioactive sulfur, S^{35} is prepared by the neutron bombardment of as CCl_4.

$$17Cl^{35} + 0n^{1} \longrightarrow 16S^{35} + 1H^{1}$$

The resulting radioactive sulfur is oxidized to sulfate ion with NaOBr and precipitated as $BaSo_4$. The details of this preparation have been recently published by M.D. Kamen(1).

Four different samples of radioactive sulfur were used in the course of this work. The specific radioactivity of these samples varied from about 0.5 millicuries to 10 millicuries(a) per 100 mg. of $BaSO_4$.

The radioactive sulfur prepared by the above procedure is always contaminated with about 5% radioactive phosphorus (half life = 14 days) as barium phosphate. Since the beta particle emitted from P^{32} is 16 times as energetic as that from S^{35}, it is a simple matter to detect minute percentages of P^{32} in the presence of S^{35}. In view of the numerous steps required to produce mustard from this barium sulfate (see 1), the resulting mustard never contained any detectable P^{32}.

iv) Some General Considerations Regarding Radioactive Sulfur.

(a) Concentration of the radioactive element.

The number of micromoles of any radioactive element present in a sample is given by the following equation:

$$\text{no. micromoles} = 7.5 \times 10^{-6} \times (\text{half-life in days}) \times \text{millicuries}$$

Even in our most radioactive sample (10 millicuries per 100 mg. $BaSO_4$), the total number of sulfur atoms potentially radioactive was only 1 in 10^8.

Thus the gross chemical, physical and physiological properties of mustard produced from $BaSO_4$ containing a trace of S^{35} are identical with pure thiodiglycol mustard.

(b) Possible pathological effects due to the emission of beta particles by radioactive sulfur.

It has been shown in the case of X-rays that the amount of energy required to produce erythema is 10^4 ergs per sq. cm.(2) The number

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(a) A millicurie is defined as 3.7×10^7 atomic disintegrations per second.

of sulfur particles emitted per sq. cm. of skin surface inside the cutaneous layer is never greater than 10^5 per minute or 20 ergs per day. Thus it would appear that injury due to the radioactivity of the sulfur is unlikely.

(c) The possibility of metabolic discrimination between S^{35} and natural occuring isotopes of sulfur.

The percentage of natural occuring isotopes in sulfur is S^{32} 95%; S^{33} 0.74%; S^{34} 4.2%; S^{36} 0.16%. To date there is no evidence that a biological system can fractionate the natural occuring isotopes of sulfur or, for that matter, the isotopes of any other element.(b) Thus S^{35} until the time of disintegration is indistinguishable in any metabolic process from the natural occuring isotopes of sulfur.

v) Glossary

(a) H, mustard:
Unless stated to the contrary, these words may denote in this report mustard containing radioactive sulfur.

(b) Radioactivity, mustard sulfur, S^{35}:
Detected as beta particles due to the disintegration of radioactive sulfur originally incorporated into the mustard molecules.

(c) H-equiv. or mustard equivalents:
The amount of mustard sulfur present in any sample in terms of the original mustard by weight (usually gammas).

(d) Extractables:
The amount of mustard sulfur extractable from a tissue by a solvent, usually chloroform. This mustard sulfur is always expressed in H-equiv.

(e) Fixed-H, fixed-mustard, fixed-material:
The amount of mustard sulfur not extractable from a tissue by either cold isotonic salt solution or hot or cold chloroform, acetone or alcohol. This mustard sulfur is always expressed in H-equiv.

(f) Gammas of fixed and/or extractable-H per sq. cm. of skin:
The amount of mustard sulfur present as fixed and/or extractable inside a skin site one sq. cm. in area.

(g) "Threshold" value of fixed-mustard:
The amount of fixed mustard in H-equiv. per sq. cm. of skin surface in a skin site associated with minimum irreversible injury.

(b) Schoenheimer and Rittenburg[16] have shown that relative abundance of the stable nitrogen isotopes is identical in amino acids, proteins and air; Dole[17] has shown that the ratio of Deuterium to Hydrogen is the same in water, cholesterol and honey.

(h) Exposure Time, exposure period, application time (period):
The period for which the skin was exposed to mustard.

(i) Post application time (period), post-exposure period (time):
The interval between termination of the exposure period and the excision of the exposed site.

(j) Carrier, ordinary, non-radioactive:
The use of a compound (usually mustard and thiodiglycol) not containing any radioactive sulfur.

SUMMARY

Introduction.

(1) There is no evidence that the injury producing propensity of mustard is in any way altered by the presence of radioactive sulfur.

I. Preparation of Mustard.

(1) Through the use of high vacuum technique, mustard of excellent purity containing S^{35} can be prepared in better than 90% yields from as little as 50 mg. of $BaSO_4$.

II. An Analytical Technique for Measuring Radioactive Sulfur.

(1) A method is described whereby radioactive sulfur can be measured to an accuracy of 2%.

IV. Penetration Rate of Mustard Through Pig, Rabbit and Human Skin.

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

(1) Mustard applied in liquid form penetrates rabbit skin faster than the skin of either man or pig. With sufficient mustard to saturate the skin surface, at a room temperature of 60°F, the amounts penetrating per sq. cm. of skin surface for man; pig; rabbit are 130/40/360 gamma per hour. At 100°F the respective rates are 330/250/850 gamma per hour. At average room temperature (70°F) these respective penetration rates become 200/100/500 gamma per hour.

(2) The temperature coefficients of penetration rates for man and rabbit are about the same (1.3/10°F of room temperature.) That for pig is considerably higher (1.6/10°F of room temperature.)

(3) At the termination of a one hour application, both pig and rabbit have an appreciable reservoir of unreacted mustard (about 50% of the "extractables") as compared to man. Man's reservoir may be considered negligible. None of the animal tissue sites has significant quantities of "extractables" 24 hrs. after an exposure.

(4) In man, pig and rabbit the amount of mustard fixed at the portal of entry is in direct relation to the amount that has penetrated. About the same percent of the amount penetrating is fixed in rabbit (10%) and in man (12%). With pig the amount fixed is 25%. These percentages are invariant with respect to environmental conditions.

(5) The reproducibility of results is greatest in man and least in rabbit. The reproducibility with man is probably due to the smooth texture and the relatively thin keratinized layer of abdominal human skin. The relatively large experimental error with rabbits is probably due to the ease with which rabbit skin is stretched and abraided with resulting differences in skin penetrability beneath the exposure cups.

(6) It is a relatively simple matter with man and pig, to obtain reproducible burns at threshold level with appropriate control of the exposure time and the environmental conditions. This is decidedly not the case with rabbits. Aside from the irreproducibility of experimental results with rabbits, an exposure to sufficient mustard to saturate the skin for 10 minutes at 70° F would result in about 8 times the amount penetrating necessary to produce a relatively mild lesion. Thus one must exercise extreme care in both the control and interpretation of all therapeutic experiments using rabbits as test animals.

V. The Short Time Exposure and Post-application Experiments with Pig, Rabbit and Man.

Va. The Short Time Exposure and Post-application Data on Pig.

(1) Immediately after the termination of a 10 minute exposure to mustard, there is a considerable reservoir of unreacted mustard in the exposed tissue.

(2) Additional fixation of mustard (and additional injury) may occur from this unreacted reservoir during the first 10 minutes after the termination of exposure (surface decontamination). After 10 minutes additional fixation injury does not occur despite the fact that a considerable part of the H is still present. This H does not react with the tissue but is slowly carried away by the body fluids.

(3) With pigs, about 70% of the fixed material is present in the epidermis after a 10 minute exposure; the remaining 30% is fixed in the upper corium.

Vb. The Short Time Exposure and Post-application Experiments with Rabbit.

(1) Immediately at the conclusion of a 10 minute exposure, there is a considerable reservoir of unreacted mustard in rabbit skin.

(2) Immediately following the 10 minutes exposure in rabbit, only 1/2 of the mustard which will ultimately become fixed has reacted.

(3) After a 15-5 minute post-application period, fixation becomes complete and the reservoir of free mustard (extractables) becomes inconsequential.

Vc. The Short Time Exposure and Post-Application Data on Human Subjects.

(1) Within 2 minutes after a 10 minute exposure period all of the H which is going to be fixed in a skin site has already done so.

(2) Compared to the amount of mustard which penetrates during the 10 minute exposure period (15±10 gamma), the amount of free mustard present at the application site 2 minutes to 3.5 minutes later is trivial. It is of the same order as the amount fixed. Thus, if even 12% of this extractable mustard were to become fixed it would not increase appreciably the amount fixed.

(3) The rate of disappearance of mustard from the application site is rapid. This process has a half life certainly no longer than 3 minutes.

(4) All therapeutic agents for human subjects based on reaction with penetrated free mustard must necessarily be valueless, for the reason that there is no significant amount of locally free mustard within the skin at any time.

(5) Since there is no significant difference between the findings with dosages of 1.1 and 10 mg. per sq. cm., all of the above conclusions hold in spite of massive contamination. Thus even massive contamination in man does not provide a source for progressive post-exposure injury.

(6) With sufficient mustard to saturate the skin (about 200 gamma per sq. cm.) only so much mustard can penetrate a given area in a given time interval. Since 1.1 mg. per sq. cm. is more than sufficient to insure a maximum penetration rate for 10 minutes, the massive dosage of 10 mg. per sq. cm. does not increase the penetration rate.

Vd. The Results of Post-exposure "Ice-Pack" Treatment of Skin (Pig and Man) in Relation to the Presence or Absence of a Local Reservoir of Unfixed Mustard.

(1) With pig the immediate application of an ice pack to an exposed site completely or partially inhibited the appearance of injury as compared to a non-iced lesion.

(2) With man the immediate application of ice to an exposed site had no effect on the intensity of the subsequent injury.

(3) Since with man mustard either reacted or was transported away from the portal of entry almost as soon as it penetrated (no reservoir), while with pig, there was a reservoir of mustard at the termination of an exposure, the differences in the effects of ice-pack treatment in pig and man were not unexpected. Thus it seems likely that ice reduced injury in the pig by cooling the exposed site to such an extent that the reservoir mustard could not react and was hence slowly carried away by the body fluids. Since in man there is never an appreciable mustard reservoir in the skin, ice-pack treatment could not be expected to reduce injury by its effect on unreacted mustard.

Ve. Comparison of Short Time Exposure and Post-application Data in Man, Pig and Rabbit.

(1) Immediately at the termination of a mustard exposure, pig and rabbit have an appreciable reservoir of penetrated mustard; man, however, does not.

(2) With pig and rabbit the time to reduce this mustard reservoir to an inconsequential amount is about 10 min.

(3) The time for total fixation of penetrated mustard in man, like the time of removal, is less than 3 minutes following termination of exposure. Fixation of penetrated mustard in pig and rabbit is not complete until about 10 min. following the termination of an exposure.

(4) In view of the above conclusions, it is suggested that in testing therapeutic agents on either pig or rabbit for ultimate use on man, that at least a 10 min. period be allowed to elapse after surface decontamination before the therapeutic agent is applied. Only under such conditions will results obtained from animal experiments be truly applicable to man.

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

VIa. Reaction of Human Skin following Application of Liquid Mustard.

(1) Despite the fact that a cell-killing concentration in the skin is usually reached within 10 minutes after exposure to liquid mustard there is an initial non-reactive period of several hours before physiological or morphological evidence of injury is recognizable.

(2) The first visible reaction on the part of the skin after exposure to mustard is usually vascular and consists of dilation of and stasis within the capillaries of the dermal papillae. If injury has been mild the hyperemia may persist for several days. If injury has been moderate or severe the erythema disappears from the central portion of the lesion and gives way to ischemia.

(3) Degenerative nuclear changes can usually be recognized in the exposed epidermis within 6 hours and subsequent alterations in the epidermis vary according to the intensity and duration of the exposure and the susceptibility of the exposed skin to injury.

(4) Mild or subvesicating injuries: If the injury has been mild it will have reached its peak so far as the epidermis is concerned within 24 to 48 hours after exposure and the damage will at no time involve a sufficient number of cells in continuity or attract a sufficient amount of edema fluid to the foci of epidermal necrosis to result in a through-and-through destruction of the epidermis.

(5) Vesicating injuries: If the injury is more severe the small vesicles which can be recognized microscopically within 12 hours have coalesced to macroscopic dimensions within 18 to 24 hours and the exposed epidermis is elevated and separated from the corium by serous fluid. Despite the relatively normal appearance of the denuded corium, the marginal ingrowth of epithelium is retarded for several weeks and not until 4 or 5 weeks after exposure is the epidermal defect likely to be completely and permanently healed.

(6) Coagulation necrosis: If the skin has sustained a still more severe injury the central portion of the lesion becomes coagulated and apparently incapable of vesicle formation and only at the peripheral

junction of dead and living skin does vesicle formation occur. Lesions of this type are deeper and result in the formation of a plate-like sequestrum which interferes with healing so long as it occupies the base of the lesion.

VIb. Comparison of pathologic changes in man with those occurring in animals.

Cutaneous mustard lesions in animals (pig and rabbit) differ from those in man in that the former manifest earlier and more severe signs of dermal injury, fail to vesicate, and heal more rapidly. Despite the fact that lesions having the same surface area show deeper necrosis in animals than they do in man, there is quicker functional recovery in the former than in the latter. A relatively normal appearing denuded dermis in man fails to attain a new epithelium for a much longer period than is the case with animals.

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

VIIa1.The Correlation of Fixed-Mustard to the Severity of the Injury in Human Skin Tissue.

(1) Direct quantitative observations of the anatomic distribution of mustard sulfur within human skin were not made. It is known that fixation does not occur in the adipose layer. It may be inferred from experiments on pig skin and from radiographs made by Dr. J.A. Hamilton(17) from human skin that the distribution of fixed-H is approximately 70% in the epidermis and 30% in the upper corium after short-time exposures.

(2) For a given exposure time the rate of penetration and severity of injury varied in direct relationship with the environmental temperature. Augmentation of injury by exposure in a hot room and reduction of injury by exposure in a cold room were probably due to increased or decreased penetration rather than to differences in the rate or nature of the reaction between the penetrated material and the tissues.

(3) The fact that thermal augmentation and reduction of injury were not altered when the exposure cups were insulated in such a manner as to keep them at the relatively constant temperature of the skin rather than at the changed temperature of environment indicated that the phenomenon was due to a general physiological alteration of the skin rather than to a temperature change in the local milieu of penetration and reaction.

(4) Differences in environmental and/or skin temperature and individual differences in the proportion of penetrated mustard to become fixed, may alter the amount fixed per unit time of application. Yet, irrespective of this, the correlation between the amount fixed and the severity of the resulting injury is remarkably constant.

(5) There are three recognizable types of mustard injury in man and each is associated with a definite range of fixed mustard. These respective injuries can be readily differentiated macroscopically.

Group I. Mild injury-"sub-vesication" (0.2 to 1.0 gamma of fixed-H per sq. cm. of skin surface). Erythema with or without edema.

Group II. Moderate injury-"central-vesication" (1.0 gamma to 2.5 gamma of fixed-H per sq. cm.). One or more isolated or confluent vesicles.

Group III. Severe injury-"marginal-vesication" (more than 2.5 gamma of fixed-H per sq. cm). Pale yellow, flat or slightly depressed centrum; marginal vesication.

VIIa-2. Comparison of "Short Time" and "Ice-Pack" Fixation Studies in Man and Pig.

(1) Irrespective of the "ice-pack" treatment, the degree of injury tends to parallel the amount of fixed-H in both man and pig.

(2) In pigs at least 0.3 to 0.4 gamma of fixed mustard per sq. cm. of skin surface are required to produce irreversible injury. With man this value is about 0.2 gamma per sq. cm. These values are designated as the "threshold" value for fixed-mustard.

(3) In the pig the immediate post-exposure application of ice prevents any further fixation despite the fact that there is present a local reservoir of unreacted mustard. It may be inferred that the temperature coefficient for fixation is high and that the thermal depression of the fixation rate enables the body fluids to carry the cutaneous reservoir of unreacted mustard away from the portal of entry.

(4) Since the half life for fixation of mustard in pig skin is about 5 min., the sites where ice-pack treatment prevented further fixation of H show significantly less injury than these which did not receive the ice pack treatment.

(5) With the human subjects, fixation of mustard is practically concomitant with penetration. Thus there is at no time a local reservoir of unreacted mustard and the ice-treatment had no effect on reducing either the amount fixed or degree of injury.

VIIa-3. Fixation Studies by Means of the Repeated Applications of Mustard to the Same Site of Pig and Man.

(1) If skin was exposed to repeated application to sub-injurious dosages of mustard, there was local accretion of fixed-mustard at the application site and injury occurred with the attainment of fixed-H levels comparable to those which resulted from single larger injury-producing doses.

VIIa-4. Rate of Disappearance of Fixed-Mustard in Human Skin Tissue

(1) The amount of fixed mustard in human skin does not change significantly during the first week after exposure.

(2) The rate of disappearance of fixed mustard tends to parallel both the rate of healing as evidenced by microscopic sections and the rate of sloughing of the dead epidermis. Thus it appears that the body cannot readily metabolize fixed-mustard.

VIIb. Certain Properties of Fixed Mustard.

VIIb-1. A Simple Protein Fractionation of Pig Skin Tissue Containing Fixed Mustard.

(1) The tissue subjected to this fractionation contained about 100 gamma per sq. cm. of skin surface. Since the amount associated with irreversible injury is 0.4 gamma per sq. cm., the data summarized below may not be a true representation of the properties of fixed mustard in lesions of average severity.

(2) Practically none of the fixed mustard is combined with skin constituents soluble in either 0.9% NaCl or acetone or an ether-alcohol mixture.

(3) Linkages split at 6°F at mild alkalinity (pH 9) to yield a dialyzable radioactive material (primarily thiodiglycol) amounts to about 40% of the total initially fixed.

(4) Linkages in addition to (2) broken by autoclaving to yield also dialyzable radioactive substances amounting to about 20% of the initially fixed mustard.

(5) The remaining 40% of the radioactivity is attached to soluble compounds after autoclaving; however, these constituents, presumably proteins, are not dialyzable.

VIIb-2. The Stability of Fixed Mustard in Pig Skin Tissue with Respect to Alkaline pH.

(1) These results pertain to tissue where the amount of fixed-mustard is about 2 gamma per sq. cm. of skin surface or 0.5 gamma per 10 mg. of dry wt. epidermis and corium. This amount of fixation is associated with a moderately severe injury.

(2) The amount of fixed-mustard soluble or decomposed in 45 hrs. at pH 7 is about 10%. This percentage increases linearly with increasing pH and is 100% in 45 hours at pH 13.

(3) In the pH range of 7 to 13, about 25% of the alkaline soluble fixed-mustard is precipitated by 80% alcohol. These fixed-mustard molecules are undoubtedly protein in nature.

(4) In the pH range 7 to 13, about 75% of the alkaline soluble mustard sulfur is soluble in 80% alcohol. About 50-65% of this mustard sulfur is in the form of thiodiglycol. Thus at pH 13, 40% of the fixed mustard linkages have been broken in such a manner as to form thiodiglycol.

(5) In sufficient time 70% of the fixed material can be removed in the pH range of 9 to 11. Apparently it requires a higher pH to remove the remaining 30%.

(6) Several types of linkages between the mustard and the tissue molecules are undoubtedly present in fixed-mustard. 40% of these linkages are very labile at pH 13, being split to form thiodiglycol. An ester linkage with phosphate or carboxyl group is indicated and possibly the sulfonium salt linkage. 60% of these linkages are not capable of being split from soluble protein or alcohol molecules to form thiodiglycol at a pH of 13. Among the stable linkages of this type are probably; C-N (by alkylation of amines); Ar-O-C (by formation of ether with a phenolic-OH); R_4N (by formation of quaternery nitrogen); and C-S-C (by formation of a thio-ether from a sulfhydryl group).

VIIb-3. The Fraction of Fixed-Mustard Present in Nuclei of Pig Skin Tissue.

(1) 70% of the H fixed in the epithelium is present in the upper layers. Since much of this layer is dead tissue, it is difficult to see how this particular H fixation could be related to any skin damage.

(2) An appreciable fraction (12.5%) of the fixed-N in the malpighian layer is present in the nuclei. On a nitrogen basis, there is about 80% less fixed mustard in the malpighian nuclei than in the entire malpighian layer.

(3) In mild to moderately severe mustard lesions, practically all of the injury is confined to the malpighian and basal layer. In dosages with pigs comparable to that necessary to produce blisters in human skin, the amount of fixed mustard is 0.25 gamma in the malpighian layer per sq. cm. of pig skin surface. This corresponds to about 25 micr moles of fixed mustard expressed in N-equivalents per gram atom of nitrogen or 10^{-9} micromoles per malpighian cell.

VIIc. The General Mechanism of Mustard Injury.

(1) The reaction of mustard with impermeable or insoluble constituents or constituent present in skin tissue is apparently responsible for injury. These impermeable constituents are probably protein in nature.

VIII. Miscellaneous Control Experiments with Animals.

VIIIa. Pathological Studies on Pigs of Regional Vulnerability and of the Reliability of Early Macroscopic Changes in Estimating the Severity of an Injury Produced by Mustard.

(1) There are striking differences in the degrees of susceptibility of different skin areas of the same animal (pig) to injury by mustard. The gross external appearance of a 24-hour post-application lesion does not necessarily provide reliable evidence as to the extent to which the exposed tissue is injured.

(2) No conclusion regarding the effect of a toxic, prophylactic, or therapeutic agent should be drawn from the appearance of skin lesions unless the lesions being compared are situated in anatomically comparable sites of the body.

VIIIb. The Relative Vulnerability of Pigmented and Non-pigmented Pig Skin to Injury by Mustard.

(1) Pigment, per se, does not alter the susceptibility of a pig's skin to injury by mustard.

VIIIc. Effect of Pre-exposure Wetness and Dryness on the Susceptibility of Pig Skin to Injury by Mustard.

(1) Pre-exposure wetness or dryness of a pig's skin does not alter its susceptibility to injury.

VIIId. The Distribution of Mustard Sulfur in the Various Organs of a Pig after a Gross Contamination by Mustard.

(1) Mustard reacts with numerous constituents present in the blood. Only a small fraction of the mustard sulfur present in the blood is thiodiglycol.

(2) There is no apparent connection between systemic effects and the distribution of mustard sulfur in various tissues.

(3) The tissue-fixed mustard bond at the site of direct application is less readily disassociated than at sites to which mustard has not been directly applied. Systemically fixed mustard can be partially dissolved in isotonic salt solution.

VIIIe. The Rate of Disappearance of Mustard from Rabbit Blood in Vitro and Vivo.

(1) In vitro mustard remains in the blood a considerable length of time; in vivo mustard disappears very rapidly.

(2) Apparently certain organs react or absorb mustard from the blood stream extremely rapidly.

IX. Certain Chemical Experiments.

IXa. The Mechanism of the Chemical Reaction which Produces Fixed-Mustard.

(1) The heterogeneous reaction of mustard with pig skin brei in vitro is of the competition factor type.

(2) The fixation of mustard in human skin in vivo is apparently by the competition factor mechanism.

IXb. The Competition Factors of Various Compounds Including Amino Acids, Blood Plasma and Plasma Proteins.

(1) The free amino group in an amino acid has a competition factor (F) of about 75. The presence of an H ion on the amino nitrogen renders its F small. Thus all amino groups involved in zwitter ion formation have a low F. The amino group in an acid amide linkage is also unreactive towards mustard.

(2) Although simple amino acids do not have a very high competition factor near pH 7, the amino acids with other reactive groups do (e.g., the N of the imidizole ring of histidine, the sulfur atom of methionine, the tryptophane indole nitrogen and the guanido group of arginine).

(3) The competition factors of a 1% solution of certain human blood plasma proteins show that the relative reactivities of proteins toward mustard are by no means identical. Thus, human plasma albumin is essentially unreactive towards mustard as compared to fibrinogen.

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Part 1

The Synthesis of Mustard Gas Containing Radioactive Sulfur

by F.C.H. and C.M.

As already indicated (see introduction) barium sulfate was the source of the sulfur used in the preparation of the mustard used in these experiments. Excellent yields of a pure product on both a semi-micro and a micro scale were obtained by the method represented schematically by the following equations:

$$BaSO_4 \xrightarrow[900^\circ C]{H_2} BaS \xrightarrow[6M]{H_3PO_4} H_2S \xrightarrow[70^\circ C]{C_2H_4O} (HOC_2H_4)_2S$$

$$(HOC_2H_4)_2S \xrightarrow[70^\circ C]{\text{Conc.HCl and Pentane}} (ClC_2H_4)_2S$$

The barium sulfate was ground with water in an agate mortar into a paste to insure a subsequent complete reduction by hydrogen. It was then thoroughly dried and transferred to a weighed platinum boat which was introduced into a long quartz tube. By heating the tube to 900°C for 4 hours and passing through a stream of dry hydrogen, a 98% reduction was obtained.(a) The boat with contents was transferred to a small gas generator, containing 1 gm. of 20 mesh Zn. Sufficient 6 M_3PO_4(b) to cover the boat was then added through a stopcock. The issuing gases were dried with P_2O_5 and the H_2S was condensed in a trap chilled by liquid nitrogen. The purpose of the hydrogen was to sweep out all trace of H_2S from the gas generator. The H_3PO_4 solution was then boiled to remove the last traces of H_2S. The H_2S was then vacuum distilled into a calibrated volume containing a mercury manometer, and then condensed into a thick-walled reaction tube (20-30cc. capacity.) Exactly twice as much redistilled ethylene oxide was measured out in the same volume, and also condensed into the reaction tube. The tube was then sealed off under high vacuum and heated with continuous shaking in a water bath at 60°-70°C for about 14 hours. The completion of the

(a) $BaSO_4+4H_2 \rightarrow BaS+4H_2O$ and (2) $BaSO_4+4H_2 \rightarrow BaO+H_2S+3H_2O$. At 900°C reaction (1) is predominantly favored. The trace of H_2S formed was caught by bubbling the issuing H_2 gas through a 1 N $ZnCl_2$ solution. The trace of ZnS formed was added to the BaS.

(b) Since it is difficult to separate by distillation gaseous HCl from H_2S, phosphoric acid was used rather than hydrochloric.

reaction was indicated by the low pressure discharge obtained in the tube when tested with a Tesla coil; the thiodiglycol formed appeared as a clear colorless, viscous liquid. By means of a hot air bath or centrifugation the liquid was removed away from the top and the tube was then opened and a 2-3cc of concn. HCl and a 2-4cc. of petroleum (BP 20 to 40) ether were added. After freezing and sealing-off, the tube was again heated with shaking at 60°-70°C for about 24 hours.(c)

The reaction tube was centrifuged, cooled with dry ice, opened, the top ether layer pipetted off into a micro sublimation tube and washed three times with pet.ether. The sublimation tube is connected to a vacuum sublimation apparatus and the majority of the pet-ether was pumped off at about -50°C in order to prevent bumping. The petroleum ether mustard was then slowly warmed to 80°C under vacuum; the mustard sublimed on the cold spot which had been kept continuously at -60°C by means of dry ice. The sublimation apparatus was then placed in a cold room and when the cold spot reached 4°C, the crystalline mustard was transferred by means of a spatula and black glazed paper to a weighed centrifuge tube provided with a ground glass stopper. Four different preparations starting with 50 mg., 65 mg., 125 mg. and 210 mg. of $BaSO_4$ produced mustard in yields of 90-95% with melting point of 14.5±0.5°C.

(c) Preliminary experiments had shown that this chlorination reaction proceeds to 98% completion under these conditions, the same yield being obtained with conc. HCl as with $ZnCl_2$ and $CaCl_2$ catalysts added to the acid. The petroleum ether provided a water insoluble solvent for the reaction product, thus removing the H from the reaction zone.

Part II
An Analytical Technique for Measuring Radioactive Sulfur

by F.C.H. and W.G.S.

The radiation from sulfur consists solely of a continuous beta ray spectrum having an upper energy limit of 100,000 electron volts and an average energy of 35,000 electron volts. These electrons are produced by the following nuclear disintegration:

$$_{16}S^{35} \rightarrow {}_{17}Cl^{35} + {}_{-1}e^{0}$$

The energies of these electrons are so low that they are 1/2 absorbed

by a 5 mg. sq.cm. layer and are totally absorbed by 20 mg.sq.cm.(a)

Two types of apparatus were used in the counting of these low energy beta rays; namely, a Geiger counter and an electroscope.

IIa The Geiger Counter:

The recent development of Geiger counters filled with helium at atmospheric pressure enables them to be constructed with extremely thin windows, thereby making them especially suited for the measurement of soft radiation. We used a Geiger counter of the end window type, an amplifier-integrator and an Esterline-Angus Recorder. The bell type Geiger Counter is shown diagrammatically in Fig. 1d. All the units were designed by R. B. Evans and A. Kimp of the Massachusetts Institute of Technology and, except for the counter itself, were obtained from them. In order to make the Geiger-counter more useful for our work two minor improvements were made:

I) The thickness of the mica windows was reduced from the usual 5 to 10 mg. sq. cm. to 1 mg. sq. cm. (10^{-4}in.) This exceedingly fragile window (1" in diameter) was protected from the radioactive sample by means of a metal guard ring 3/4" in diameter.

II) Before assembly, the copper cylinder was immersed in an ammonium polysulfide solution. The resulting black sulfide surface produced a better background and better plateau characteristics than the usual bright copper or copper oxide surfaces. With these improvements the above counter recorded about 50% of beta particles produced in a solid angle of 2 pi. When placed inside a 1" thick lead box its natural background (cosmic radiations) was 20-40 counts per minute.

IIb. The Modified Lauritzen Quarts Fiber Electroscope.

The quantity of ionization produced in the neighborhood of the gold plated quartz fiber of an electroscope is a function of the inverse square of the velocity of the beta partical. The energy of the sulfur Betas are sufficiently low as to make the sensitivity of the modified electroscope about the same as the best designed Bell type Geiger counters.

In order to obtain maximum sensitivity for sulfur with the Lauritzen quartz fiber electroscope, it was decided to place the samples inside the ionization chamber. This was accomplished in the following manner: First, the region of maximum sensitivity within the electroscope chamber was determined by placing a radioactive sample at various positions inside the chamber. Then a simple device was constructed which combined the usual cover of the ionization chamber and a sliding bar device provided with an air lock. This sliding bar device enabled brass disks

(a) The "thickness" of a beta absorber is always expressed as mg./sq.cm. in radioactive absorption work, since when expressed in this manner the amount of beta absorption is roughly independent of the nature of the absorber. Mg/sq.cm. is of course the product of thickness times density.

containing the radioactive samples to be introduced into the most sensitive location within the chamber in a reproducable manner. In addition, a small boat filled with the drying agent, anhydrous magnesium perchlorate, was kept continuously inside the chamber; this resulted in much lower and steadier backgrounds (cosmic radiations). A diagramatic representation of the modified case of the Lauritzen quartz fiber electroscope is given in Fig. Ia.

IIc. The Preparation of the Radioactive Sulfur for Measurement.

In order to obtain an accuracy of better than 5% (2% under most favorable circumstances) in determining amounts of radioactive sulfur, it was found necessary to isolate a sulfur containing compound in the pure state. This was accomplished by ultimately oxidizing all sulfur compounds to the sulfate ion through the use of a micro Carius method and precipitation as benzidine sulfare. (3), (b).

The precipitate (16.5 mm. in diameter) was collected on a weighed filter paper (7/8" in diameter) that had been placed previously over a fritted glass filtering disk of coarse porosity. The paper (c) was held in place by means of a glass cylinder; thus when assembled, the filtering apparatus resembled an Allihn filter tube (see Fig. Ib.) This method was somewhat similar to that described by Tarver and Schmitt (4)

After reweighing the filter paper, it was placed on a brass disk (19 mm in diameter). The paper was then permanently mounted by pressing on a brass ring which fitted snugly over the disk. (see Fig. Ic.) The activity of the precipitate was then determined by placing the assembled disk inside the electroscope chamber by means of the sliding bar, or face down on to the guard ring of the Bell counter mica window.

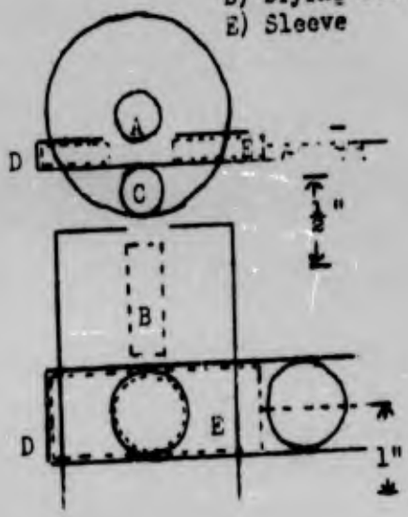
Through experiment it has been found that 6 to 7 mgs. was a convenient amount of precipitate to handle. Thus enough inactive sulfur was always added so as to keep the benzidine sulfate weight above 6 mg.

(b) Benzidine hydrochloride was advantageous over barium chloride as a precipitant due to the uniformity and reproducibility of the particle size of the resulting precipitate. Thus self-absorption (caused by the low energy of the β particle emitted from sulfur 35) was reduced to a reproducible factor.

(c) Since it was necessary to have the precipitate adhere firmly to the filter paper and still be of uniform thickness, the type of filter paper was of importance. Munktell's No. 0K has been found quite satisfactory.

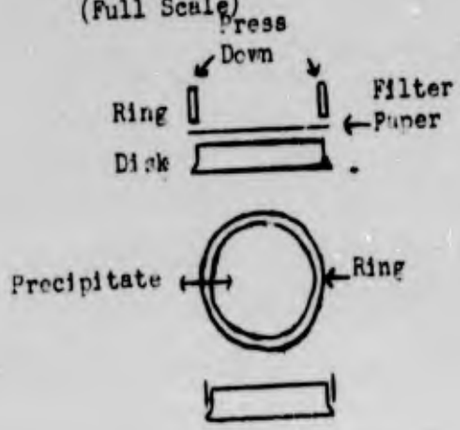
1a
Modified
Electroscope Case
(1/2 scale)

A) Light Window
C) Hole for Boat
D) Bar
B) Drying Boat
E) Sleeve

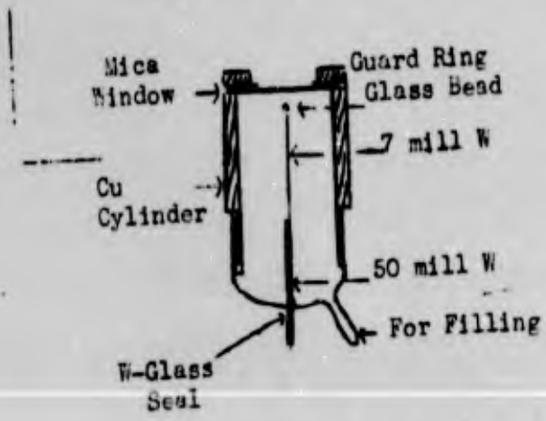


1b
Benzidene Sulfate
Precipitating Apparatus
(Full Scale)

1c
Measuring Disk
(Full Scale)



1d
Bell Counter
(1/2 scale)



11d. Determination of the Accuracy and Sensitivity of the Radioactive Analytical Method for Both Electroscope and Geiger Counter. (Dilution Experiments).

The appearance of a beta particle inside a Geiger counter produced a descrete impulse or "count" which was recorded by an auxiliary apparatus. Thus, the number of counts per minute minus the natural background was a measure of the amount of radioactivity present. This quantity will be denoted as $(c/m)_S$; c is counts, m is minutes, and S is sample.

The appearance of a beta particle in the neighborhood of the charged gold plated quartz fiber of an Lauritzen electroscope resulted in ionization; this ionization discharged the fiber at a faster rate than its natural (background) rate of discharge. Thus the reciprocal of the time for the fiber to move a certain number of scale divisions in the presence of a radioactive sample minus the reciprocal of the time for the fiber to move the same number of scale divisions in the absence of the radioactive sample (background) was a measure of the amount of radioactivity present. This quantity will be denoted as $(d/t)_S$ where d is the distance traversed by the fiber in the time, t in seconds, and S is the sample.

The sensitivity of the Geiger counter for comparative reasons may be defined as:

(1) $(c/m)_s \Big/ (c/m)_b$; while the sensitivity of the electroscope may be defined as

(2) $(d/t)_s \Big/ (d/t)_b$; where b denotes background.

Thus for a given radioactive sample the determination of the numerical values for equation (1) and (2) enabled the computation of the sensitivity of the electroscope relative to the Geiger counter. The sensitivity as defined by equation (2) is essentially the same for all Lauritzen Quartz fiber electroscopes.(d)

(d) The Lauritzen electroscope, purchased from the Fred C. Hensen Co., Pasadena, Calif. possessed an absolute sensitivity such that a millicurie of radium at a distance of one meter produced a motion of two to five scale divisions (depending upon the electroscope) per minute; however the comparitive sensitivity of these electroscopes was quite constant. Under the above conditions <u>all Lauritzen electroscopes discharge at a rate of about 60 times "natural" background</u>. Thus the absolute sensitivity was of small import, since there was no appreciable loss in accuracy in allowing the fiber of the electroscope possessing the low <u>absolute</u> sensitivity to discharge 10 scale divisions instead of the 20 scale divisions one would use with electroscopes of greater absolute sensitivity.

TABLE I

Radioactivity Measurements as of a Function of S^{35} Concentration

Ia - Electroscope

Radioactivity Measurements as a Function of S^{35} Concentration
Numbers in Matrix are (activities/background) = $(d/t)_s/(d/t)_b$

Value along principle diagonal is experimentally determined at that concentration.

Conc.	1	2	4	5	8	10	20	40	50	80	100	200	400	500	800	1000
1	0.20															
2	0.22	0.44														
4	0.22	0.44	0.87													
5	0.22	0.44	0.87	1.09												
8	0.21	0.42	0.85	1.06	1.69											
10	0.22	0.44	0.88	1.10	1.76	2.20										
20	0.21	0.42	0.84	1.05	1.68	2.11	4.21									
40	0.20	0.41	0.82	1.02	1.63	2.04	4.07	8.15								
50	0.21	0.41	0.82	1.03	1.65	2.11	4.11	8.22	10.3							
80	0.22	0.43	0.85	1.06	1.70	2.15	4.25	8.50	10.6	17.0						
100	0.21	0.42	0.84	1.05	1.67	2.09	4.18	8.36	10.5	16.7	20.9					
200	0.21	0.42	0.83	1.05	1.67	2.10	4.20	8.30	10.5	16.7	21.0	42.0				
400	0.21	0.42	0.83	1.04	1.66	2.07	4.15	8.30	10.4	16.6	20.7	41.5	83.0			
500	0.21	0.41	0.82	1.03	1.65	2.06	4.12	8.24	10.3	16.5	20.6	41.2	82.4	103		
800	0.21	0.40	0.82	1.03	1.64	2.05	4.10	8.21	10.3	16.4	20.5	41.0	82.1	103	164	
1000	0.21	0.41	0.82	1.03	1.64	2.05	4.10	8.20	10.3	16.4	20.5	41.0	82.0	103	164	205
Ave	0.21	0.42	0.84	1.05	1.67	2.09	4.15	8.27	10.4	16.6	20.7	41.3	82.4	103	164	205
Prop* % Dev	2.0	2.0	1.7	1.5	1.5	1.5	1.0	1.0	0.5	1.0	0.5	0.5	0.5	0	0	-
Max % Dev	5.0	5.0	4.5	5.0	5.0	5.5	2.5	1.5	2.0	2.5	1.5	1.7	0.7	0	0	-

* Prob. % Dev. is prob. Dev. of any <u>one</u> experimental value from the average, <u>not</u> the prob.dev. of the average value.

TABLE I

1b- Geiger Counter

Radioactivity Measurement as a Function of S^{35} Concentration Numbers in Matrix are (activities/background) = $(c/m)_s/(c/m)_b$. Value along principle diagonal is experimentally determined at that concentration.

Conc.	1	2	4	5	8	10	20	40	50	80	100	200	400	500	800	1000
1	0.33															
2	0.25	0.50														
4	0.27	0.49	0.97													
5	0.26	0.52	1.04	1.30												
8	0.25	0.49	0.99	1.24	1.98											
10	0.25	0.50	0.99	1.24	1.98	2.48										
20	0.27	0.53	1.07	1.33	2.13	2.67	5.33									
40	0.27	0.53	1.07	1.34	2.14	2.65	5.35	10.7								
50	0.24	0.48	0.96	1.20	1.92	2.40	4.80	9.6	12.0							
80	0.24	0.49	0.99	1.24	1.97	2.46	4.92	9.9	12.4	19.7						
100	0.25	0.51	1.02	1.27	2.03	2.54	5.08	10.2	12.7	20.3	25.4					
200	0.25	0.51	1.01	1.26	2.03	2.53	5.06	10.1	12.6	20.3	25.3	50.6				
400	0.25	0.49	0.98	1.24	1.97	2.46	4.92	9.8	12.4	19.7	24.6	49.2	98			
500	0.25	0.50	1.01	1.26	2.02	2.52	5.06	10.1	12.6	20.2	25.2	50.4	101	126		
800	0.26	0.52	1.04	1.30	2.08	2.60	5.20	10.4	13.0	20.8	26.0	52.0	104	130	208	
1000	0.25	0.51	1.02	1.27	2.03	2.54	5.08	10.2	12.7	20.3	25.4	50.8	102	127	203	254
Av. Value	0.25	0.51	1.01	1.27	2.02	2.53	5.07	10.1	12.6	20.2	25.3	50.6	101	128	206	
Prob* % Dev	2	2.5	2	2	2	2.5	2	1.5	1	1	1.5	1.5	1	1		
Max (30%) % Dev	8%	6%	6%	5.5%	6%	5.5%	5.5%	6%	5%	3%	3%	3%	3%	1.5%	1%	

* Prov. % Dev. is prob. Dev. of any <u>one</u> experimental value from the average, <u>not</u> the prob. dev. of the average value.

The determination of the numerical values of equation (1) and (2) as function of concentration of radioactive sulfur at constant weight of benzidine sulfate was a measure of the accuracy of the radioactive analytical method for S^{35} with both the electroscope and Geiger counter.

Table Ia and Ib tabulate the results of these determinations in matrix form for both electroscope and Geiger counter. The concentration of the weakest radioactive sample measured has been arbitrarily taken as unity. The values along the principle diagonal of the matrix were the average of duplicate experimentally determined radioactivities (measures as so many times background) at the given concentration; the remainder of the tabulated values were computed from all samples containing a higher concentration of radioactivity.

These results show that a satisfactory analytical method was developed for the determination of the relative amounts of radioactive sulfur. Further, on the basis of accuracy and sensitivity, there was little to choose between the Geiger counter and electroscope.

The absolute sensitivity of both instruments was such that 10^{-4} microcuries of S^{35} will be the order of background in activity. Thus 10^{-3} gamma of mustard can be readily determined if the specific activity of the radioactive mustard is 1 millicurie per 10 mg. of H.

IIe. The Correction for the Weight of the Benzidine Sulfate Precipitate (Self Absorption of the Beta Particles)

Since the beta particles emitted from S^{35} have a maximum range of only about 0.11 million electron volts, a considerable fraction of these beta particles was absorbed by the benzidine sulfate precipitate itself.

Thus it was necessary to refer all radioactivity measurements to predetermined weight. This weight was arbitrarily chosen as 7.00 mg. or 3.25 mg. per sq. cm.

A correction chart was obtained by determining the radioactivity at constant concentrations of S^{35} as a function of the weight of the benzidine sulfate precipitate; the range was 1-25 mg. in duplicate 1 mg. intervals.

The results are given in Table II. It will be noticed that values were not quite the same for both the electroscope and the Geiger counter. Since the beta particles were far from monochromatic and the Geiger counter counted each particle while the electroscope measured the ionization resulting from each particle, the difference in the above results was to be expected.

A consideration of the magnitude of these correction factors showed that the error from this source was less than 1%, since the benzidine sulfate precipitate could be weighed to better than 0.1 mg.

TABLE II

Reciprocal Correction Factors for Weight of Benzidene Sulfate Precipitate for both an Electroscope and Geiger Counter. (Measured-activity is to be divided by these factors.)

Electroscope Reciprocal Wt. Correction Factor	Wt. of B_2SO_4 Ppt. in milligrams	Wt. of Ppt. mg./sq.cm.	Geiger Counter Reciprocal Wt. Correction Factor
1.595	1	0.46	1.370
1.465	2	0.93	1.290
1.350	3	1.40	1.205
1.245	4	1.85	1.150
1.155	5	2.30	1.095
1.070	6	2.80	1.050
1.000	7	3.25	1.000
0.935	8	3.70	0.955
0.880	9	4.15	0.910
0.830	10	4.65	0.865
0.780	11	5.10	0.825
0.750	12	5.55	0.790
0.715	13	6.00	0.754
0.680	14	6.50	0.715
0.650	15	6.95	0.680
0.625	16	7.40	0.650
0.595	17	7.90	0.625
0.570	18	8.35	0.600
0.545	19	8.80	0.575
0.520	20	9.25	0.555
0.500	21	9.75	0.535
0.480	22	10.20	0.520
0.465	23	10.70	0.500
0.450	24	11.10	0.480
0.435	25	11.60	0.465

Table III

Sample Computation Sheets for an Electroscope and Geiger Counter

Electroscope #37

Exp. No. 19-P Date 3/24/43 (gamma/Eu) = 0.566

Cerius No.	Disk No.	Weight Ppt.	Initial Read.	Final Read.	Time Sec. (T)	1000X 10 div/T (Total Act.)	Net activity (Eu.)	Table II Wt. Corr. Factor	H-equiv in gammas	Gammas Eu.
			45	55	173.1					
	Std-1	7.00	45	55	174.4	5.74	5.68	1.000	3.200	0.563
			45	55	292.4					
	Std-2	7.00	45	55	293.4	3.41	3.35	1.000	1.900	0.567
			45	55	312.1					
12	7	8.70	45	55	311.0	3.21	3.15	0.895	1.99	
			45	55	100.5					
14	9	6.07	45	55	101.1	9.91	9.85	1.065	5.27	
17	11	11.25	45	50	1840	0.273	0.214	0.780	0.16	
21	12	8.18	45	50	7100	0.070	0.011	0.925	0.007	
	Background		45	50	8500	0.059	nil			
			45	55	175.6					
	Std-1		45	55	174.6	5.71	5.65		3.200	0.566
			45	55	295.7					
	Std-2		45	55	292.1	3.40	3.34		1.900	0.569

Geiger Counter #C

Exp. No. 19-P Date 3/24/43 (counts/gamma) = 1070

Cerius No.	Disk No.	Weight Ppt.	Scale	Reading	Total counts/min.	Net counts min.	See Table II Wt. Corr. Factor	H-equiv. in gammas	Counts gamma
	Std-1		3	69	3450	3420		3.200	1070
	Std-2		3	41	2050	2020		1.900	1060
	Background		7	15	30	nil			
12	7	8.70	3	41	2050	2020	0.925	2.04	
14	9	6.07	2	58	5800	5770	1.045	5.16	
17	11	11.25	7	82	164	134	0.815	0.15	
21	12	8.18	7	20	40	10	0.945	0.01	
	Std-1		3	68	3400	3370		3.200	1050
	Std-2		3	42	2100	2070		1.900	1090

It is of interest to note that the self-absorption is expressed to an excellent approximation by the following equation:(e,5) $I/I_o = (1-e^{-ad})/ad$. Where I_o is the "true" total activity present in the benzidine sulfate precipitate; d, is the thickness of the sample in mg. per sq. cm.; and a, is an average exponential absorption coefficient of numerical value 0.27 sq.cm. per mg.

11f. A Comparison of the Geiger Counter and Electroscope for the Measurement of Radioactive Sulfur.

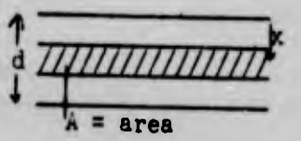
The results of section 11e and 11f show that the analytical method developed in section 11c will give accurate results (within 5%) with either the Geiger counter or electroscope described in sections 11a and 11b respectively. In fact, on the basis of accuracy and sensitivity they are essentially equivalent. Both instruments will detect readily 10^{-4} microcuries of the S^{35} (the activity producing the same effect as the "natural" background.) Both instruments have been used interchangeably in the course of the investigations.

In laboratories where there are a limited number of personnel trained in electronics, an electroscope is to be preferred in view of its simplicity of design.

The average life of an electroscope under the somewhat drastic conditions of placing the radioactive samples inside the ionization chamber is about 1 year. Thus it is advisable to own two electroscopes. In view of the price of an electroscope as compared to a Geiger counter, namely $50.00 and $500.00, an electroscope is nevertheless preferable.

Since the sensitivity with respect to background of the modified electroscope and bell-type Geiger counter are essentially identical, there can be no difference in the time required to measure any radioactive sample to the desired accuracy with either instrument.

(e)



Let s be a specific activity per unit volume. Assuming exponential absorption $I=I_o e^{-ax}$), then measurable activity of from volume element Adx is $sAe^{-ax}dx$. Total measurable activity $=I=sA\int_0^d e^{-ax}dx = sA/a[1-e^{-ad}]$ Since "true" total activity $= I_o = sAd$, we have $I/I_o = [1-e^{-ad}]/ad$.

All of the approximations made in the above derivation are trivial as compared to the assumption of exponential absorption for the Beta particles. Although the absorption of high velocity monochromatic electrons does not follow an exponential law, the energy distribution curve of beta particles arising from radioactive disintegrations is such that to within 2-5% an exponential equation is valid.

It is of interest to indicate the actual time of measurement. Samples with radioactivity the order of background necessitate one to two hours to measure accurately (5%); however with these samples fifteen minutes will suffice for most investigations.

The maximum accuracy for both Geiger counter and electroscope will be with samples 50 to 150 times background. The time required to measure these samples is a matter of a few minutes.

With neither instrument should samples greater than 250 times background be measured.(g) Table III shows typical sample calculation sheets for both an electroscope and Geiger counter.

(g) Due to the time required to equilibriate the position of the quartz fiber, accurate results with samples containing radioactivity in excess of 250 times background cannot be expected; depending upon the electroscope an activity of this order will discharge at a rate of 10 to 20 divisions per minute.

PART III

Details of the Experimental Procedures

Animal Methods; H.L.B., A.R.M.
Chemical Methods; F.C.H., L.A.P., W.G.S.
Human Surgical Methods; R.S.M.

The types of animal experiments which were performed can be divided into three groups as follows:

(1) Determination of the penetration rate of the skin by mustard.
(2) Determination of the fate and distribution of the penetrated mustard.
(3) Investigation of the significance of that portion of penetrated mustard that reacted with the skin and remained at the site of exposure as "fixed" mustard.

IIIa. Preparation of animals.

Two to 24 hours before exposure the hair was clipped. In the case of rabbits it was necessary to supplement clipping by an application of barium sulfide. When a chemical depilatory was employed it was used 24 hours prior to experimentation. All animals, pigs and rabbits, were anesthetized with nembutal before exposing the skin to mustard. This procedure was necessary to insure the degree of immobility that was required to prevent leakage of mustard from the shallow application cups.

IIIb. Acclimatization of subjects.

Preliminary investigations showed that temperature has a profound effect upon the rate at which mustard penetrates the skin. Thus in all penetration experiments, the subjects were placed in a constant temperature and humidity room for at least one hour before experimentation. Human subjects were required to remove all clothes.

IIIc. Temperature determinations.

Environmental temperature (Wet and Dry bulb) and skin temperature were recorded in most experiments. The skin temperatures were obtained by means of a calibrated three junction copper-constantan thermocouple contained in an insulated glass cup (2 sq.cm.); the glass cup fitted tightly onto the skin.

IIId. Preparation of Applicator Cups.

IIId-1 Preparation of Applicator-Cups for Penetration Experiments.

By means of a mercury driven micropipette[b], 475±15 gamma of radioactive mustard was delivered onto a filter paper which fitted the base of an elliptical cup (12x4x1mm.) of internal height of 0.5 to 0.7 mm. and of inside area 0.43 sq.cm. These cups were provided with a greased cover. The approximate concentration of H. was 1.1 mg. per sq.cm.

The cups with and without mustard were weighed on a microbalance (Ainsworth) to within 3 gamma and prior to the time of application were stored on dry ice.

IIId2. Preparation of Applicator Cups for Certain Other Experiments.

In most experiments it was not necessary to know the amount of mustard contained in the cup to an accuracy greater than 30gamma. The

(b) These mercury driven micro-pipettes were constructed from a metric scale micrometer caliper and a "Nurnbergs Aseptic 360°C" thermometer. The micrometer part of the caliper was removed and then machined to drive a 1/32" rod through an adapter. Into the other end of the adapter was waxed one end of the thermometer tubing. The other end of the thermometer had been previously drawn down to a fine tip. By sucking the entrance of the 1/32" rod into the adapter, the micro-pipette could be evacuated through the thermometer tubing, and thus be filled with mercury. The pipette was then filled with mustard in such a manner that there was no air block between the mustard and the mercury. The total capacity of the pipette was about 10 mg. of H; depending upon the thermometer, 20° was equal to 450± 50 gamma. Each thermometer was calibrated by means of a mercury thread. It was necessary to dismantle and clean each pipette before it was refilled.

procedure in preparing these cups was identical with that described in IIId-1, except that the cups were neither weighed nor stored on dry ice. About 1 hour before the animal experiment, they were filled and immediately covered.

IIId-3. Preparation of Large Applicator Cups for Certain Pig Experiments.

Circular cups 2 sq.cm. in area and 1 mm. in internal height containing 1 mg. of mustard were used on all pig work conducted prior to the beginning of our human experimentation.(c) The procedure for the preparation of these cups was identical with that described in either IIId-1 or IIId-2.

IIIe. Technique of Application of Mustard to Skin and the Subsequent Decontamination of the Exposed Site.

The period for which the skin was exposed to the mustard in an applicator cup is defined as exposure time.

IIIe-1 Technique of Application and Decontamination in Penetration Rate Experiments.

The applicator cups were removed from the dry ice storage vessel (see IIId-1) and placed on a block of dry ice. At the time of application, while the mustard is still frozen, the cover was removed and dropped into a special nitric acid solution (see section IIIk. The cold cup was then placed on the skin and held there by means of a rubber dam stretched over a cork on top of the cup; the rubber dam was fastened to the skin with adhesive tape.

Upon the termination of the exposure time the cup was chilled with dry ice and dropped into the nitric acid solution containing the corresponding cover. The chilled skin was then swabbed three times with cotton soaked in petroleum ether (30-60). The cotton swabs were also dropped into the nitric acid solution.(d)

The mustard applications were always made at time intervals such that the time for excision of all exposed sites would coincide.

(c) The size of the elliptical applicator cups (0.43 sq.cm.) was chosen with the human experiments in mind. This size was considered to be optimal for experiments involving the excision of a full-thickness segment of human skin. The fact that the filter papers in these cups made contact with the skin has been shown numerous times by saturating the paper with a colored dye.

(d) By placing a cup on the skin and immediate removal by the above procedure, it was shown that 98±% of the applied mustard could be accounted for.

IIIe-2 Technique of Application and Decontamination in All Other Experiments.

The cover of an applicator cup (see IIId-2 or IIId-3) was removed; the cup was placed either on the side of the pig or rabbit or on the lower abdomen of the human subject. Since these exposure times were usually of short duration (5-20 min.), these cups were held in place manually.

At the conclusion of the exposure period, the cup was removed and dropped into nitric acid. (e) The skin site was then swabbed three times with cotton soaked in petroleum ether (30-60).

The mustard cups were always applied at time intervals such that all sites could be excised at the same time.

IIIf. Post-Application Treatment.

The post-application period is the interval between termination of the exposure and the excision of the exposed site.

IIIf-1. The Short Time Post-application Periods with Pigs and Rabbits.

In these experiments there was no post-application treatment and the exposed skin was allowed to remain undisturbed for a pre-determined period of time (0-60 min.) and at the same environmental temperature at which the experiment was conducted. These sites were then frozen rigid with dry ice and excised.

IIIf-2. The Short Time Post-Application Periods with Human Subjects.

Since it was not practical to freeze the skin of human volunteers the shortest post-application period in human experiments was about 3 min. This was the shortest time that the surgeon could prepare and remove the exposed site upon the exposure. The short time post-application periods in human experiments ranged from 2-10 minutes.

IIIf-3. The Long Time Post-Application Periods.

These post-application treatments consisted of allowing the lesions to remain in situ for (1 to 38 days) before excision. No control of the temperature was attempted during these periods.

(e) The radioactivity in these nitric acid solutions was subsequently recovered as barium sulfate; at appropriate intervals the barium sulfate was reconverted to mustard. (See 1).

IIIf-4. The Ice-Packed Post-Application Treatment.

Immediately at the termination of the exposure period, the sites were kept continuously cold for a period of 3 to 6 hours by means of an ice cube in a latex bag. A cylindrical section of cardboard just large enough to accommodate the piece of ice was strapped in place so as to keep the ice cube from moving. At intervals of about 20 minutes thereafter the ice cubes were removed so that a skin area larger than that of the application site was kept constantly chilled. The first 3 to 6 hours of the post-application period, therefore, consisted of this ice-pack treatment.

IIIg. Excision of the Exposed Sites.

Immediately after the termination of the post-application period, the pig or rabbit was sacrificed. The exposed sites were excised.

The human skin sites were anethezitized with novocain and then excised. The excised skin comprised a section of integument measuring 20x10 mm. and included a small amount of subcutaneous fat.

Upon the removal of the tissue, the abdominal incision was closed with sutures. In 98% of subjects, the wounds were completely healed in 7 days.

IIIh. Storage of Excised Skin Sites.

IIIh-1. Storage of Exposed Sites for Histological or both Histological and Chemical Analysis.

After excision the specimen was placed in a 10% formalin. In a few instances human tissues were fixed in Zenker's fluid. Practically all of the tissues subjected to this treatment were associated with post-application periods of at least 1 hour.

IIIh-2. Storage of Excised Sites Subjected to Radio-Chemical Analysis Only.

These sites consisted of most of the short post-application period tissues and some of the long post-application tissues. The sites were frozen by means of dry ice, placed in small circular metal boxes and kept continuously cold until subjected to chemical analysis.

Certain of these sites were sectioned by hand on a freezing microtone into three horizontal layers designed as epidermis, corium and fat. The sections were cut in such a manner that the first layer (1/3 to 1/2 mm) contained all of the epidermis and some corium; the second, the corium and enough fat to insure the removal of all the hair follicles; and the third layer consisted of the fat. These hand sectioned layers were all kept continuously frozen until subjected to chemical analysis.

1111. Preparation of Tissues for Histological Study:

After fixation (formalin or Zenker's) the tissues were washed in running water, dehydrated, and embedded in paraffin. The remainder of the paraffin block after six satisfactory sections were obtained together with all drippings and fluids used for fixation and dehydration were saved for chemical analysis. It was estimated that the amount of mustard sulfur lost incident to the preparation of microscopic sections comprised less than 1% of the total.

1111j. Chemical Analysis of the Skin Sites.

1111j-1. Determination of S^{35} in Skin Sites from which Histological Sections have been Prepared.

The tissue was received in two parts, 50-90% of the original skin site in the formaldehyde solution; and the remainder embedded in paraffin wax. The paraffin was was removed from the skin tissue by hot benzene filtration. The formaldehyde solution was then filtered through the same filter.

The tissue was placed in a carius tube 19x25x600 mm., 1/2 cc. of fuming nitric was added and the tube was steamed for about 15 min., one cc. of red fuming nitric, a crystal of KBr and 1.5 mg. of anhy. sodium sulfate added and the tube was sealed. It was then placed in an iron casing and heated in an electric furnace for 4 hours at 250°C. The remainder of the procedure is described in section 11c. The resulting benzidine sulfate precipitates weighed between 6 and 10 mg. (f)

The solutions(g) in which the tissues were fixed and dehydrated were boiled down, transferred to a carius tube and treated according to the procedure described in section 11-c. The addition of 3 mg. of anhydrous sodium sulfate resulted in benzidine sulfate precipitates weighing 6-7 mg. In no case was the radioactivity found in the formaldehyde washings more than 10% of the activity in the tissue. The usual value was 2 to 5%.

(f) The Zenker's fixed tissue contained a considerable amount of mercury and other salts. It was not possible to precipitate benzidine sulfate in the presence of these salts. Even when barium ion was used instead, there was considerable difficulty in quantitatively precipitating the $BaSO_4$. It was necessary to bring the solution to complete dryness numerous times. If it is at all possible the use of Zenker's solution as a tissue fixative is to be avoided.

(g) It was not possible to precipitate any sulfate ion from the Zenker solution due to the large mercury and other salt concentration. It was assumed that the Zenker's solution could not dissolve out any more activity than the formaldehyde fixative.

IIIj-2. Chemical Analysis of Skin Sites Stored on Dry Ice (see IIIh-2) "The Short Time Post-Application Period Tissues".

The procedures employed to recover mustard and its various extractable and non-extractable derivatives from excised skin is shown schematically in Table IV. Explanation for the steps designated by capital letters in Table IV are enumerated below:

(A) Since very small amounts (0-10 gamma) of mustard and glycol evaporate rapidly, carrier "inactive" mustard and glycol were added at this point. As a further precaution the tissue was always kept moist with chloroform. The tissue was ground to break up the cell structure. Chloroform was chosen for the extraction since it is very slightly miscible with water, and is as well a solvent for mustard and glycol. Further, mustard will not react in a non-aqueous media.

(B) In order to insure complete extraction in an actual animal experiment, the extract residues, in most instances, were reground and reextracted before being carbused.

(C) The petroleum ether insured a quantitative removal of the glycol to the aqueous layer; no appreciable hydrolysis of the mustard during this liquid-liquid extraction took place. An emulsion only resulted when a large amount of fat is present.

(D) The nitric acid added during the evaporation oxidized the glycol to a less volatile compound and thus prevented any loss.

(E) Since the sulfoxide formed was appreciably soluble in chloroform, the solvent was evaporated off. The 1 cc. of solvent subsequently added was for the purpose of dissolving the fat, the 0.2 cc. of chloroform being necessary to keep the fat in solution.

(F) Since the mustard has been converted to the sulfoxide, there was no appreciable loss of activity during the evaporation at 100°C.

(G) No significant amount of activity has ever been found in this layer.

The applicability of this procedure was shown in Table V; the capital letters heading the columns correspond to those of Table IV. In these experiments known quantities of radioactive mustard and glycol were added to about 1 gm. of pig skin tissue during the course of grinding. These results prove that all of the free mustard and glycol can be extracted, and can be quantitatively determined to within 5%.

In the early stages of this work a simplified version of the above procedure was used but only semiquantitative results could be obtained; all the precautions described above were then gradually introduced.

TABLE IV

Separation Scheme for Radioactive Mustard and Thiodiglycol in Animal Tissue

(A) Tissue-Cut into small pieces and grind with 60-80 mesh quartz. Keep moist with 2 cc. chloroform containing enough inactive mustard and thiodiglycol to ultimately give 7 mg. of Benzidine sulfate. Then extract with 3 cc. of hot chloroform in a continuous micro-extractor for one hour.

(B) Non-extractable: Carius with red fuming nitric acid. Pot. SO_4 as Benzidine sulfate. See II-c

(C) Chloroform Extract: Make up to 6 cc with Pet. Ether (35° to 60°). Take 5 cc. aliquot and shake with 3 cc. H_2O. Break emulsion with Tesla coil discharge.

(D) Aqueous layer contains glycol. Pipette 2 cc into micro-Carius tube and add Red Fuming nitric. Evaporate to near dryness. Add KBr and more fuming nitric acid. Then Carius. See II-c.

(E) Organic Solvent layer: Contains the mustard. Shake 4 cc. aliquot with 5 cc of conc. nitric acid. Evaporate off solvent; then add 0.2 cc of chloroform and 0.8 cc of pentane; shake.

(F) Nitric acid layer: Contains Sulfoxide. Pipette 4 cc into a micro-Carius tube and treat as aqueous layer.

(G) Organic Solvent layer: Contains primarily fat. Discard.

TABLE V

Recovery of Radioactive Mustard and Glycol from Pig Skin Tissue in Vitro. The Capital Letters titling the columns correspond to those in Table IV. (All values in Table in gammas in H-equiv.)

Activity added to tissue as Mustard	Glycol	(B) Non Extractable	(D) Aqueous Layer glycol	(F) Acid layer Mustard	(G) Solvent Layer	Total Recovered
85.0	0					
85.0	0	<0.1	1.0	75.0	4.0	80.0
		<0.1	1.0	84.0	4.0	89.0
0	25.5	.1	26.0	.8	nil	27.0
0	25.5	.1	23.0	1.0	nil	24.1

IIIj-3. Determination of the Amount of Mustard and Thiodiglycol Present in the Tissue Extract (see IIIj-2) of Some of the Short Time Post-Application Period Tissues.

By exposing the tissue sites to mustard of high specific activity (1 millicurie/10mg. of H), it was possible to determine the percent of the chloroform "extractables" (IIIj-2) that was mustard and thiodiglycol; the method used was essentially the "isotope dilution procedure"(6)

100 mg. of non radioactive mustard was added as carrier to the organic solvent layer (Layer E of Table IV). The sulfoxide was then prepared by nitric acid oxidation; the sulfoxide was repeatedly recrystallized until the radi activity per mg. became a constant. A comparison of the amount of activity present per mg. of sulfur as benzidine sulfate of the original organic solvent layer with the radioactivity per mg. of the recrystallized sulfoxide as benzidine sulfate enabled the computation of the amount of mustard present in the tissue chloroform "extractables".

Fifty mg. of nonradioactive thiodiglycol was added to the water soluble fraction (Layer D of Table IV). The solution was extracted several times with chloroform to remove the thiodiglycol.

The di-p-nitrobenzoate derivative of thiodiglycol was now prepared. Recrystallizations of the derivative were then carried out until constant radioactivity per mg. of this derivative was obtained; three crystallizations usually sufficed. Similarly to above, the amount of thiodiglycol originally present in the tissue chloroform "extractables" could now be calculated.

The preparation of these samples for radioactivity determinations was done by the method described in section IIc.

IIIj-4. Chemical Analysis of Skin Sites Stored on Dry Ice. (see IIIh-3) "The Long Time Post-Application Period Tissues".

It was soon discovered that tissues with relatively long post-application periods (>1 hr.) did not contain enough "extractable" activity to warrant the separation scheme given in IIIj-2. These tissues were treated in two ways:

(1) Carlused directly and then subjected to the procedure given in section IIc.

(2) Extracted with chloroform; and then treated as in (1). A great many of these chloroform solutions were analysed for radioactivity. In no case was the activity present in this chloroform tissue extract more than 5% of the activity present in the corresponding tissue.

IIIk. Determination of the Amount of Mustard Penetrating by the Radiochemical Analysis of the Contents of Applicator Cups before and after Exposure. (see IIIe-1)

The procedure of determining the amount of mustard that had penetrated

was based on a difference determination. Through standardization, the weight of mustard originally in the applicator cups predetermined the radioactivity; thus the subsequent radiochemical analysis of the applicator cups permitted the calculation of the amount that had penetrated.

This radioactivity determination was performed on the special nitric acid solution containing the applicator cup and three cotton swabs. The nitric acid solution was filtered and three 1 cc. aliquots were pipetted into micro-carius tubes and subjected to the procedure described in section II-c.

Originally this nitric acid solution consisted of exactly 5 cc of concn. nitric acid containing enough non-radioactive mustard sulfoxide in 1 cc. to produce exactly 7.00 mg. of benzidine sulfate. Since all of the weighable sulfur was due to the "carrier" sulfoxide, the weight of the benzidine sulfate precipitate (6.80-7.20 mg.) determined extremely accurately the aliquot used for the radioactivity measurement.

During the course of the penetration experiments on one type of animal, five standardizations were made. These consisted of analysing known weights of radioactive mustard (300 to 500 gammas) by the above procedure. The specific activity of mustard used in these particular experiments (5 micr curies/mg. H) was such that 500 gammas subjected to the above procedure would produce 7 mg. of benzidine sulfate with a radioactivity 150 times background (see section IIf).

In view of the determination of aliquots by weight, the triplicate analyses, the five standardizations, and the rigid specifications for the specific activity of the mustard, it was possible to have the measured radioactivity determine the weight of mustard with maximum error of 3%, or buble error of 1%.

IIIm. Special Chemical Procedures:

IIIm-1 Procedure for the Determination of the Amount and Rate of Hydrolysis of Fixed mustard in Pig Skin Tissues as a Function of Alkaline pH.

i) Preparation of the Tissue: The epidermis and the upper part of the corium of pig skin that had been exposed to mustard were removed to a depth of 25 microns by means of a freezing microtome. A fine tissue pulp was obtained. This material was extracted five times with acetone at -5°C to remove all acetone soluble bodies.(g) The tissue was freed of acetone under reduced pressure at 4°C and ground in a mortar. Subsequent analysis of portions of this tissue showed it to be remarkably homogeneous in regard to its radioactivity per unit of weight.

ii) Methods of Determining the Effect of pH on Hydrolysis of Fixed H* in Tissue; a 10 mg. sample of the tissue containing fixed H* was

(g) No appreciable amount of acetone soluble material containing fixed mustard was ever found.

placed in a 12 cc. centrifuge tube and 2 cc. of the hydrolyzing solution containing a drop of toluene was added. Borate buffers and 0.1 N NaOH were used. The tubes were shaken for 45 hours at room temperature (23°C). At the conclusion of the shaking, a one cc. aliquot of the supernatent hydrolysate was removed and analyzed for radioactivity. The one cc. of solution remaining in the tube was diluted to 6 cc. with 95% alcohol. A 5 cc. aliquot was removed and analyzed for radioactivity. The value obtained here represents substances containing fixed H* soluble in 80% alcohol. The residue containing the material not soluble in the hydrolyzing solution and the material precipitated by the alcohol was washed well with 95% alcohol and analyzed for radioactivity.

iii) Determination of the Amount of Thiodiglycol in the Alkaline Hydrolysates: 100 mg. portions of the radioactive tissue were hydrolyzed with 3 cc. of the hydrolytic solution (containing merthiolate) for 70 hours at 23°C. Ordinary thiodiglycol was added to the hydrolyzate and the radioactivity per unit weight of sulfur as benzidine sulfate (see IIc), determined (99% of the sulfur present being due to carrier thiodiglycol). The solution was extracted several times with chloroform to remove the thiodiglycol. The crystalline di-p-nitrobenzoate of the thiodiglycol was prepared and purified until the radioactivity per unit of weight of sulfur as benzidine sulfate was constant. This activity divided by the activity in the original hydrolysate gave the percentage of thiodiglycol in the hydrolysate.

iv) Determination of the Rate of Hydrolysis of Fixed H*: In a series of centrifuge tubes, were placed 20 mg. of the tissue and 4 cc of the buffered solution (containing merthiolate). The tubes were shaken at room temperature. At various time intervals a tube was removed from the shaker and the hydrolysate and residue analyzed for radioactivity.

IIIm-2. Procedure for Determining the Fraction of the Fixed-Mustard Present in the Nuclei of Pig Skin Tissue.

i) The separation of the Nuclei from Pig Skin; of the several methods which have been developed for the isolation of nuclei from tissue, the procedure of Marshak (10) seemed the most useful for our purpose. In this procedure the nuclei were liberated by treating the tissue with 5% citric acid and the nuclei isolated by fractional centrifugation. Although this method gave excellent results with liver, bone marrow, spleen and heart muscle, it was not directly applicable to skin. Preliminary work on the separation of nuclei from skin showed that the main difficulty was the presence of extraneous material insoluble in 5% citric acid and of about the same density as skin nuclei. These extraneous substances were primarily collagen and keratin (cornified epithelial layer).

The following procedure avoided these difficulties: the skin from one side of a pig was excised in one piece and spread out flat. Most of the cornified layer of the skin was removed by scraping with a sharp knife, until the further recovery of material was negligible. Microscopic examination showed that little or none of the nucleated malpighian layer was removed by this treatment.

A 5% solution of citric acid was then poured over the skin and after 10 to 15 minutes the excess was removed. The skin was then scraped with a sharp knife until the yield of tissue was negligible. This process was repeated and it was found that three or four scrapings were usually sufficient to remove all of the epidermis. Excessive scraping will remove fibers of collagen and this was avoided by paying close attention to the appearance of the brei.

Histological preparations of the original skin, of the skin after the dry scraping, and of the skin after the citric acid scraping treatment were made and disclosed that the material comprising the citric acid scrapings had been derived almost entirely from the malpighian layer.

The brei of the malpighian layer was then ground with 5% citric acid in a potter-Elvehjem homogenizer until microscopic examination showed separation of most of the nuclei from the tissue. The brei was then centrifuged according to the method of Marshak and the top layers of nuclei were separated. The bottom layers consisted of masses of nucleated cells and strands of collagen and keratin. This brei was reground and recentrifuged to increase the yield of nuclei.

The nuclear fraction was purified by repeated centrifugation from 5% citric acid, which served to remove most of the cytoplasm. Further purification was obtained by washing the nuclei several times with isotonic saline and centrifugation and then passing the nuclei through four layers of cheese cloth. Thereafter further purification by 5% citric acid and centrifugation with rejection of debris at the bottom of the centrifuge tube yielded nuclei(h) contaminated only with occasional threads of collagen and a few cornified cells. The residual citric acid was removed by washing with isotonic saline.

Thus the above procedure separated the original pig epidermis quantitatively into three layers, namely; cornified epithelial layer, malpighian layer, and corium. A certain fraction of the nuclei from the malpighian layer were obtained in a relatively pure state.

(ii) The Determination of the Amount of Thymonucleic Acid in the Nuclear Brei.

The preparation of nuclei was analyzed for thymonucleic acid by use of the Beckman ultraviolet spectrophotometer. The major part of the absorption in the ultraviolet was due to the thymonucleic acid present. The nuclei were dissolved by heating in a boiling water bath with

(h) Microscopic smears were essential to the following of the purification.

10 ml. of 0.5 N sodium hydroxide. One ml. of the resulting solution was removed and made up to a volume of 10 ml. This solution was measured in the ultraviolet spectrophotometer, the absorption at 2600A° being recorded. This wave length represents the maximum for pure thymonucleic acid prepared from large amounts of pig skin nuclei. Furthermore the changes in absorption spectrum of thymonucleic acid in alkaline solution observed by Heyrouth and Loofbourow (11) were negligible in this range.

(iii) The Determination of the Amount of Thymonucleic Acid Originally Present in the Malpighian Layer of the Exposed Skin.

An aliquot of the citric acid scrapings was analyzed for thymonucleic acid by the method of Dische.(12) This method consisted of peptic digestion to remove a large amount of the cytoplasmic material, solution of the residue by heating with sodium hydroxide, neutralization with acetic acid, and precipitation of thymonucleic and other compounds(j) by alcohol. The determination depended upon the development of a color by reaction of the desoxyribose, present only in thymonucleic acid, with diphenylamine. Although the solution was green the maximum deflection on the Klett colorimeter was obtained with the 540 filter and this was used to determine the absorption. The amount of thymonucleic acid present was proportional to the number of nuclei.

(iv) Standardization of Method (ii) with Respect to Method (iii).

The relation of the ultraviolet absorption of the thymonucleic acid of the purified nuclei (ii) to the intensity of color developed with diphenylamine by the desoxyribose (present only in thymonucleic acid) (iii) was determined by analyses on aliquots of large amounts of pure nuclei prepared from the malpighian layer of pig skin.(k)

(v) Determination of the Amount of Nitrogen Present in the Malpighian Layer and the Isolated Nuclei.

The nitrogen was determined by Kjehldahl digestion and direct Nesslerization.

(j) These other compounds, principally rib nucleic acid of cytoplasm would interfere with the use of method (ii) on the malpighian layer. The yield of pure nuclei was not sufficient to enable the use of method (iii) with isolated nuclei. Thus the two different procedures for determining thymonucleic acid content were necessary.

(k) The standardization was initially done with isolated rabbit liver nuclei. There was no appreciable difference in the standardizations.

Part IV

RATE OF PENETRATION OF SKIN (ANIMAL AND HUMAN) BY MUSTARD. LOCAL FATE OF PENETRATED MUSTARD.

by H. L. B., F. C. H., A. R. M. and L. A. P.

The rate at which mustard penetrated the skin was determined by measuring the amount of mustard in the application cup before and after contact with the skin. The amount of mustard represented by the difference less the amount recovered from the pentane washings of the exposed skin represented the amount penetrated. To establish differences sufficiently large to be statistically reliable it was necessary that cups be kept in contact with the skin for an hour or longer. It was not possible therefore to make direct measurements of penetration rate in the case of short (less than 1 hour) exposures. It was found, however, that there was a fairly constant relationship between the amount penetrated and the amount that became fixed in the tissues at the site of exposure. Since it was possible to make an accurate determination of the amount fixed, even in exposures as brief as 10 minutes, the rate of penetration for such exposures could be interpolated from the amounts of fixed mustard recovered from the tissues.

Thus the penetration data provided information regarding the amount penetrated, the amount fixed, the amount extractable, and the amount that had been transported from the site of exposure.

IV (a). Penetration Rate Experiments with Pigs.

IVa-1. Penetration data with pigs

A complete compilation of the initial penetration studies with pigs has been published.(7) At the time of these experiments the extent to which environmental temperature affects the penetrability of the skin was not known. Although these data are of little value so far as the rate of penetration is concerned, the results pertaining to the fate of the penetrated mustard are valid. These latter results are in excellent accord with the following data.

Purpose: to determine penetration rate and fate of penetrated mustard in pig skin under controlled environment conditions.

Experimental Procedure: See III (a-1, b, c, d-1, e-1, f-1, f-3, g, h-2, j-2, j-4, k). Two white shoats were used (A-362 and A-371), one at an environmental temperature of 60° F and the other at 103° F, (mustard conc. was about 1.1 mg. per sq. cm.). All exposure times were of one hour duration. Post application periods were either 0 or 24 hrs.

Results: The experimental results are given in Table VI.

Discussion: See IVa-4.

TABLE VI

Penetration and Fate of Penetrated Mustard in Pig Skin
One Hour Exposures to 1.1 mg. per sq. cm. Skin Surface.
(All calculations based on gammas of H-equiv.)

Pig A-362 Environmental Temp. of 60° F; Skin Temp. = 92.5° F

Gammas Appl.	Gammas* Penet. ±35	Post-applic. Time, hrs.	Gammas* Fixed ±1	Gammas* Ext. ± 1	Average % of Amt. Penet.
1080	35	24	5.6	nil	
1120	12	24	8.8	nil	
1130	23	24	9.3	nil	
1180	80	0	10.4	15.5	
1120	35	0	6.8	13.7	
1100	60	0	8.8	11.8	
Average					
					Fixed Ext.
1120	40	24	8.5	13.5	21 nil
		0			35

Pig A-371 Environmental Temp. = 103° F; Skin Temp. = 105° F

1140	200	24	37	nil	
1180	300	24	63	nil	
1140	280	24	75	nil	
1130	280	0	68	44	
1120	280	0	70	42	
1160	245	0	83	28	
Average					
					Fixed Ext.
1130	270	24	65	35	24 nil
		0			14

*The values to the right of the plus or minus sign represent maximum possible error due to the analytical procedure.

IVa-2. Nature of the Chloroform Pig Skin Tissue Extractables

Purpose: To determine the percentages in pig skin of the chloroform extractables that were mustard and thiodiglycol.

Procedure: See III (a-1, d-3, e-2, f-1, g, h-2, j-3). Six skin sites of pig A-290 received applications of mustard (1 mg. per sq. cm.) for 4 hrs. The post application period was of 0 hr. duration.

A radioactivity equivalent to 1000 counts per gamma-H enabled the determination of the amount of mustard and thiodiglycol in the chloroform extract of the entire tissue site by the procedure given in Section IIIj-3.

Results: The results in duplicate are given in Table VII.

Table VII

The Percentage of the Chloroform Extractables That Were Mustard and Thiodiglycol

Activity in Original Chloroform Phase = 100%

Chloroform Pet. Ether Phase				Aqueous Phase			
Aliquot of	No. of Crystal	% Activity (a)	(b)	Aliquot of	No. of Crystal	% Activity (a)	(b)
Original layer		70	73	Original layer		30	27
Crude sulfoxide	0	70	73	Crude derivative*	0	28	26
Sulfoxide	1	60	65	Derivative*	1	27.5	24.5
Sulfoxide	3	54	56	Derivative*	3	27	24.5
Sulfoxide	5	52	56	Derivative*	6	27	24.6
Sulfoxide	6	52	55	Derivative*	8	27	24.3

*The p-nitrobenzoyl derivative of thiodiglycol

Thus 75% of the activity in the chloroform pet. ether phase is H
90% of the activity in the aqueous phase is thiodiglycol

Based on the original chloroform extractables, 50% is mustard, 25% is thiodiglycol and 25% of the activity is in unknown constituents.

Calculations are based on the original activity found in the chloroform extract.

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

(1) With sufficient mustard to saturate pig skin, mustard penetrated at a rate of about 40 gamma/hour at an environmental temperature of 60° F and 230 gamma/hour at 105° F. It was estimated that at room temperature, 70° C, the rate was approximately 90 gamma/hour.

(2) The percentage of penetrated mustard which became fixed in pig skin was markedly constant, 25 ± 5%,[a] over a range of penetrated mustard values from 40 gammas to 2.2 mg. Further, the fraction of the penetrated mustard fixed was independent of the environmental temperature.

(3) At least 95 ± 5% of this fixed mustard was always in the epidermis and corium of pig skin.[a]

(4) Zero hour post-application period sites have extractable radioactivity varying from 15 to 35% of the penetrated mustard depending somewhat upon the exposure time. A greater fraction of extractable activity is to be associated with a low environmental temperature.

(5) About 50% of the chloroform extractables was mustard, 25% thiodiglycol and the remaining 25% was comprised of unidentified substances.

(6) 24 hour post-application period sites had a negligible amount of radioactivity in the chloroform extractable fraction.

These data give a general picture of the penetration of the pig skin by mustard. In view of the human data to be presented, a more detailed exposition of the experiments on pigs is not justified.

IVb. Penetration of Rabbit Skin by Mustard

Purpose: To study the penetration rate and the fate of penetrated mustard in rabbit skin.

Experimental Procedure: See III (a-2, b, c, d-1, e-1, f-1, f-3, g, h-2, j-2, j-4). Two rabbits (A-415 and A-417) were used, one at an environmental temperature of 60° F and the other at 100° F. The concentration of the applied mustard was about 1.1 mg. per sq. cm. All exposure times were one hour; post-application periods were either 0 or 24 hours.

Results: The experimental results are given in Table VIII.

(a) These conclusions are based in part upon data published in a formal report.[7]

Table VIII

Penetration and Fate of Penetrated Mustard in Rabbit Skin
One Hour Exposures to 1.1 mg. per sq. cm. Skin Surface.
(All calculations based on gammas of H-equiv.)

Rabbit A-417; Environmental Temp. = 60° F; Skin Temp. = 91° F.

Gammas Appl.	Gammas Penet. ±35	Post-applic. Time, hrs.	Gammas Fixed ± 1	Gammas Ext. ±1	Average % of Appl. Penet.
1025	200				
1025	315	0	14	23	
1025	280	0	30	54	
1060	500	0	23	54	
1060	420	24	28	0	
1060	540	24	26	0	
Average		24	33	0	
1040	340	0			Fixed Ext.
		24	26	45	8 16
					0

Rabbit A-413; Room Temp. 100° F; Skin Temp. 106° F.

1010	665				
1010	750	0	74	77	
1010	720	0	60	37	
1010	695	0	79	56	
1110	720	24	81	0	
1100	885	24	93	0	
Average		24	83	0	
1060	770	0			Fixed Ext.
		24	75	56	10 8
					0

Discussion: (1) With rabbits at an environmental temperature of 60° F, approximately 35% of the mustard applied penetrated in 1 hr. (application of 1.1 mg. per sq. cm. under a closed cup). At 100° F approximately 75% penetrated in 1 hr. It was estimated that with sufficient mustard to saturate the skin at room temperature (70° C), about 45% would penetrated (500 gamma per sq. cm. per hour).

(2) After an exposure of 1 hr., approximately 10% of the mustard that had penetrated the rabbit's skin was fixed locally. Immediately after a 1-hr. exposure at 60° F, approximately 16% in H-equivalents of the penetrated mustard was extractable. At 100° F approximately 8% in H-equiv. of the penetrated mustard was extractable.

IVc. Penetration Studies Using Human Subjects; the Subsequent Fate of the Penetrated Mustard.

R. S. M. Surgical Consultant

Purpose: Determination of the penetration rate of mustard through human skin and the fate of this mustard in the skin as a function of environmental and/or skin temperature.

Procedure: See III (a-3, b, c, d-1, e-1, f-2, f-4, g, h-2, j-2, j-4, k). Each of eight human subjects were exposed to two applications of mustard (1.1 mg. per sq. cm.) for 1 hr. With three subjects a 24-hr. post-application period elapsed before the exposed sites were excised; the exposed skin sites of the other five subjects were excised immediately after the 1-hr. exposure.

Results: Observations of the injury to the skin 24 hrs. after a 1-hr. exposure disclosed elliptical areas of epidermal necrosis slightly larger than the size of the cup that was used. The lesions were raised above the surface of the surrounding skin. There was a faint zone of erythema at the periphery. The border comprised a confluent, narrow linear zone of vesication about 1 mm. wide. The centrum, measuring about 4x10 mm., was grayish-yellow and appeared macerated.

The penetration studies are given in Table IX. It must be kept in mind that the skin penetration rates are determined by difference in the radioactivity of the contents of the cup before and after application and have an over-all accuracy of ±35 gamma per sq. cm. It is obvious that at the lower environmental temperatures the experimental error is considerable.

Plates II and III are graphical representations of the amount penetrating in relation to environmental and skin temperatures respectively.

The amounts of penetrated mustard in H-equiv. which became fixed and were extractable after either a 0 or 24 hour post-application period are given in Table X.

Discussion: (1) The penetration of human skin in an environmental temperature of 100° F (skin 100° F) was approximately three times as rapid as it was at 50° F (skin 85° F).

(2) With sufficient mustard to saturate the skin, mustard penetrated at a rate of about 200 gamma/hour/sq.cm. of skin surface at average room temperature (70° C); 350 gamma/hr. in a 100° F environment; and 90 gamma/hour in a 50° F environment.

(3) About 12% of the total amount penetrated in one hour was fixed locally and the proportion fixed was independent of temperature. The amount of fixed mustard was unchanged during the first 24 hours after the exposed skin was decontaminated.

TABLE IX

Penetration of Human Abdominal Skin by Mustard as a Function of Environmental and/or Skin Temperature

One Hour Exposures to 1.1 mg. per sq. cm. of Skin Surface

Exp. No.	Environ. Temp., °F	Skin Temp. ± 10° F	Amt. H Appl. in Gammas	Amt. H Penet. in Gammas ±35	Average Amt. Penet. Gammas/Ft.
H360	101	99	1130	300	350
			1050	410	
H359	102	97	1100	250	260
			1140	310	
H370	100	98.5	1090	320	320
			1130	320	
H358	75	95	1070	245	270
			1120	290	
H357	72	93	1470	230	220
			990	220	
H369	60	90.5	1100	70	110
			1080	140	
H364	52	83.5	1120	95	110
			1120	130	
H363	49	84.5	1080	70	70
			1090	70	

TABLE X

Local Fate of Penetrated Mustard in Human Skin

Skin Exposed to 1.1 mg. per sq.cm. of H for one hour

(All calculations based on gammas of H-equiv. per sq.cm. of skin surf.

Exp. No.	Skin Temp., °F	Post Appl. Pd. Hrs.	Penet. of H (Gammas)	Amount Evap.* (Gammas)	Amount Fixed (Gammas)	% of Penet. H-Fixed
H-360	99.0	0	330	0.0	43	13.0
H-370	98.5	0	385	3.9	37	10.0
H-359	97.0	24	280	0.0	26	9.0
H-357	93.0	0	210	0.0	7?	3.0?
H-358	95.0	24	270	0.0	33	12.0
H-369	90.5	0	130	1.9	18	13.5
H-364	83.5	0	105	0.0	15	14.5
H-363	84.5	24	70	0.0	9.5	13.5
Average		0		1.0**		12 ± 3 (max. dev.)
		24		nil		

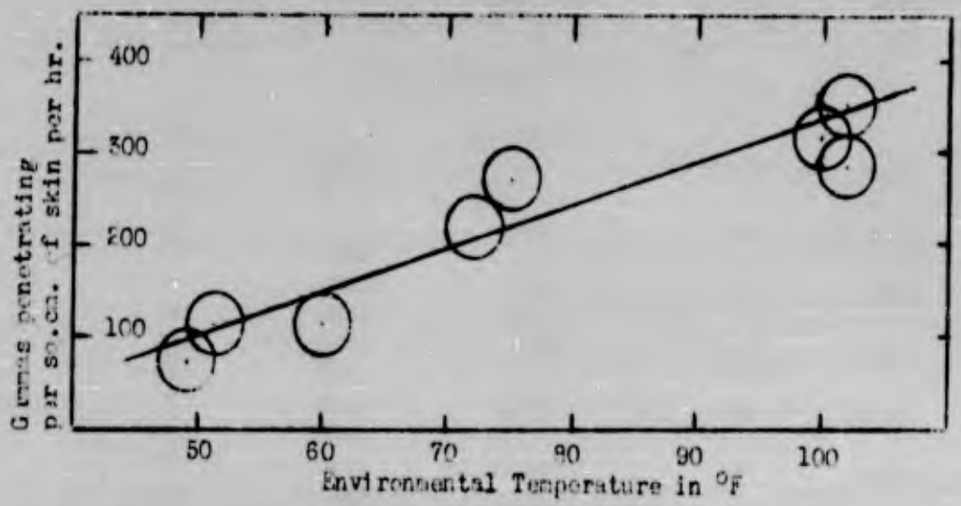
*Humidity of room, 50%; humidity under cup, 100%.

**In all zero-hour application experiments except H-369 and 370, a separation of the extractable activity into water-soluble and pentane-chloroform soluble fractions was made. Due to this separation and since the specific activity of the penetration study mustard was only 500% background per mg (see IIIk) an extractable activity of the order of 1.5 gammas might not be detected; however, 2.5 gammas certainly would have been.

33 Effect of Temperature on the Penetration of Human Abdominal Skin by Mustard

Plate II[a]

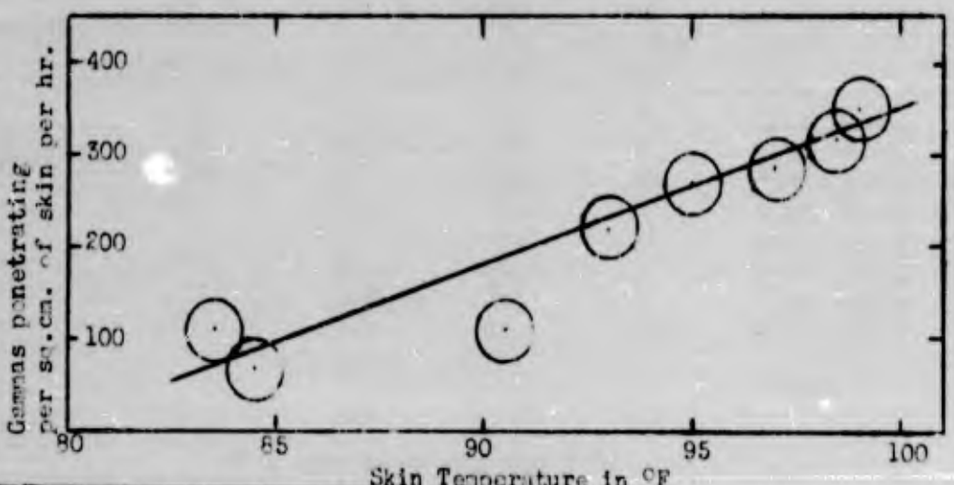
Skin Penetration vrs. Environmental Temperature



All Sites Exposed to 1.1 mg. of mustard per sq.cm. of Skin Surface for one hour

Plate III[a]

Skin Penetration vrs. Skin Temperature



(a) Size of circles indicate maximum experimental error. Due to short temperature range, straight lines have been drawn; extrapolation in either direction is not warranted.

(4) Immediately at the termination of a one-hour exposure, only 1% of the penetrated mustard in H-equiv. was extractable. Since this amount (1 to 4 gammas per sq. cm.) was trivial as compared to the total amount penetrated, the determination of what portions of the "extractable" were mustard and thiodiglycol would have had little significance (see IVa-2).

(5) It appeared that mustard was either fixed or transported almost as rapidly as it penetrated human skin and that the reservoir of free mustard that was present in the skin at the conclusion of an exposure was very small.

IVd. Comparison of the Penetration and Rate of Penetrated Mustard Data for Pig, Rabbit, and Man.

The comparative data are summarized in Table XI.

TABLE XI
Summary of Average Penetration Data for Man, Pig, and Rabbit
One-hour Exposure to 1.1 mg. per sq.cm. of Mustard

Room Temp. °F	Subject	Skin Temp. °F	Post-Applic. Time, hrs.	Amt. Penet. Gamma/Cm.	% Penet.	% of Penet. Fixed	% of Penet. DH Extract
60	Man	90.5	0	130	12	12	1
			24				0
60	Pig	92.5	0	40	3.5	21	35
			24				0
60	Rabbit	91	0	360	33	6	16
			24				0
102	Man	98	0	330	30	12	1
			24				0
103	Pig	105	0	250	23	24	14
			24				0
100	Rabbit	106	0	850	75	10	8
			24				0

At environmental temperature of 60° F, ratios of penetration rates for man: pig: rabbit were 1.0/0.3/2.8

At environmental temperature of 102° F, ratios of penetration rates for man: pig: rabbit were 1.0/0.75/2.5

Discussion: The following comparative conclusions can be drawn:

(1) Mustard penetrated rabbit skin faster than either man or pig. With sufficient mustard to saturate the skin surface, at a room temperature of 60° F, the amounts penetrating per sq. cm. of skin surface for man: pig: rabbit were 130/40/360 gamma per hr. At 100° F the respective rates were 330/250/850 gamma per hr. At average room temperature (70° F) these respective penetration rates became 200/100/500 gamma per hr. (Conditions of constant humidity.)

(2) The temperature coefficients of penetration rates for man and rabbit were about the same (1.3/10° F of room temperature). That for pig was considerably higher (1.6/10° F of room temperature).

(3) At the termination of a 1-hr. application, both pig and rabbit had an appreciable reservoir of unreacted mustard (about 50% of the "extractables" as compared to man. Man's reservoir was negligible. None of the animal tissue sites had significant quantities of "extractables" 24 hrs. after an exposure.

(4) In man, pig and rabbit the amount of mustard fixed at the portal of entry depended only on the amount that penetrated. About the same percent of the amount penetrating was fixed in rabbit (10%) and in man (12%). With pig the amount fixed was 25%. These percentages were invariant with respect to environmental conditions.

(5) Consideration of conclusions (1) and (4) showed that at 70° F, in a given time interval, rabbit under the same conditions will fix 2.5 times as much mustard in the exposed site as either man or pig. In view of the correlation between the amount fixed and the severity of the ensuing injury (see section VII) it is apparent why the rabbit is more sensitive than man or pig.

(6) The data presented in Table VI, VIII and IX show that the reproducibility of results was greatest in man and least in rabbit. The reproducibility with man was probably due to the smooth texture and the relatively thin keratinized layer of the abdominal skin. The relatively large experimental error with rabbits was probably due in part to the ease with which the skin was stretched and abraded and in part to the numerous and large hair follicles.

(7) In view of the findings described in (5) and (6), it was to be expected that in man and pig, reproducible burns were obtained at threshold level when the exposure time and the environmental conditions were controlled. This was decidedly not the case with rabbits. Aside from the irreproducibility of experimental results with rabbits, an exposure to an excess of mustard for 10 min. at 70° F would result in the penetration of about 8 times as much as was necessary to produce a threshold lesion.(a) Thus extreme care must be exercised both in the control and interpretation of all therapeutic experiments using rabbits as test animals.

(a) This calculation is based in part on data presented in section VII.

Studies of penetration, fixation and transport of mustard during and after short time (less than 1 hour) exposures of pig, rabbit and man.

The observations described in the preceding section were made after exposing the skin to mustard for an hour or longer. The experiments here described were undertaken to obtain information regarding the immediate fate of mustard after penetrating the epidermis. Hence it was necessary to reduce the time of application to a minimum.

With pig and man, a 10 minute exposure at 70°F and with rabbit, a 10 minute exposure at 60°F was the shortest possible time in which reproducible amounts penetrated could be expected. Exposures of this duration in man usually resulted in vesication, in pig in intense erythema with or without hemorrhage; and in rabbit in a relatively circumscribed hemorrhagic lesion. The lesions were characterized as moderately severe.

By excising the skin at different times after completion of 10-minute exposures information concerning (i) the rate at which penetrated mustard became fixed, and (ii) the rate at which the chloroform extractables and/or mustard were carried away from the exposed skin site was obtained. Attention has already been called to the fact that it is not possible to make a direct determination of the amount of mustard that penetrates the skin in a 10 minute period. (See Section IV) It was assumed that either the percent of the penetrated mustard fixed is invariant to time during the first hour, or the amount of mustard penetrating is simply 1/6 of that penetrating in one hour at the same skin temperature. Neither assumption is supported by direct evidence.

V a The Short Time Exposure and Post-application Data on Pigs.

By F.C.H., G.B.K., C.M., A.R.M., L.A.P., L.G.S.

Purpose: To determine the rate of fixation and transport of penetrated mustard.

Experimental Methods: See III (a-1, d-3, e-2, f-1, g, h-2, j-2) Two pigs (A-306 and A-707) were exposed for 10 minutes to 0.5 mg. per sq.cm. of mustard. The post-application periods varied from 0 min to 6 hrs. The environmental conditions of these experiments were not recorded.

Results: See Table XII

Discussion: (1) Immediately after the termination of a 10 minute exposure to mustard, there was a considerable reservoir of unreacted mustard in the skin at the exposure site. (2) No further local fixation of it took place

after a 10-minute post-application period in spite of the fact that a considerable amount of H was still present (75% of the activity)[a] This H was slowly carried away by the body fluids. (3) With pigs after an exposure of 10 minutes about 70% of the fixed material was present in the epidermis and 30% in the upper corium. (4) The constancy at each post-application period of the individual ratios of extractable/fixed indicated that the variations in the chemical results were due to differences in rate of mustard penetration.

V b. The Short Time Exposure and Post-application Experiments with Rabbits.

by H.L.B., F.C.H., C.M., A.R.M., L.A.P.

Purpose: To obtain the rate of fixation and transport of penetrated mustard.

Experimental Methods: See III (a-2, b, c, d-3, e-2, f-1, h-2, j-2)

The rabbits (A-466 and A-467) were exposed for 1.1 mg. per sq.cm. of mustard at a room temperature of 60°F. The post-application periods varied from 0 minutes to 24 hours.

Results: See Table XIII.

Discussion: (1) Immediately at the conclusion of a 10 minute exposure, there was a considerable reservoir of unreacted mustard in rabbit skin. (2) Immediately following the 10 minute exposure in rabbit, only about half of the mustard that would ultimately become fixed had already done so. (3) After a 15±5 minute post-application period, fixation was complete and the reservoir of free mustard (extractables) had become inconsequential.

V c. The Short Time Exposure and Post-application Data on Human Subjects.

By H.L.B., F.C.H., F.S.M., A.R.M., L.A.P.

Purpose: To determine the fate and distribution of the mustard in abdominal skin after short time exposures to 1 and 10 mg. per sq.cm. of mustard.

(a) The explanation of this phenomenon may be as follows: After penetrating the skin, H has two alternatives, namely either (1) finds a reactive molecule and becomes fixed or (2) finds a fat cell or fat molecules and becomes immobilized by solvent action. The above takes place in about 10 minutes, and there is a 25% chance for (1) to happen and a 75% chance of (2) taking place. The H which is immobilized by mechanism (2) is slowly extracted by the body fluids with which it partly reacts.

TABLE XII

Short Time Experiments for Pig
Rate of Fixation and Transport from Application Site
Both Pigs Exposed to 0.5 mg per sq.cm. of Mustard for 10 min.
(Amounts in gammas of H-equiv.)

Pig A-307

Post Appl Time	Gammas Fixed[1]		% of Fixed in epid ½ mm thick	Gammas Extract-able	% of ext. H	Ext./fixed
	1.10		65	9.55		8.7
0	1.50	1.15	70	8.37	94	5.6
	0.80		60	10.60		13.2
	2.75		70	4.85		1.8
10	3.30	3.00	65	5.90	85	1.8
	3.30		65	3.50		1.1
20	3.30	3.30	80	3.30	79	1.0
	3.50		70	2.10		0.60
60	2.25	2.90	60	1.30	73	0.58
	2.40		60	0.85		0.35
2 hr	3.90	3.15	70	1.20	70	0.31
	3.10		75	1.00		0.32
6 hr	3.90	3.00	80	1.05	65	0.36

Pig A-306

0	-	-	-	-	-
10	2.65	3.30	8.35	95%	3.2
10	3.90		9.60		2.4
20	2.15	2.05	3.44		1.6
20	1.95		3.40		1.8
30	2.20	2.50	2.75		1.2
30	2.95		3.85	84%	1.3
40	2.45	2.25	3.10	78%	1.15
40	2.00		2.30		1.25
60	3.00	2.85	1.90		0.65
60	2.70		1.20		0.45

TABLE XIII

Short Time Experiments for Rabbit

Rate of Fixation and Transport from Application Site. Both rabbits exposed to 1.1 mg per sq.cm. of H in elliptical cups (0.43 cm^2) for 10 min.

(Amounts in gammas of H equiv.)

RABBIT A-466 Room Temp. 60°F Skin Temp. 93°F				RABBIT A-467 Room Temp 60°F Skin Temp 94°F			
Fixed/cm^2 in gammas	Ext./cm^2 in gammas	Ratio of Ext./Fixed	Post-appl. period	Fixed/cm^2 in gammas	Ext./cm^2 in gammas	Ratio of Ext./Fixed
5.6	37		0 min.	3.3	43	
4.6 (4.6)	40 (37)	8.0	0 min.	4.2 (4.2)	36 (40)	9.5
3.5	33		0 "	5.3	40	
4.9	2.9		10 "	8.1	2.2	
7.0 (6.5)	2.7 (3.1)	0.50	10 "	9.3 (8.4)	3.7 (2.7)	0.32
7.7	3.8		10 "	7.9	2.1	
12.1 (9.5)	1.0 (0.9)	0.10	20 "	7.9 (7.2)	1.0 (1.2)	0.17
7.0	0.8		20 "	6.5	1.4	
6.8 (8.1)	1.3 (1 2)	0.15	40 "	11.2 (9.8)	0.6 (0.7)	0.07
10.0	1.1		40 "	8.6	0.7	
8.4	0.4		24 "	9.8	0.5	
8.6 (8.3)	0.3 (0.5)	0.06	24 "	8.1 (8.6)	0.4 (0.5)	0.05
11.9	0.6		24 "	9.5	0.5	
7.0	0.6		24 "	7.0	0.4	

Experimental Methods: See III (a-3, b, c, d-2, e-1, f-2, f-3, g, h-1, h-2, i, j-1, j-2)

Two 10 minute abdominal exposures of 1.1 mg. per sq. cm. of mustard were made on each of seven men. One site had a 24 hour post-application period, while the post-application period of the other site varied from 2.5 to 10 minutes.

Six sites (3 subjects) received 10 min. exposures to 10 mg. per sq.cm. of mustard. Contrary to our usual practice (exposures under a shallow closed cup) the exposures in these experiments were made in open rings. The ring was employed to prevent the mustard from spreading. The inside area of the elliptical ring was identical with that of the cup, namely 0.43 sq.cm. 4.3 mg. of Dn was delivered by means of a micro-pipette into the elliptical ring. One site of each man was excised as quickly as possible (2-3 minutes) after the termination of the exposure time. The other site was excised 24 hours later.

The environmental temperature in these experiments ranged from 70 to 76°F.

Results: The experimental data are given in Table XIV. The macroscopic and microscopic appearance of the 24 hour post-application period lesions will be found in Section VI.

Discussion: (1) It will be noted from Table XIV, that the variation of the amount fixed for different subjects was considerable. The differences may be due in part to individual variations in penetration rate and in part to differences in fixation. It is to be noted however that the amounts fixed in the two skin sites of the same subject (identical H number) were usually about the same.

(2) With post-application periods longer than 2 minutes, there was no appreciable difference between the amounts fixed for comparable sites (See the fixed column for each individual subject). Thus, within 2 minutes after a 10 minute exposure period all of the H which was going to be fixed in a skin site had already done so.

(3) Compared to the amount of mustard which penetrated during the 10 min. exposure period (15±10 gamma)(b) the amount of free

(b) This figure is calculated as follows: The data given in Section IVc show that about 12% of the penetrating mustard is fixed. Thus for H 411, the amount penetrating is $\frac{.60}{.12}$ = 5 gamma, and for H 416 = $\frac{1.2}{.12}$ = 10 gamma. On the basis of 200 gamm/hour per sq.cm. penetrating man at 70° C (Sect.IVc.), one obtains 30 gamma in 10 min.

mustard present at the application site 2 min. to 3.5 min. later was trivial. It was of the same order as the amount fixed. Thus, if even 12% of this extractable mustard were to become fixed it would not increase appreciably the amount fixed.

(4) The rate of disappearance of mustard from the application site was rapid. This process had a half life certainly no longer than 3 minutes. (See Section IXa-2)

(5) To be consistent with the above data, all therapeutic agents for humans based on reaction with penetrated free mustard must necessarily be valueless, for the reason that there was no significant amount of locally free mustard within the skin at any time.

(6) Since there was no significant difference between the findings with dosages of 1.1 and 10 mg. per sq.cm., all of the above conclusions hold in spite of massive contamination. Thus even massive contamination in man did not provide a source for progressive post-exposure injury.

(7) With sufficient mustard to saturate the skin (about 200 gamma per sq.cm.) only so much mustard can penetrate a given area in a given time interval. Since 1.1 mg.per sq.cm. was more than sufficient to insure a maximum penetration rate for 10 minutes, the massive dosage of 10 mg. per sq.cm. did not increase the penetration rate.

Vd. The Results of Post-exposure "Ice-Pack" Treatment of Skin (Pig and Man) in Relation to the Presence or Absence of a Local Reservoir of Unfixed Mustard

The experiments in which immediately following a 10 minute mustard application and subsequent decontamination, an ice pack was applied continuously for three to six hours, were known as the "Ice-Pack" experiments and are described in detail in Section VIIa-2.

The gross and microscopic appearance of these skin lesions after one to three day post-application periods corroborated the data regarding the respective reservoirs of unreacted mustard in pig and man at the exposed site immediately following an application. These observations (See Table AAI. Section VII) were, namely:

(1) With pig the immediate application of an ice pack to an exposed site completely or partially inhibited the appearance of injury as compared to a non-iced lesion.

(2) With man the immediate application of ice to an exposed site had no effect on the intensity of the subsequent injury.

TABLE XIV

Rate of fixation and transport from application site in human skin
All subjects exposed to either 1 or 10 mg. per sq.cm. for ten min.
(All calculations based on gammas of H-equiv.per sq.cm. of skin surface.)

Subject	H conc.	Post-appl. period	Fixed/cm^{-2} in gammas	Extract/cm^{-2} in gammas	Ext./Fixed
H-396-13	1.1 mg/cm^2	24 hrs	1.0	nil	0.00
-29	"	2 min	1.1	1.0	0.93
H-415-1	"	10 min	0.70	0.35	0.50
-2	"	2.5 min	0.60	0.81(a)	1.35
H-416-3	"	9 min	1.20	0.49(a)	0.40
-4	"	2.5 min	1.10	0.95	0.87
H-411-3	"	24 hrs	0.60	nil	0.00
-49	"	3 min	0.56	0.60	1.00
H-390-69	"	3.5 min	0.71	1.15	1.62
-62	"	2.5 min	0.90	0.87	0.97
H-410-1	"	24 hrs	0.86	nil	0.00
-21	"	10 min	0.86	0.26	0.32
H-454-1	10 mg/cm^2	24 hrs	2.8	nil	
-2	"	3 min	2.3	4.3(b)	1.5
H-462-1	"	24 hrs	0.75	nil	
-2	"	2.5 min	0.77	1.9(b)	2.5
H-463-3	"	24 hrs	1.75	nil	
-4	"	2.5 min	1.70	3.1	1.8

(a) We had available a small amount of mustard about 5 times as radioactive as that usually used for short time experiments. In order to determine the portion of the extractable activity which is separable into pentane and water-soluble fractions this extremely potent mustard was used in the above two cases. The results are as follows:

	% of Extractable in Pentane	Water
H-415-2	87	13
H-416-4	84	16

Presumably at least 95% of the pentane-soluble activity is mustard; thus about 80% of the chloroform extractables is mustard.

(b) Due to the large excess of Di on the skin surface there is an excellent chance of leaving 1 gamma (out of 10 mg) on the skin surface after thorough washing. This of course would make the amount extractable apparently greater than it actually was.

TABLE XV

Summary of Rate of Transport and Fixation of Mustard at Site of Application for Man, Pig and Rabbit. All 10 min. exposures at conc. of 1.1 mg per sq.cm.

Subject	Post-applic. period.	Ratio of Ext./Fixed	Ratio of Fixed after stated post-applic.time to Fixed after 24 hr. post-period.
Man	2 min.	0.5	1.1
	2.5 "	1.1	1.0
	3 "	1.0	1.0
	3.5 "	1.0	1.0
	10 "	0.4	1.0
	24 Hrs.	0.0	1.0
Pig	0 min.	9.3	0.4
	10 "	1.8	0.9
	20 "	1.1	1.1
	60 "	0.6	1.0
	120 "	0.3	1.0
	24 Hrs.	0.0	1.0
Rabbit	0 min.	8.8	0.5
	10 "	0.4	0.9
	20 "	0.1	1.0
	40 "	0.1	1.0
	24 Hrs.	0.05	1.0

(3) Since with man mustard either reacted or was transported away from the portal of entry almost as soon as it penetrated (no reservoir), while with pig, there was a reservoir of mustard at the termination of an exposure, the differences in the affects of ice-pack treatment in pig and man were not unexpected. Thus it seems likely that ice reduced injury in the pig by cooling the exposed site to such an extent that the reservoir mustard could not react and was hence slowly carried away by the body fluids. Since in man there is never an appreciable mustard reservoir in the skin, ice-pack treatment could not be expected to reduce injury by its effect on unreacted mustard.

(4) In view of the experimental findings with respect to the quantity of "extractables" in human skin in Section V-c and V-d and the confirmatory data presented in this section, there can be little doubt of the absence of a mustard reservoir in man.

V-e. Comparison of the Short Time Exposure and Post-Application Data in Man, Pig and Rabbit.

The comparative data are summarized in Table XV.

Discussion: (1) Immediately at the termination of a mustard exposure, pig and rabbit had an appreciable reservoir of penetrated mustard; man, however, did not.

(2) With pig and rabbit the time to reduce this mustard reservoir to an inconsequential amount was about 10 min.

(3) The time for fixation of penetrated mustard in man, like the time of removal, was less than 3 minutes. Fixation of penetrated mustard in pig and rabbit was not complete until about 10 minutes following the termination of an exposure.

(4) In view of the above conclusions, it is suggested that if therapeutic agents are to be tested on animals for ultimate use in man a period of at least 10 minutes should be allowed to elapse between decontamination and application of the therapeutic test agents. Only under such conditions will results obtained from animal experiments be truly applicable to man.

CONFIDENTIAL
PART VI

by

Herbert S. Breyfogle -- Alan R. Moritz

THE PATHOLOGY OF CUTANEOUS INJURY FOLLOWING EXPOSURE TO MUSTARD

VIa Reaction of human skin following application of liquid mustard.

1. Kinds and numbers of experiments: See III (a-3,b,c,d-1 and 2, e-1 or e-2, f-2, f-3, i)

Ninety-nine applications of liquid mustard were made to the skin of the abdomen of 54 human volunteers. The amount applied varied between 0.5 and 10 mg. per sq.cm. of skin surface. 1.1 mg. per sq.cm. was the most commonly employed dosage. The duration of exposure varied between 2 min. and 1 hour. The most commonly employed exposure period was 10 min.

Sixty-one of the application sites were excised after post-exposure periods ranging between 1 hour and 38 days and were studied chemically and microscopically. The other 38 lesions were observed for periods ranging from 3 minutes to 6 months and were either not excised or were excised and used entirely for chemical investigation. In each instance the biopsy included the entire exposed area and extended to the subcutaneous fat.

One series of specimens representing post-exposure periods ranging from 1 hour to 38 days were fixed in Zenker's fluid and sections were stained with hematoxylin and eosin, with phloxine methylene blue, by Feulgen's technic, and by a modification of Masson's light green trichrome technic. Sections of some specimens which represented late stages of repair were stained by Verhoeff's method for elastic tissue. All other specimens were fixed in 10% formalin and stained with hematoxylin and eosin.

2. The Prodromal Phase of Latent Injury:

Regardless of the ultimate severity that a mustard lesion may attain there is an initial post-exposure interval of several hours during which time there is neither clinical nor macroscopic evidence that injury has been sustained. Exactly what transpires during this period is not known. The fact that a cell-killing concentration of mustard in the tissues at the portal of entry is usually reached within 10 minutes after exposure together with the fact that once such a concentration has been reached there is no known means of preventing cell death indicate.

(a) That the actual interval between exposure and the initiation of irreversible changes, presumably of a biochemical nature, is short (probably less than 10 minutes).

(b) That the remainder of the initial non-reactive period after the exposed skin has been decontaminated should be regarded as an interval between injury and reaction rather than as an interval between exposure and injury.

3. The Early Reaction to Injury:

Normally the first perceptible reaction at the site of exposure is erythema. Erythema usually develops within 2 to 6 hours and once developed the further course of the lesion varies according to the duration and intensity of the exposure and the susceptibility of the exposed tissue to the injurious agent. The ultimate injury may be mild, moderate, or severe. The appearance of the lesion between 24 and 48 hours after exposure usually indicates the extent and nature of the damage that has been sustained.

4. Mild or Subvesicating Injuries:

If the exposure has been made at a relatively low environmental temperature and if the exposed skin (abdominal or inner surface of the forearm) has been decontaminated within 5 to 7 minutes after exposure it may be expected that in an average subject the lesion will reach its acme with little or no vesication. At the most only a few miliary blisters will develop. (Fig.1-Plate II). In resistant subjects, particularly those whose skin is heavily pigmented, longer exposure may be tolerated without vesicle formation.

Microscopic examination of mild injuries excised 3 to 6 hours after exposure show swelling of nuclei throughout the malpighian layer of the epidermis and hyperemia and edema of the tips of the dermal papillae. (Fig. 1 - Plate I). The central portions of the affected nuclei become homogeneously pale and the chromatin becomes scanty in amount and peripheral in position.

When stained by Feulgen's technic the affected nuclei show an apparent loss of thymonucleic acid. A comparison of microscopic changes in biopsies excised 6, 24, and 72 hours after mild exposures indicates that a certain degree of nuclear swelling can occur without necessarily indicating that irreversible damage has been sustained. If the injury is mild some of the cells having swollen nuclei recover and others die.

Even though the injury has been mild, groups of irreversibly injured cells in the deepest portion of the malpighian layer usually undergo liquefaction necrosis. Twelve to 18 hours after exposure the swollen nuclei of some of the cells become pyknotic and lysis and disintegration of their cytoplasm takes place. The liquefaction of groups of cells produces small defects in the continuity of the deeper portions of the epidermis. (Fig. 2-Plate I). The first sites at which such epidermal foci of liquefaction necrosis appear are immediately above the tips of the dermal papillae. If the injury is to remain mild these foci of necrosis do not enlarge to any significant degree and either remain invisible to the naked eye or at the most lead to the formation of miliary blisters.

If the injury has been mild it will have reached its peak so far as the epidermis is concerned within 24 to 48 hours after exposure. At its peak there will be (1) diffuse nuclear swelling throughout the malpighian layer with varying degrees of loss of chromatin, (2) pyknosis of nuclei within some cells that otherwise appear to be intact, (3) foci of liquefaction necrosis in the deepest layer of the epidermis immediately above the dermal papillae, and (4) hyperemia, edema, and perivascular mononuclear infiltration of the dermal papillae with extravasation of edema fluid into the interstial spaces.

The injury may be so mild that liquefaction necrosis fails to occur and whatever irreversible damage is sustained involves individual rather than groups of cells. On the other hand a so-called mild injury may be of sufficient intensity that the microscopic foci of liquefaction necrosis enlarge sufficiently to become macroscopically perceptible. They do not, however, involve a sufficient number of cells in continuity or attract a sufficient amount of edema fluid to result in through-and-through destruction of the epidermis.

- Characteristic of the mild lesion between 3 and 4 days after exposure at which time it has usually passed its fastigium is (a) augmented desquamation by which dead cells are disposed of, and (b) augmented mitotic activity in the basal layer by which the destroyed cells are replaced. (Fig. 5-Plate II). Desquamation and regeneration may be seen for as long as a week after exposure. In some individuals there is a marginal accretion of pigment around the lesion and a persistent state of irritability of the vessels of the dermis beneath the lesion.

5. Vesicating Injuries:

The usual reaction of the skin after exposure to mustard under non-experimental conditions is vesication. (Plate I). Exposure of abdominal skin to liquid mustard at an environmental temperature of 70 degrees or above for between 8 and 15 minutes usually leads to massive vesicle formation. During the early (6 to 12) hours of the reactive period the microscopic changes are similar to those seen in the case of mild injuries. If, however, the exposure is sufficient to produce a vesicating injury the microscopic foci of liquefaction necrosis present at the end of 12 hours continue to enlarge and coalesce with one another so that eventually all or most of the exposed epidermis becomes separated from and elevated above the dermis. As fluid collects within the epidermis and between the epidermis and dermis the central portion of the lesion becomes pale and erythema gives way to ischemia. The blister fluid is comprised to a small extent by the detritus of broken down cells and to a large extent by edema fluid. Although most of the disruption of epidermis from dermis occurs at the site of liquefaction necrosis there is evidence that the traction exerted on living and relatively undamaged cells at the margins of vesicles frequently enlarged the lesions beyond the limits of the original cytotoxic damage. There is more pronounced exudation of mononuclear and polymorphonuclear cells in the dermis than occurs in mild lesions

and subsequently there is desiccation and coagulation necrosis of all or most of the corium. Beginning ingrowth of epithelium from the normal epidermis at the margin can usually be recognized within 72 hours after exposure.

Despite this early evidence of regenerative activity the damaged corium is at first incapable of maintaining the newly regenerated epidermis. Not until about the second week is the marginal ingrowth of epithelium able to survive and not until between 4 and 5 weeks after exposure is the epidermal defect completely and permanently healed. The slowness with which permanent epithelialization is accomplished apparently depends on the functional state of the dermis. In the beginning new epithelium grows over a dermis that is dead or dying. Frequently a brightly acidophilic zone of necrotic collagen can be recognized between viable dermis and new epidermis. Later the tentative epidermis is supported by a dense cicatrix. Finally the last and successful crop of regenerated epithelial cells is supported by a vascularized layer of loose connective tissue which bears a close anatomic resemblance to the original corium. (Fig. 7 - Plate I)

6. Coagulation Necrosis:

The most severe type of mustard injury of the skin leads to the formation of a lesion which shows vesication only at its outermost margin. (Fig. 3-Plate II). When such injuries were observed they were usually after exposures of 15 minutes or longer. Throughout the centrum of the lesion the skin together with the underlying dermis is tanned or coagulated in such a manner that fluid never accumulated in sufficient amounts to separate the dead cells from the living. In the early stage of reaction such an injury is indistinguishable from the milder lesions. In some instances abortive foci of liquefaction necrosis appear and in other instances nuclear swelling and pyknosis occur throughout the entire thickness of the epidermis without immediate, morphological changes in cells or in intercellular relationships. Macroscopically the center of such a lesion becomes pale and takes on an opaque, yellow-gray parchment-like appearance.

The necrotic epidermis retains all or a large part of its connection with the dermis, (Fig.4-Plate II), and a week or more is required before a zone of demarcation between the dead and the living tissue is recognizable. The epidermis together with the adjacent zone of necrotic dermis remains in situ and desiccates to form a plate-like sequestrum which apparently interferes with healing so long as it remains interposed between the margins of the defect. Whereas the final epithelialization of a vesicating type of injury is usually accomplished within 4 or 5 weeks, this type of lesion may require several weeks longer to heal.

PLATE I: The Pathology of Cutaneous Injury in Man following Exposure to Liquid Mustard

The central panel shows 7 stages in the pathogenesis and repair of a vesicating injury in human skin. To either side of the central panel are photomicrographs showing the salient microscopic changes at each stage of the lesion. (Photographs X 230)

Photomicrograph #1: Hydropic degeneration of cells and pyknosis of nuclei in the deeper portion of the malpighian layer 6 hours after exposure to mustard. There is accompanying edema of the corium.

Photomicrograph #2: Foci of liquefaction necrosis in the deeper portion of the epidermis are coalescing at the end of 12 hours to form a miliary vesicle. The epidermis is being separated from the dermis by an accumulation of serous fluid.

Photomicrograph #3: The attentuated and necrotic epithelium forming the roof of a large vesicle 24 hours after exposure.

Photomicrograph #4: The denuded corium showing edema, perivascular lymphocytic infiltration, and pyknosis of connective tissue nuclei 24 hours after exposure.

Photomicrograph #5: Central portion of the base of a lesion 7 days after exposure. The vesicle has collapsed and the denuded corium is covered by a thin membrane of necrotic kerato-hyalin material. The corium appears acellular and non-viable despite the presence of dilated lymphatics. Occasional basal cells such as those shown in the upper right hand corner which were not dislodged incident to vesication will probably not survive or participate in the regenerative process.

Photomicrograph #6: During the first 3 weeks several crops of regenerated epithelial cells have grown in from the margins and perished, leaving a thick crust of dead epidermis over the base of the lesion. Coming in from the right hand side there may be seen a layer of hypertrophic epithelial cells between the crust and the dense collagen of the base of the lesion. Separating these cells from the collagen is a thin zone of brightly acidophilic hyalin material which probably means that they too will fail to survive.

Photomicrograph #7: 5 weeks have elapsed and the entire lesion which was less than 4 mm. wide in the beginning is now healed. The new epidermis is hypertrophic and basophilic, has prominent intercellular bridges, and is for the first time separated from the densely collagenous layer of the dermis by a zone of loose and richly vascularized connective tissue resembling the original corium.

PLATE I

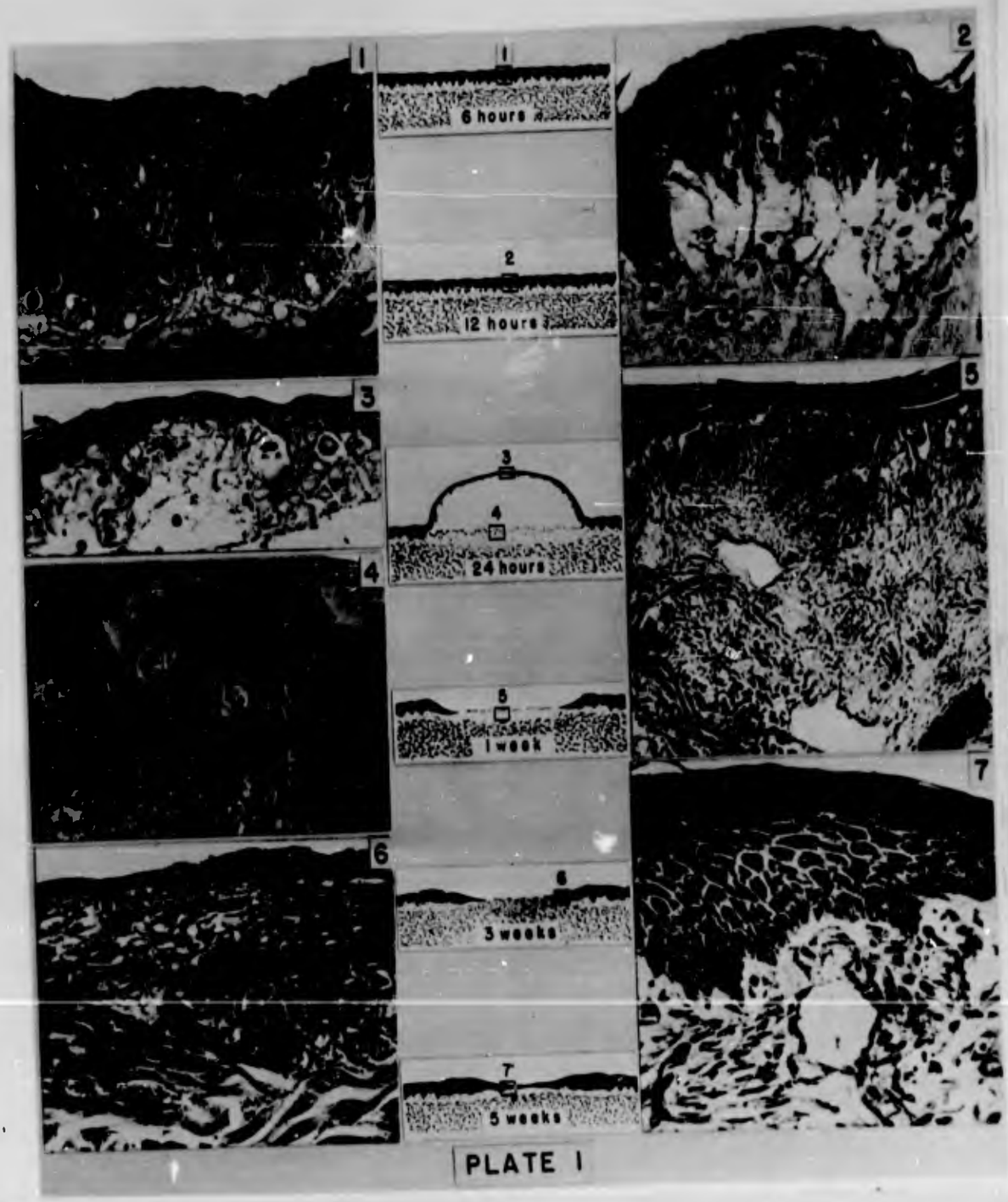


PLATE I

PLATE II: The Pathology of Cutaneous Injury in Man following Exposure to Liquid Mustard

Photographs 1, 2, and 3 show mild moderate, and severe cutaneous injuries 48 hours after exposure to mustard.

Photograph #1: Depicts a mild or subvesicating type of lesion.

Photograph #2: Shows a typical vesicating injury.

Photograph #3: Shows a so-called doughnut blister, the central portion of which is not vesicated and is the seat of coagulation necrosis.

Photomicrograph #4: Shows the appearance of the central portion of a severe injury 3 days after exposure. There is necrosis of the entire thickness of the epidermis but insufficient fluid has collected beneath the dead epidermis to result in the formation of a blister. The dermal capillaries appear empty. Their walls have a thick hyalin appearance and their endothelial nuclei are pyknotic. (X 400)

Photomicrograph #5: The appearance of a mild subvesicating injury 4 days after exposure. Dead cells are being discussed of by exfoliation and although irreversible injury to isolated cells is apparent throughout the epidermis it has nowhere been destroyed in its entire thickness and nowhere has sufficient fluid collected at sites of focal necrosis to result in macroscopic vesicle formation. (X 400)

PLATE 11

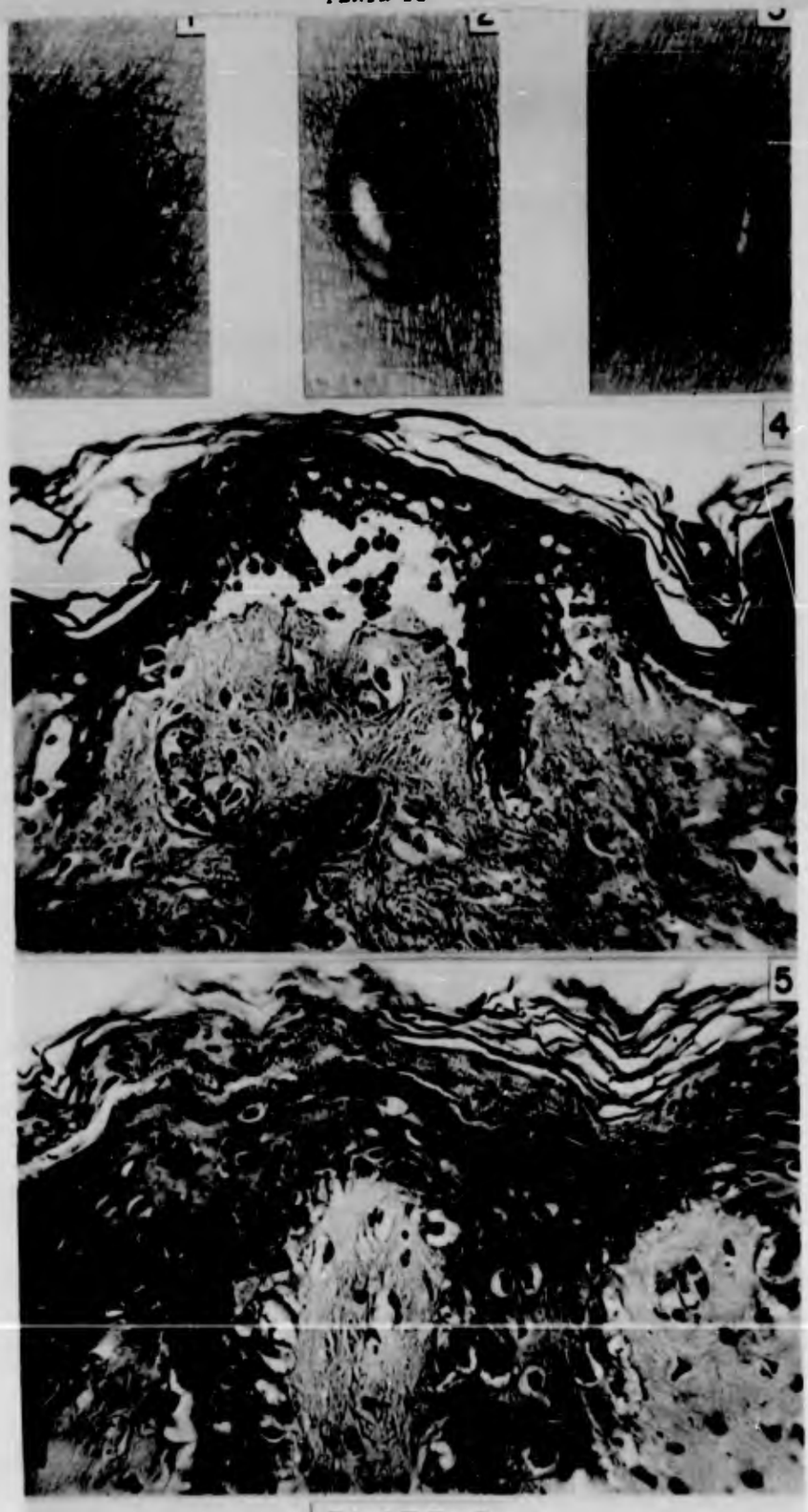


PLATE 2

PLATE III: Pathology of Cutaneous Injury in the Pig following Exposure to Mustard

The photograph depicts 4 stages of a moderately severe injury.

Photomicrograph #1: Shows the appearance of the epidermis and corium 24 hours after exposure. Focal areas of liquefaction necrosis may be recognized in the deeper portion of the intact epidermis and at the right hand side of the photograph the epidermis being separated from the corium by edema. Such separation never becomes sufficiently extensive to result in macroscopic vesicle formation. The dermis is diffusely infiltrated by exudative cells and extravasated erythrocytes. (X 200)

Photomicrograph #2: One week following exposure to mustard the dead epidermis lies on a lake of exudative cells and the underlying dermis appears to be undergoing progressive degeneration. (X 200)

Photomicrograph #3: Two weeks after exposure the epidermis and superficial portion of the corium have been sloughed off and the base of the lesion is occupied by a sequestrum of dead collagen which is undergoing liquefaction and organization. (X 200)

Photomicrograph #4: Four weeks after exposure the dermal defect has filled in by granulation tissue and new permanent epidermis has completed the repair. (X 200)

PLATE III

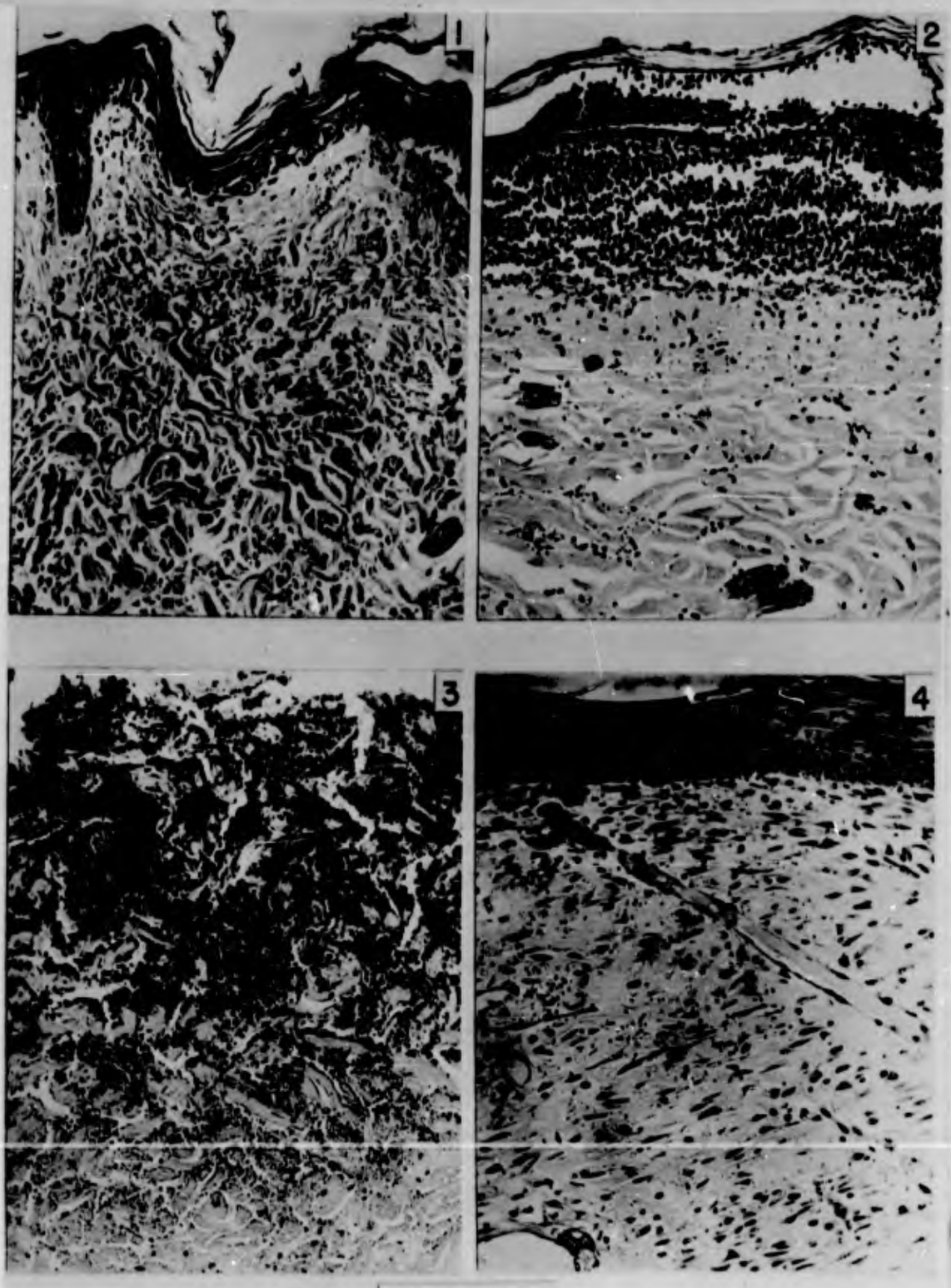


PLATE 3

VIb Comparison of the Pathologic Changes in Man with those Occurring in Animals (Pig and Rabbit)

Dermal injury in the form of hyperemia, hemorrhage, exudate, and necrosis is more pronounced in animals than in man. The more severe the lesion in man the more quickly the hyperemia of the central portion of the lesion gives way to ischemia, whereas in animals the reverse is true. Rarely does any significant amount of bleeding occur from the capillaries of human skin following exposure to mustard. In animals dermal hemorrhage occurs early and during the first three days provides the most reliable evidence as to the severity of the injury.

Exudation is ordinarily an inconspicuous feature of cutaneous mustard injuries in man unless infection supervenes. In animals the damaged epidermis is often extensively undermined by mononuclear and polymorphonuclear cells by the end of 72 hours. In man perivascular infiltration is ordinarily sparse and confined to the dermal papillae whereas in animals it is likely to form a prominent feature of the reaction and to include the entire thickness of the dermis.

Rarely does a human lesion (within the limits of our experiments) result in more than superficial necrosis of the dermis. Although the dermis beneath the exposure site may remain incapable of supporting a permanent new epithelium for several weeks after injury the recognizable zone of dermal necrosis rarely exceeds a few mm. in thickness. In animals and particularly in the rabbit necrosis of the dermis frequently progresses throughout the first week with the formation of a thick sequestrum of coagulated collagen beneath the base of the epidermal defect. Granulation tissue penetrates between the necrotic masses of collagen and eventually the entire thickness of the dermis is replaced by new connective tissue. In man only the superficial portion of the affected dermis is replaced by granulation tissue and the deeper portion appears to recover without being replaced by new tissue.

Macroscopic vesication does not occur in animals. The early degenerative changes in the epidermis in man and animal are similar. Focal liquefaction necrosis occurs in the deeper layers of epidermis in both. In man the accumulation of serous fluid in and the coalescence of these miliary foci of necrosis leads to macroscopic vesication. In animals serous fluid does not accumulate to any significant extent in the epidermal defects and the necrotic epidermis is not elevated to form vesicles. Whether the absence of vesication in animals is due to the elicitation of a different kind of exudative response or to a firmer and less destructible anchorage between epidermis and corium was not disclosed. Human lesions of the mild or subvesicating and the severe or coagulation necrosis types bore a close resemblance to corresponding animals lesions. Epithelium in the hair follicles is destroyed to about the same depth in man and animals but in animals

there is greater tendency on the part of surviving hair follicle epithelium to participate in the healing process.

Healing occurs more rapidly in animals than in man. In lesions involving comparable areas it was noted that deep defects in the rabbit were likely to be healed within 2 weeks whereas similar defects in the pig required between 3 and 4 weeks and in man between 5 and 7 weeks. One possible explanation for these differences in rapidity of healing is a quicker functional recovery on the part of the dermis in animals than occurs in the dermis of man. A relatively normal appearing denuded dermis in man fails to support new epithelium for a much longer period than is the case with animals.

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

In section IV it was shown that upon the application of mustard to the skin of an animal, a certain fraction (man 0.12, pig 0.25, rabbit 0.10) of the mustard penetrating reacted with the tissue at the application site and became "fixed"; the remainder, as indicated by mustard sulfur was carried away by the body fluids.

This fixed material was not extractable in appreciable quantities (5%) from the excised quartz ground tissue by cold water and isotomic salt solution, or by either hot or cold chloroform, acetone and alcohol. In fact the inability to extract this fixed-H by the above solvents may be considered the definition of "fixed".

This section will be divided into three parts with the view in mind of ellicudating the possible biological significance of the fixed material.

VII-a. The Correlation of Fixed-Mustard to the Severity of the Injury in Human Skin Tissue.

VII-a-1. Fixation Studies with Man.

By H.L.B., F.C.H., R.S.M., A.R.M., L.A.P.

Considerable evidence, primarily pig experimentation, has accrued during the past 18 months indicating a direct relationship between the amount fixed and severity of the skin lesion. This observation finally culminated in a series of human experiments purposely undertaken to bring out this relationship.

Purpose: To provide data regarding the relation of the injury produced in human skin by DH to the amount of fixed H recovered from the application site and to correlate these findings with temperature factors (environmental and body) and the selected exposure periods.

Procedure: See III (a-3,b,c,d-2,e-2,f-3,g,h-1,i-1,j-1). Bilateral abdominal exposures were made on each of 13 human volunteers (1.1 mg. mustard per sq.cm.) The exposed skin was excised 24 hours later. From eight of the subjects (16 application sites) the excised tissue from each side of the abdomen was transferred to 10% formalin and microscopic sections were prepared before chemical analysis was done. Experiments were conducted either in an environment of 60°F or 85°F. The exposures to mustard ranged from 3 min. to 25 min. The relative humidity in each environment was 50%.

Clinical observations were made of each application site at the end of 24 hours and prior to excision. During the course of these experiments it was discovered that excision permitted recognition of miliary vesicles that had not previously been visible to the naked eye.

Histological Observations: The exposures were at first limited to ten minutes or less and were subsequently prolonged to as long as twenty-five minutes. It soon became apparent that mild and moderate forms of skin injury with certain uniform features were being produced. As the exposure time was lengthened there appeared a third group corresponding to the lesion observed previously after one-hour exposures in the human penetration studies. (sec IV-c) The features of each group were as follows:

Group I
Mild Injury

This group was characterized macroscopically by erythema with or without edema and in some instances by miliary vesicles. Microscopically the principal changes were hyperemia and edema of the corium with insufficient epidermal injury to cause death of more than occasional isolated groups of basal cells. (Fig.1-Plate 2) (See Table XVI)

Group II
Moderate Injury

This group was characterized macroscopically by the formation of one or more readily recognizable, isolated or confluent vesicles containing clear fluid and having erythematous margins. (Fig. 2-Plate 2). Microscopically these vesicles separated the epidermis from the corium. The elevated epidermis was necrotic. (See Table XVI)

Group III
Severe Injury

This group was characterized macroscopically by an injury which was manifested by circumferential rather than central vesiculation, and by central necrosis. (Fig. 3-Plate 2). The centrum was flat, pale or yellow and in most instances free of visible vesiculation. Microscopically the marginal vesicles measured up to 1 mm. in width. The central epidermis was generally necrotic and although there were focal areas of liquifaction, the microscopic vesicles thus formed failed to coalesce and failed to collect enough fluid to become apparent to the naked eye. (See Table XVI)

TABLE XVI

The Type of Injury Produced by Mustard in Human Skin Application of 1.1mg.per sq.cm. of Mustard Tabulated in Progressive Order of Severity (Observations Made 24 Hours after Exposure)

GROUP I

MILD INJURY (Fig. 1 - Plate 2 Pg.52)

Exp.No.	Skin Temp. °F	Room Temp °F	Exp. Time (min.)	Amount Fixed H per sq.cm. (gammas)
H-383-7	94	85	3	0.25
H-385-13	94	85	5	0.21
H-382-10	95	85	5	0.49
H-381-13	87	60	8	0.63
H-380-1	97	60	5	0.77
H-385-1	95	85	5	0.52

GROUP II

MODERATE INJURY (Fig.2 - Plate 2, Pg.52)

H-385-11	95	85	7	1.28
H-382-1	95	85	7	1.01
H-384-10	95	85	10	1.54
H-388-13	95	85	10	1.1
H-384-7	95	85	7	1.21
H-380-10	87	60	15	2.30
H-375-13	88.3	60	10	1.05
H-375-14	88.3	60	12	1.20
H-377-2	86.7	60	12	1.10
H-377-5	86.7	60	15	1.78
H-379-3	87.0	60	15	1.63
H-378-6	87.5	60	15	1.80

GROUP III

SEVERE INJURY (Fig.3 - Plate 2, Pg.52)

H-387-10	97	85	20	5.56
H-387-6	97	85	15	2.62
H-388-12	95	85	25	3.60
H-381-7	97	60	20	2.45
H-378-3	87.5	60	18	3.60
H-379-4	87.0	60	20	4.16

On the basis of the macroscopic alterations the mild injury (Group I) represented a "sub-vesication" lesion; the moderate injury, (Group II) a "central vesication" lesion; while the severe injury, (Group III) was associated with central coagulation necrosis and "marginal vesication".

Microscopically, the difference between the most severe lesions in Group I and the least severe lesions of Group II was not striking. Exposure H-383-7 represented the mildest type of injury observed. H-385-11 included a lesion, the severity of which was midway between Group I and Group II. In the case of H-385-11 there was some question whether to include it with Group I or Group II. Group III comprised lesions that were strikingly more severe than those of Group I and II. The reason for non-vesication of the central position of the lesion was not apparent.

The gradation of individual lesions within Group II and Group III was based partly on changes in the corium, marginal epiderm and associated skin structures. The changes in the corium were restricted to exudation, which was not severe in the majority of the sections, hyperemia, and an altered staining reaction of the papillary layer. The reticular layer of the corium and the subcutaneous fat showed no conspicuous change. Only in Group III did the accessory skin structures bear any evidence of change and this was limited to early cell degeneration.

Results: The correlation between the amount of "fixed-mustard" and the associated pathological injury is given in Tables XVI. Table XVII summarizes the data.

The effect of temperature and exposure time on the character of the injury are given in Table XVIII.

Discussion: It was observed that with a given exposure to mustard, the lower the room and/or skin temperature during the exposure, the less severe would be the resulting injury. Exposures from 3 to 25 minutes produced three types of injury; mild, moderate, or severe. Generally speaking the longer the exposure, the more severe the injury. The irregularities within each group were probably due to individual differences in rate of skin penetration.

It was noted that both the environmental and/or skin temperature, individual variation in mustard penetration rate, and perhaps individual differences in fraction of penetrated mustard fixed, markedly effected the amount fixed per unit time of application. Yet, irrespective of this, it was possible to predict quantitatively, from the amount found fixed, the severity of the resulting injury, as shown in Table XVI. These data are summarized in Table XVII.

TABLE XVII

The Type of Injury Produced in Human Skin correlated with the amount of Fixed H per square centimeter of skin surface recovered from the tissue 24 hours after the application of 1.1 mg per sq.cm. of H in controlled environments

Room Temp	Skin Temp.	Amount of Fixed H per cm^2 (in gammas)		
60°F	81.3--88.3°F	0.12--0.77	1.05--2.30	2.45--4.16
No Exposures		4	7	3
85°F	94 --97°F	0.21--0.52	1.01--1.54	2.62--5.56
No Exposures		4	5	3
Character of Inj.		MILD GROUP I	MODERATE GROUP II	SEVERE GROUP III
Rounded off values for Amt. fixed per cm^2		0.1--1.0	1.0--2.5	2.5--

TABLE XVIII

The Type of Injury Produced in Human Skin after 24 hours by various Exposure Periods to 1.1 mg. per sq.cm. of H in controlled environments

Room Temp	Skin Temp.	Exposure Time in Minutes		
60°F	81.3--88.3°F	5--10	10--15	18--20
No exposures		4	7	3
85°F	94--97°F	3--5	7--10	15--25
No exposures		4	5	3
Character of Inj.		MILD GROUP I	MODERATE GROUP II	SEVERE GROUP III

There were three recognizable types of mustard injury in man and each was associated with a definite range of fixed mustard. These respective injuries can be readily differentiated macroscopically.

Group I. Mild injury - sub-vesication. (0.2 to 1.0 gamma of fixed-H per sq.cm. of skin surface). Erythema with or without edema.
Group II. Moderate injury - central-vesication. (1.0 gamma to 2.5 gamma of fixed-H per sq.cm. One or more isolated and confluent vesicles).
Group III. Severe injury - central coagulation necrosis and marginal-vesication (more than 2.5 gamma of fixed-H per sq.cm.) Pale yellow, flat or slightly depressed centrum; small vesicles at margin.

VIIa-2 A Comparison of "short Time" and "Ice-Pack" Fixation Studies in Man and Pig.

By H.L.B., F.C.H., C.M., A.R.M., L.A.P. and W.G.S.

Purpose: To provide further data bearing on the correlation between fixation of mustard and injury by means of short-time exposures and post-application experiments in conjunction with the ice-pack treatment.

Procedure: (1) Short time exposures of man and pig. These experiments were considered in detail in sections Va and Vc.

(2) Ice pack treatment of exposed human skin. See III (a-3,b,c,d-2,e-2,f-3,f-4,g,h-1,i-1, j-1). Bilateral abdominal exposures to 1.1 mg. per sq.cm. of mustard for 10 min. were made on eight human subjects. One site of each pair received a 3 hour "ice-pack" treatment (See IIIf-4). The post-application period for six pairs of skin sites was 24 hours. The other two pairs were excised 3 days after the mustard exposure. The room temperature was either 70 or 85 C.

(3) Ice pack experiments with pig. See III (a-1,d-3, e-2,f-4,g,h-2,j-4) Skin sites of two pigs (A210 and A211) were exposed to 0.5 mg. per sq.cm. of mustard for 10 minutes. Three sites of each pig received a 6 hour "ice-pack" treatment (IIIf-4). The post-application period for all of the sites was 26 hours.

Results: The chemical and pathological ice-pack results are given in Table XIA. The short time data are obtained from Table XV, Section V-e.

The grading of the human injuries was according to the method given in detail in VIIa-1. In no case was there any significant difference either macroscopically and microscopically in the degree of injury in the iced and non-iced skin sites of human subjects.

In the case of pigs, the sites receiving the ice-pack treatment showed consistently less injury than the non-iced sites.

TABLE XIX

Ice Pack and Short Time Data for Man and Pig. All Exposures for 10 min. Duration to 1.1 mg. per sq.cm. of Mustard for Man and 0.5 mg. per sq.cm. for Pig. (Amounts Fixed Expressed in Gammas of H-equiv.)

Rate of Transport & Fixation of Mustard at Portal of Entry for Man and Pig.

Subj.	Post-appl. Pd.	Ratio of Ext/ Fixed	Ratio of Fixed after Stated Post appl.time to Fixed after 24 hr.post period.
Man	2 min.	0.9	1.1
	2.5 "	1.1	1.0
	3 "	1.0	1.0
	3.5 "	1.0	1.0
	10 "	0.4	1.0
	24 "	0.0	1.0
Pig	0 "	9.3	0.4
	10 "	1.8	0.9
	20 "	1.1	1.1
	60 "	0.6	1.0
	120 "	0.3	1.0
	24 hrs.	0.0	1.0

Ice Pack Experiments

Subj.	Room Temp.	Duration Ice Pack	Post appl. Pd.	Gammas fixed per sq.cm.	Injury Type Group
Man					
H-419	70°	3 hr.	24 hr.	0.65	I
		0 "		0.64	I
H-422	85°	3 "	24 "	1.15	II
		nil		1.10	II
H-421	85°	3 "	24 "	1.9	II
		nil		2.0	II
H-428	85°	3 "	24 "	2.3	II
		nil		2.5	II
H-429	85°	3 "	24 "	5.2	III
		nil		5.5	III
H-427	78°	3 "	96 "	0.70	II
		nil		0.67	II
H-438	78°	3 "	96 "	0.37	I
		nil		0.40	I
Pig					
310		6	26 "	0.27	0
		6	26 "	0.22	0
		6	26 "	0.39	±
		0	26 "	0.79	++
		0	26 "	0.82	++
		0	26 "	0.76	++
		0	26 "	1.00	++
311		6	26 "	0.25	0
		6	26 "	0.34	±
		6	26 "	0.24	0
		0	26 "	0.58	+
		0	26 "	0.77	++
		0	26 "	0.62	+

Discussion: See Vd

(1) Irrespective of the "ice-pack" treatment, the degree of injury paralleled the amount of fixed-H in both man and pig.

(2) In pigs at least 0.3 to 0.4 gamma of fixed mustard per sq.cm. of skin surface were required to produce irreversible injury. With man this value was about 0.2 gamma per sq.cm. (See VIa-1). These values were known as the "Threshold" value for fixed mustard.

(3) The immediate application of ice prevented any further fixation (probably due to a high activation energy and thus a high temperature coefficient for fixation; hence the slowing down of the fixation rate enabled the body fluids to slowly carry the cutaneous mustard reservoir away from the tissue site.

(4) Since the half life for fixation of mustard in pig skin was about 5 minutes, the sites where ice-pack treatment prevented further fixation of H showed no appreciable injury, while those receiving no ice-pack showed readily recognizable injury. With the human subjects, the short time experiments of Table XIX indicated that fixation of mustard happened essentially immediately after penetration. Thus ice-treatment had no effect on reducing either the amount fixed or degree of injury.(a)

(a) Collins and others have found in experiments of a practical rather than mechanistic nature that in subjects treated with ice for periods of 1 to 12 hours the injury by mustard was considerably lessened. In the experiments conducted by them 2 skin sites were exposed to 10 mg. of mustard for 1 minute. Both sites were then blotted with filter paper. One site was treated with ice for 15 minutes to 12 hours and the other was left undisturbed.

Their technique provided a local reservoir of mustard on the surface of both the ice-treated and non-ice-treated skin. In analyzing their results it appeared probable that at the end of a 1 minute exposure to 10 mg. of mustard, an amount A penetrated the skin, and an amount B remained on the surface. Thus, at the non-ice-treated site, amounts A plus B ultimately penetrated. Presumably, about 12% of this total amount was fixed.

At the ice-treated site it may be assumed that an amount A penetrated the skin from the 10 mg. of mustard applied for 1 minute. An amount B remained on the skin, but because of the application of ice and the correspondingly low temperature of the skin, it was likely that little or none of B as it penetrated became fixed, but is transported from the site. Thus, in the ice-treated skin, the amount of fixed H was 12% of the amount A instead of 12% of A plus B and therefore the reaction or injury was less or was entirely prevented by the ice-pack treatment.

(5) Thus it appears that fixation of mustard and injury are manifestations of the same reaction, that one does not occur without the other and that there is a quantitative relationship between the two so far as the skin is concerned.

VIIa-3 Fixation Studies by Means of the Repeated Applications of Mustard to the Same Site of Pig and Man.

By H.L.B., F.C.H., C.M., A.R.M., L.A.P.

Purpose: To determine the relationship between fixed-mustard and severity of injury in the repeated application experiments.

(1) Pig

Procedure: See III (a-1,d-3,e-2,f-3,g,h-2,j-4) At an environmental temperature of 65°F, a series of 5 min. applications of mustard were made; the concentration of the mustard was 0.5 mg. per sq.cm. The following day, certain of these sites were again exposed under identical experimental conditions. This process was repeated at 24 hour intervals for a period of four days. Thus at the termination of the experiment, various sites had received from 1 to 4 applications. The tissue was excised one day after the termination of the final application.

On the same pig another series of experiments were run which were identical in every respect to that described above, except that after the termination of the 5 min. application an ice-pack was applied for six hours.

The purpose of the environmental temperature of 65°F and the 5 min. application time was to reduce the amount fixed by any single application to just threshold value and still get fair reproducibility. The purpose of ice-packs was to further reduce the amount fixed by any single application.

A sufficient number of single exposures were made to control the above series of experiments.

The severity of the resulting lesions was judged macroscopically.

(11) Man (H-523)

Procedure: See III (a-3,d-2,e-2,f-3,g,h-1,j-1) At an environmental temperature of 70°F a series of three exposures to 1.1 mg/sq.cm. of mustard were made to two abdominal sites.

TABLE XX

Repeated applications on successive days of 0.5 mg.per sq.cm. of H to Pig 735 to the same site at 65°F. Correlation of severity of injury with amount fixed. Tissue excised 24 hrs. following termination of final application.

Appl time	No.successive appl.at site.24-hr.intervals	Duration ice-pack in hrs.	Fixed H/cm² in gammas	Severity* of injury	Correlation of Injury with Fixed	
					Deg.of Injury	Fixed
5 min	1	6	0.2[a]	0	0	0.2
5 "	2	6	0.4[b]	1	0	0.3
5 "	3	6	0.3[b]	0	1	0.7
5 "	4	6	0.5	1	1	0.4
5 "	1	0	0.3[a]	1	1	0.35
5 "	2	0	0.4[b]	2	1	0.5
5 "	3	0	0.6[b]	2	2	0.4
5 "	4	0	0.75[b]	3	2	0.65
10 "	1	6	0.35	1	2	0.6
10 "	1	0	0.65	2	3	0.75
5 " at 80°F	1	0	1.00	3	3	1.0

a) Average of six experiments
b) Average of two experiments

Numbers indicate order of increasing severity of lesions.
Macroscopic observations only.

0 - No reaction
1 - Minimal reaction
2 - Intermediate reaction
3 - Most reaction

Site #1 received a 1.5 min. exposure the first day, a 3 min. exposure the tenth day and a 3.5 min. exposure the twelfth day.

Site #2 received a 1.5 min. exposure on the first day, a 1 min. exposure on the third day and a 5 min. exposure on the twelfth day.

Thus both sites had the total of an eight min. exposure and were excised on the fourteenth day.

Results: Site #1 contained 0.85 gamma of fixed mustard per sq.cm. and showed a high type I (sub-vesication) injury. Site #2 showed a mild type II (vesicating injury) and contained 0.55 gamma per sq.cm. of fixed-H.

Discussion: (1) If skin was exposed to repeated applications to subinjurious dosages of mustard, there was local accretion of fixed-mustard at the application site and injury occured pari passu with the attainment of fixed-H levels comparable to those which resulted from single larger injury-producing doses.

(2) The above result would be expected if the death of cells is dependent upon the same reaction that results in the fixation of mustard.

VIIa-4 Rate of Disappearance of Fixed Mustard in Human Skin Tissue.

By H.L.B., F.C.H., C.M., R.S.I., A.R.M., L.A.P.

Purpose: To study the rate of removal of fixed mustard from the application site.

Procedure: See III(a-3,

Eight volunteers received two 10 min. exposures to 1.1 mg. per sq.cm. of mustard. The experiment was so conducted that one site had a short post-application period (1 hour to 3 days), while the other site had a relatively long exposure period. Thus the ratio of the amounts fixed in each site gives the fraction of fixed material which has been transported from the site during the long post-application period. The similar appearance in each individual case of the two lesions during comparable post-application periods served as a further check that the amounts originally fixed were about the same.

Results: The data are given in Table XXI

TABLE XXI

Rate of Disappearance of Fixed-H from Injured Site. 10 min. applications of 1.1 mg. per sq.cm. of Mustard.

Exp.No.	Post-application period	% of originally fixed-H still at the site.
H-472	5d	100
H-474	7d	100
H-473	14d	50
H-465	21d	45
H-447	38d	25

Discussion: (1) These results were certainly subject to large experimental error; but they did indicate that fixed-mustard remained at the tissue site for a considerable time. In fact the amount of fixed mustard in human skin following an application was constant for the first week.

(2) The rate of disappearance of fixed mustard tended to parallel both the rate of healing as evidenced by microscopic sections and the rate at which the dead epidermis was desquamated. Thus it appears that the body could not readily metabolize fixed-mustard.

(3) Although the skin site of H-447 (38 day post-application period) appeared grossly to be completely healed, there was some injury still present in the corium. Since the epidermis at the site was entirely new, the 25% activity was probably in the corium.

VIIb Certain Properties of Fixed Mustard.

VIIb-1. A Simple Protein Fractionation of Pig Skin Tissue Containing Fixed Mustard.

By F.C.H., C.B.A., W.G.S.

In collaboration with Dr. R. A. Ormsbee and Dr. E. G. Bell the fixed mustard present in pig skin 24 hours after application was subjected to a simple protein fractionation scheme. The experimental method and data pertaining to this experiment has been described in detail in a Formal Progress Report.(9)

Results: The results may be summarized as follows:

a)Practically none of the fixed mustard was combined with skin constituents soluble in either 0.9% NaCl or acetone or an ether-alcohol mixture.

b) Linkages split at 6°F at mild alkalinity (pH 9) to yield a dialyzable radioactive material (primarily thiodiglycol) amounts to about 40% of the total initially fixed.

c) Linkages in addition to (a) broken by autoclaving to yield also dialyzable radioactive substances amounting to about 20% of the initially fixed mustard.

d) The remaining 40% of the radioactivity was attached to soluble compounds after autoclaving; however, these constituents, presumedly proteins, were not dialyzable.

Discussion: (1) These results certainly indicated that the mustard became fixed by reacting with numerous groups of numerous skin constituents.

(2) A considerable percentage (40%) of the fixed mustard was alkali (pH 9) labile going to thiodiglycol.

(3) Unfortunately, at the time of these investigations, mustard containing radioactive sulfur of high specific activity was not available. Thus, in order to obtain sufficient radioactivity for analysis of the various fractions, it was necessary to fix about 100 gamma of H per sq.cm. of skin surface. Since the amount of fixed mustard required to just produce irreversible injury was 0.2 to 4 sq.cm., the above data may not be a true representation of the properties of fixed mustard in lesions of usual severity.

VIIb-2 The Stability of Fixed Mustard in Pig Skin Tissue with Respect to Alkaline pH.

By F.C.H., and L.A.P.

Purpose: To study the effect of alkaline pH on the solubility and/or hydrolysis of fixed-mustard.

Procedure: Sec III (a-1,d-3,e-2,f-3,g,h-2,m-1) Both sides of a pig received 15 applications of 0.5 mg. per sq.cm. of mustard for a period of 15 minutes. At the time of excision, one side had had a post application period of 24 hrs. and the other side one of one-hour duration.
The skin from each side of the pig was removed and subjected to the procedure given in detail in Section III m-1. Radiological analyses were made according to Section II-c.

Results: The experimental data are given in Table XVII

Discussion: (1) Unlike the data presented in VIIb-1, these results pertained to tissue where the amount of fixed-mustard was about 2 gamma per sq.cm. of skin surface or 0.5 gamma per 10 mg. of dry wt. epidermis and corium. This amount of fixation was associated with a moderately severe injury.

(2) There were probably no differences in the chemical properties of fixed-mustard after a one-hour or 24-hour post-application period.

(3) The percent of fixed-mustard soluble or decomposed in 45 hours at pH 7 was about 10%. This percentage increased linearly with increasing pH and is 100% in 45 hours at pH 13.

(4) In the pH range of 7 to 13, about 25% of the alkaline soluble fixed-mustard was precipitated by 80% alcohol. These fixed-mustard molecules were undoubtedly protein in nature, since it was inconceivable that any substance originally soluble in 80% alcohol could be rendered insoluble by reacting with mustard in the minute concentrations of this experiment.

(5) In the pH range 7 to 13, about 75% of the alkaline soluble mustard sulfur was soluble in 80% alcohol. About 50-60% of this mustard sulfur was in the form of thiodiglycol.

(6) The rate of solution of fixed-mustard became nearly negligible at pH 11 after 16 hours; at pH 9 the rate of solution was much slower and was apparently not negligible even after 136 hours. The differences in the amount of hydrolysis in a given time between pH 9 and 11 may be merely a matter of reaction rate.

(7) In sufficient time 70% of the fixed material can be removed in the pH range of 9 to 11. Apparently it requires a higher pH to remove the remaining 30%.

(8) Several types of linkages between mustard and the tissue molecules were undoubtedly present in fixed-mustard. 40% of these linkages were very labile at pH 13, being split to form thiodiglycol. An ester linkage with phosphate or carboxyl group was indicated and possibly the sulfonium salt linkage. 60% of these linkages were not capable of being split from soluble protein or alcohol molecules to form thiodiglycol at a pH of 13. Among the stable linkages of this type were probably: C-N (by alkylation of amines); or-O-C (by formation of ether from phenolic OH; R_4N^+ (by formation of quaternary nitrogen); and C-S-C (by formation of a thi-ether from a sulfhydryl group).

(9) The quantitative determination of the molecules or groups present in the tissue which react with mustard could best be determined by adding non-radioactive mustard derivatives of the different tissue constituents (e.g., amino acids) to a hydrolysate of the mustard treated skin tissue; this hydrolysate should be obtained so as not to rupture the linkages between the mustard and the reacted molecules. The derivatives would be isolated, purified and analyzed for radioactivity.

TABLE XXII

Temperature 23°C
Time 45 hours

THE EFFECT OF pH ON SOLUBILITY AND/OR HYDROLYSIS OF FIXED-MUSTARD DERIVED FROM PIG SKIN

pH	% mustard sulfur in Hydrolysate		% of mustard sulfur in Hydrolysate soluble in 80% alcohol		% of alcohol soluble Hydrolysate as thiodiglycol
	1 Hr. Post-application Tissue	24-Hr. Post application Tissue	1 Hr. Post application Tissue	24-Hr. Post application Tissue	
7	12	8	58	87	-
9.0	42	42	74	79	47
10.0	52	65	73	71	-
11.18	68	69	72	78	50
13.	95	97	67	65	67

Temperature 23°C

RATE OF SOLUTION AND/OR HYDROLYSIS OF FIXED-MUSTARD

Time Hrs.	Percent mustard sulfur in Hydrolysate pH 9.1	Percent mustard-sulfur in Hydrolysate pH 11.18
16	36	62
23	42	65
40	46	67
64	52	69
88	55	71
136	60	72

VIIb-3 The Fraction of Fixed-Mustard Present in Nuclei of Pig Skin Tissue.

By F.C.H., C.M., J.R.W.

In view of the observations of decreased mitotic rate in mustard treated tissue, of the delayed healing of mustard lesions, and of the microscopic observation of nuclear degeneration in skin tissue exposed to mustard, a determination of the fraction of fixed-mustard in cell nuclei was of interest.

Purpose: To determine the percentage of fixed-mustard present in nuclei prepared from the malpighian layer of pig skin.

Procedure: See III (a-1,d-3,e-2,f-3,m-2) Two pigs (A502 and A545) were used.

(a) Pig A502: Nine application cups each containing 250 gamma per sq.cm. of mustard were applied to the side of a pig (A) for 10 minutes. The cups were then moved to new sites and left for 12 minutes after which they were applied to other sites for 15 minutes. Thirty minutes after the removal of the last cup the pig was sacrificed and the skin of the entire side of the pig was removed and frozen. The skin was removed and separated into cornified layer, malpighian tissue, brei and corium, by the procedure given in IIIm-2. The amounts of fixed-mustard present in the corium and cornified layers were determined (Section IIc). Sixty-two percent of the malpighian layer (citric acid scrapings) were used for preparation of nuclei, 16.2% for determination of radioactivity (section IIc), and 21.8% for determination of thymonucleic acid content. 10% of the isolated nuclei were used for the determination of thymonucleic acid and 90% for the determination of fixed-mustard.

(b) Pig A545: Four application cups containing 250 gamma per sq.cm. of mustard were applied to the flank for 10 minutes. The cups were then transferred successively to new sites for 15 minutes and for twenty minutes. The opposite flank of the pig was treated in a similar manner. Two hours later, the pig was sacrificed, the skin excised and separated into cornified layer, malpighian tissue, brei, and corium.(See IIIm-2). The malpighian layer brei was divided into five aliquots of 3.2 and 3.3% for fixed-mustard determination, 35.7% for determination of thymonucleic acid, 1.5% for nitrogen determination, and 56.5% for preparation of nuclei. The purified nuclei were divided into three aliquots of 80% for fixed-mustard, 10% for thymonucleic acid, and 10% for nitrogen determination by Kjeldahl digestion and direct Nesslerization.

Results: The experimental findings are given in Tables XXIII and XXIV.

TABLE XXIII

The Fraction of Fixed Mustard in the Nuclei Obtained from the Malpighian Layer
(Fixed-H expressed in H-equiv)

	Gammas of Fixed-H per thymonucleic acid unit.		Micromoles of fixed-H per gram-atom of nitrogen in exposed tissue.
	Pig A502*	Pig A545	Pig A545
Original malpighian layer of epithelial tissue	11.8	3.2	67 micromoles
Nuclei from malpighian layer	1.45	0.42	37 "
Percent of fixed-H in nuclei	12%	13%	

TABLE XXIV

Comparison of Amount of Fixed-Mustard in the Separated Layers
(Fixed Mustard expressed in gammas of H-equiv.)

	Gammas of Fixed-H per sq.cm. of exposed skin		% of total fixed mustard	
	Pig A502*	Pig A545	Pig A502	Pig A545
Cornified Layer (1st. scrapings)	3.5	1.4	59%	51%
Malpighian Layer (5% citric acid tissue brei)	1.3	0.70	23%	25%
Corium (residue after scrapings)	1.1	0.65	19%	24%
Total Epidermis	4.8	2.1	82%	76%
Total Skin	5.9	2.7		

Discussion: (1) Table XXIV shows that 70% of the H fixed in the epithelium was present in the cornified layer. Since this layer was dead tissue, it was difficult to see how this particular H fixation could be responsible for any skin damage.

(2) The results given in Table XXIII show that an appreciable fraction (12.5%) of the fixed-H in the malpighian layer was present in the nuclei. On a nitrogen basis, there was about 80% less fixed mustard in the malpighian nuclei than in the entire malpighian layer. This difference is not believed to have any qualitative significance.

(3) In mild to moderately severe mustard lesions, practically all of the injury was confined to the malpighian layer. In dosages with pigs comparable to that necessary to produce blisters in human skin, the amount of fixed mustard is 0.25 gamma in the malpighian layer per sq.cm. of pig skin surface. This corresponds to about 25 micromoles of fixed mustard expressed in H-equivalents per gram atom of nitrogen. This also corresponds to 10^{-9} micromoles of mustard per malpighian cell.(b)

VII-c. The General Mechanism of Mustard Injury.

By F.C.H.

Let us for the moment examine in quite general terms all of the chemical reactions of penetrated mustard at the portal of entry which could lead possibly to injury. They are the following:

(b) The average thickness of the malpighian layer is about 200 microns. Assuming that the 90% of the malpighian layer is water and 15% of dry weight is nitrogen, one obtains 75 micrograms of fixed mustard per gram atom of nitrogen. In view of the approximations this value is an excellent check since the malpighian layer is probably somewhat more dessicated than other tissues. (70% water would give the value in the text).

By a rough count the number of cells per sq.cm. of pig malpighian layer is about 2.8×10^5.

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(1) H - X ⟶ HX (fixed) (a)

Where X denotes impermeable or insoluble constituent or constituents present in tissue which remains impermeable or insoluble when reacted with mustard.

(2) H - Y ⟶ HY (fixed) (a)

Where Y are permeable substances or substance (may include constituents of lymph and blood) present in the tissue which become insoluble or impermeable when reacted with H.

(3) H - Z ⟶ HZ (permeable) (a)

Where Z are constituents or constituent present in tissue, which are carried away by the body fluids.

One is interested in deciding which of the above three reactions are ultimately responsible for injury: whether or not the presence of HX or HY or the absence of Z are in themselves primarily responsible for injury, or initiate a series of reactions one or some of which may be primarily responsible for injury is not to be considered at present.

Let us first consider mechanisms (1) and (2): In Section VIIa-3, it was demonstrated that an accumulation of 25 micromoles of fixed-mustard as H-equiv. per gram-atom of nitrogen suffices to produce a blister in human skin.

It is unlikely that any molecule, large or small, reacting with mustard at these concentrations could be changed from a permeable state to such an impermeable state (b) that it would remain at the tissue site for at least 7 days (see VIIa-4). Further, no large impermeable or insoluble molecule could have its gross physical properties altered so as to be rendered permeable by mustard at these concentrations.

These considerations based on the minute concentration of mustard reacting at the skin site immediately rule out mechanism (2). Hence the fixed material must be produced by mechanism (1); namely the reaction of H on impermeable or insoluble molecules probably protein in nature. Incidentally a restriction was put on Z in mechanism (3); namely, Z must be permeable constituents.

(a) The reactions (1), (2), and (3) are merely the alkylation by mustard of the specific groups of tissue constituents X, Y, Z: viz.

$$H + COO^- = COOCH_2\text{-}CH_2\text{-}SCH_2\text{-}Cl + Cl^-$$

$$H + R\,NH = R_2N\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CH_2Cl + H^+ + Cl^-$$

The possibility of the mustard reacting with certain groups of tissue constituents due to the coordination properties of the mustard sulfur cannot be rigorously excluded; however all of the chemical evidence renders this possibility exceedingly implausible.

(b) 25 micromoles of fixed-mustard per gram atom of nitrogen is about equivalent to 75 micromoles of fixed mustard per liter of tissue brei. Even a substance as insoluble as mercuric sulfide would not be precipitated out at these concentrations. For the most part membrane permeability is primarily a function of molecular size, the addition of one mustard molecule to any constituent could not possibly change it from a permeable molecule to an impermeable molecule.

It is,however, somewhat more difficult to decide between mechanism (1) and mechanism (3) as the injury producing reaction. Due to the competition factor mechanism (see section IX) for the reaction of H in skin, the amount of H reacting by mechanism (3) would be proportional to the amount of fixed-mustard formed by mechanism (1); thus as experimentally shown in section VIIa, the fixed material would be an indicator of injury rather than the iniator of injury.

Let us reexamine some of the conclusions given in section VIIa, with the view in mind of deciding between these mechanisms.

VIIa-1 The Correlation of Fixed-Mustard to the Severity of Injury in Human Skin Tissue.

(4) It was possible to predict quantitatively from the amount fixed, the severity of the resulting injury as judged by histological study.

VIIa-2. A Comparison of "Short-Time" and (Ice-Pack" Fixation Studies in Man and Pig.

Since the half life for fixation of mustard in pig skin is about 5 minutes, the sites where ice-pack treatment prevented further fixation of H showed no appreciable injury, while those receiving no ice-pack showed readily recognizable injury. With the human subjects, the short time experiments of Table XXI indicate that fixation of mustard happens essentially immediately after penetration. Thus, ice-treatment had no effect on reducing either the amount fixed or degree of injury.

Thus, irrespective of the "ice-pack" treatment, degree of injury parallels the amount of fixed-H in both man and pig.

VIIa-3. Fixation Studies by Means of the Repeated Applications of Mustard to the Same Site of Man and Pig.

If skin is exposed to repeated subinjurious dosages of mustard, there is local accretion of fixed-mustard at the application site and injury occurs "pari passu" with the attainment of a fixed-H level comparable to that which resulted from a single application of an injury-producing dose.

VIIa-4. Rate of Disappearance of Fixed Mustard in Human Skin Tissue.

The rate of disappearance of fixed mustard tended to parallel both the rate of healing as evidenced by microscopic sections and the rate sloughing off of the dead epidermis. Thus apparently the body could not readily metabolize fixed-mustard.

Although the skin site of H-447 (38 day post-application period) appeared grossly to be completely healed, there was some injury still present in the corium. Since the epidermis at the site was entirely new, the 25% activity was probably in the corium.

The assumption that mechanism (1) is the initiating injury producing reaction explains all the above data. Let us consider the restrictions necessarily placed on mechanism (3).

(i) The amount of mustard reacting by mechanism (3) is proportional to the amount of fixed-mustard produced by mechanism (1).

(ii) The temperature coefficient for the reaction rate of mechanism (3) is essentially equal to that of mechanism (1).

(iii) The body is unable to replenish the exposed tissue site with permeable constituents Z in a 24 hour period even after the application of subinjurious dosages of penetrated mustard; or once Z is destroyed the reappearance of Z has no effect upon the subsequent history of the mustard lesion.

While these three restrictions are surmountable they make mechanism (3) implausible as the initiating step in producing injury.

Thus the experiments described in section VIIe indicate that mechanism (1) is responsible for injury; namely

(1) $H + X \longrightarrow HX$ (Fixed Mustard)

Where X denotes impermeable or insoluble constituents or constituent present in tissue which remain impermeable or insoluble when reacted with H.

These impermeable or insoluble constituents are probably protein in nature. The minute concentration of mustard (25 micromoles per gm.atom of N) required to react with these proteins to produce moderately severe injuries (blister dosages) is indicative of reactions with groups of highly functional proteins, namely enzymes.(b) Thus the formation of fixed-mustard is probably breaking the chain of some metabolic process.

(b) It is not possible to rigorously exclude the formation of H ion as the ultimate cause of injury. The most likely formation of H ion would be due to a reaction such as:

R_2NH + Mustard ---- > $R_2N\text{-}CH_2\text{-}CH_2\text{-}S\text{-}CH_2\text{-}CHCl + H^+ + Cl^-$

Again in view of the 25 micromoles of mustard per gm.atom of N sufficient to produce considerable injury, it is difficult to believe that H^+ would be liberated in sufficient quantity to alter locally the pH. Further the known vesicancy of divinyl sulfone renders the H ion hypothesis still more improbable.

This leaves two possibilities for the actual cause of cell death: (1) The initial chain breaking step due to the formation of fixed mustard or (2) The subsequent prevention of a reaction or a series of reactions further along the metabolic chain.

From the point of view of therapy, there are two important questions regarding fixed-mustard, namely:

(i) In order to alleviate the injury, how early after fixation takes place would it be necessary to alter or remove the mustard residue present in the fixed-mustard?

Thus determinations of the rates at which these secondary reactions take place would be of utmost importance. Unfortunately, the radioactive sulfur studies offer no solution to this problem. The relative slowness of the appearance of cell death does afford some indication that if the mustard grouping in fixed-H could be altered or removed in reasonable time so that the normal metabolic processes could continue the injury would be alleviated.(d)

(ii) Would the altering or removal of fixed-H after the appearance of injury shorten the time of healing? i.e., Fixed-H is intrinsically toxic. To date there is no real evidence regarding this point. Experiments which may yield some evidence regarding this point are now in progress.

It is unfortunate that it is necessary to leave the problems considered in this section in such an inconclusive state.

(d) Namely a substance (T) that would catalyze the decomposition of fixed mustard (HX) liberating X and thiodiglycol; or a reaction such as:

$$HX + T \longrightarrow HT + X$$

Professor P.D. Bartlett and others have suggested the oxidation under biological conditions of the mustard residue to either the sulfoxide which may render fixed-H innocuous, or to the sulphone for which it may be easier to find the substance T.

There is not enough information (see VII-b) available at present regarding the properties of either fixed-H or the linkages to which the mustard is attached. If there were it might be possible to make reasonable guesses regarding the necessary properties of T. Certainly substances which will penetrate cells and are mildly alkaline should be tried as therapeutic agents. (see VII-b-2).

PART VIII
MISCELLANEOUS ANIMAL EXPERIMENTS

VIIIa. Miscellaneous Control Experiments with Animals
by H.L.B. and A.R.M.

During the course of these studies, and particularly during the early phase of the work, various experiments were undertaken to determine the extent to which certain biological and other variants might alter the chemical and/or pathological findings.

One group of such experiments had to do with the effect of environmental temperature. These proved to be of such basic importance to an understanding of the mechanism of vesication that they have been described in detail in an earlier section of the report. Others provided useful information as to how experiments should or should not be performed, but aside from this made relatively little contribution to the problem as a whole. Of the many questions explored for control purposes, the following have been selected for brief mention.

VIIIa-1. Pathological Studies on Pigs of Regional Vulnerability and of the Reliability of Early Macroscopic Changes in Estimating the Severity of an Injury Produced by Mustard.

Procedure: A white pig (305) was used and four areas of its body were chosen for comparative studies of skin reaction following 10 min. exposures of 0.5 mg. per sq.cm. of mustard. Exposures were made in each region in such a manner that when the animal was killed, each region would contain two one-day post-application lesions, two 7-day post-application lesions, and two 14-day post-application lesions.

The four chosen regions were:

(1) Mid-Abdomen. Here the epidermis is usually least compact, the intercellular bridges are most prominent, and the intercellular interstices seen in microscopic sections of paraffin embedded material are widest. The corium is of loose texture and is richly vascularized. This region is relatively hairless.

(2) Inner Aspect of the Thigh. Here the epidermis is slightly more compact than in (1), the corium is denser and hairs are few in number.

(3) Lateral Aspect of Abdomen. Here the epidermis is most compact and the corium densest and least vascularized; hairs are most numerous.

Experimental Results: At the end of 24 hours the gross appearance of the lesion in the various above-described regions were of similar appearance. In all instances there was a sharply delineated, cup-size area of intense erythema with superficial hemorrhage in the corium. Edema was slight or absent. The color of the application sites varied from red to purple.

Although the external appearance of the lesions was similar at the end of 24 hours, there were striking microscopic differences. The mid-abdomen and the inner thigh regions showed necrosis of the entire thickness of the epidermis. Although the necrotic epidermis was intact, it was almost completely undermined by shallow confluent lakes of exudate which separated it from the edematous, hyperemic, and superficially hemorrhagic corium.

The lateral abdominal region showed less severe microscopic injury than was observed in the previously described sites and on the dorsum of the animal only focal damage to the epidermis was observed. Both regions showed hyperemia and superficial hemorrhage in the corium.

At the end of 7 days it was apparent in the mid-belly region that not only the entire thickness of the epidermis had been destroyed but also one half and two thirds of the corium. The exposed sites were deeply ulcerated and showed only a small amount of marginal healing. The lesions on the inner aspects of the thighs showed ulceration which was less severe and shallower than was observed in the mid-abdomen region. The lesions on the lateral abdominal region were almost completely healed but in places the epithelial regeneration had partially covered still-necrotic corium. The lesions on the dorsum of the animal showed no residual damage to the corium and almost complete regeneration of the epidermis.

At the end of 14 days the mid-belly lesions were still deeply ulcerated. The ulcers on the inner aspects of the thighs and on the lateral aspects of the abdomen were epithelialized but beneath the regenerated epithelium the superficial layer of the corium showed considerable residual injury, was relatively anuclear and avascular and had an unusually compact hyalin appearance. The lesions on the dorsum of the animal were completely healed and the underlying corium appeared normal.

Discussion: (1) This experiment corroborates previously held impressions (a) that there are striking differences in the degrees of susceptibility of different skin areas of the same animal (pig) to injury by mustard and (b) that the gross external appearance of a 24-hour post application lesion does not necessarily provide reliable evidence as to the extent to which the exposed tissues have been injured.

(2) Absence of reaction at the end of a 24-hour post-application period probably indicated that no significant injury has been sustained. However, what appears grossly to be a severe injury in the skin of a pig at the end of 24 hours may actually turn out to be relatively mild so far as the epidermis is concerned.

(3) No conclusion regarding the effect of a toxic, prophylactic, or therapeutic agent should be drawn from the appearance of skin lesions in the pig unless the lesions being compared are situated in anatomically comparable sites of the body.

VIIIa-2. The Relative Vulnerability of Pigmented and Non-Pigmented Pig Skin to Injury by Mustard.

Procedure: See III (a, d-3, e-2, f-3)

A black and white, piebald pig (306) was used. Pigmented and non-pigmented areas on the dorsum of the animal were exposed to 0.5 mg. per sq.cm. of mustard for 5, 10, 15 and 30 minutes. The exposure sites were examined grossly and microscopically at the end of 24 hours.

Results: The resulting injuries varied from mild to severe in relation to the duration of exposure but no differences were observed in the reactions in pigmented and non-pigmented areas.

Discussion: This experiment indicated that pigment, per se, did not alter the susceptibility of a pig's skin to injury by mustard.

VIIIa-3. Effect of Pre-Exposure Wetness and Dryness on the Susceptibility of a Pig's Skin to Injury by Mustard.

Procedure: and Results: See III (a, d-3, e-2, f-2)

Three areas on the dorsum of a white pig (305) were exposed to 0.5 mg. per sq.cm. of mustard for 10 minutes. 24 hours prior to exposure the skin was shaved. 30 minutes prior to exposure one site was scrubbed with soap and water and kept moist until the exposure was made. 30 minutes prior to exposure another site was scrubbed with ether and alcohol and kept in contact with alcohol until the exposure was made. The third exposure was made on untreated and unwashed skin. 24 hours after exposure the three sites were excised and examined microscopically. Moderately severe injury was observed at all three sites and there were no differences in the extent or character of the reactions.

Discussion: This experiment provided no evidence that preliminary maceration or drying of a pig's skin altered its susceptibility to injury by mustard.

VIIIb. Miscellaneous Animal Experiments Involving the Use of Radioactive Mustard.

VIIIb-1. The Distribution of Mustard Sulfur in the Various Organs of a Pig after a Gross Contamination by Mustard
by F.C.H., H.R.M. and W.G.S.

Procedure: See III (a, d-3, e-2, f-3, h-2)

About 15 mg. of mustard (activity = 1500 counts/gamma of H/min), penetrated the skin of pig A-290. After a 24 hour post-application period, the animal was sacrificed and aliquots of the blood and certain organs were obtained.

The blood was allowed to clot and the clot and the serum were separated. The serum was subjected to dialysis. The radioactivity of all fractions and the amount of thiodiglycol (see IIIj-3) in the serum permeate were determined.

Certain organs of the body were subjected to radiological analysis. Thus the bone marrow, spleen and thymus were chosen since they are susceptible systemically to the cutaneous absorption of mustard, while liver, kidney and skeletal muscle are considered to be representative of the organs where no pathological changes are observed.

These organs were subjected to a simple protein fractionation scheme, namely:

Tissue subjected to acetone extraction
I. Acetone soluble (mostly thiodiglycol)
Acetone residue extracted with isotonic salt solution.
IIa. .9% NaCl soluble, alcohol insoluble (proteins)
IIb. .9% NaCl soluble, alcohol soluble.
Isotonic salt residue extracted with .03M NaOH
IIIa. .03M NaOH soluble
IIIb. .03M NaOH residue
Mustard sulfur analysis were made on all of the above fractions.

Experimental Results: (all results in gammas of H-equiv.)
(1) Blood (estimated at 1 litre)
1.5 gamma per cc. of which 35% was in the formed elements and fibrinogen, 35% in the non-dialyzable (protein) portions of the serum, 25% in the dialyzable serum constituents other than thiodiglycol, and 5% as mustard thiodiglycol.
(2) The results on the organs are given in Table XV.

Discussion: All data pertain to 24 hrs. following mustard application.
(1) Only a small fraction of the mustard sulfur present in the blood was thiodiglycol.
(2) Mustard was reacting with numerous constituents present in the blood.
(3) There was no apparent connection between systemic effects and the distribution of mustard sulfur in various tissues.
(4) In all of the tissues except skin, an appreciable fraction of the fixed-mustard was soluble in isotonic salt solutions. Thus there were marked differences between the types of skin tissue constituents and those of other organs which contain mustard sulfur.

VIIIb-2. The Rate of Disappearance of Mustard from Rabbit Blood in Vitro and Vivo.

by F.C.H. and L.A.P.

Procedure: (1) In vitro experiments.
About 5 gamma of radioactive mustard in .02cc of alcohol was added to 1cc. of heparinized rabbit blood. 2 cc. of pentane (containing 1 mg. of non-radioactive mustard as carrier) was now carefully pipetted on top of the blood. After a predetermined interval of time, the test tubes were shaken. The mustard sulfur in the blood and pentane layers were determined. Previous experiments had shown that the radioactivity present in the pentane layer was essentially pure mustard.

Table XXV

Distribution of Mustard Sulfur in Various Organs of Pig A290 Following Penetration of 15 mg. H
(All activities in H-equiv.)

Organ	Wt. of Organs	Activity gamma/gm.	Percent of Total Activity Fractionated into				
			Acetone Soluble (I)	Acetone Residue .9% NaCl Soluble Alcohol Ppt. (IIa)	Acetone Residue .9% NaCl Soluble Alcohol Soluble (IIb)	Salt Residue .03 M NaOH Soluble (IIIa)	.03 M NaOH Residue (IIIb)
Blood Serum	500 gm[b]	1.50	10%	55%	35%	---	---
Bone Marrow	350 gm[b]	0.60	8%	70%	6%	1%	15%
Spleen	16 gm	0.50	30%	25%	35%	5%	5%
Thymus	7 gm	0.30	10%	40%	10%	10%	30%
Kidney	50 gm	1.50	15%	33%	10%	2%	40%
Liver	25 gm	0.95	8%	43%	38%	1%	10%
Skeletal Muscle	2000 gm[b]	0.20	45%	Insufficient activity to fractionate			
Skin[a]	(at Portal of Entry)		2%	1%	2%	75%	20%

(a) Inserted for Comparative Purposes (See Section VIIb)

(b) Computed on Basis of Animal Weight

(2) In vivo experiments.(a)

About 2 mg. and 4 mg. of mustard contained in 0.2 cc of alcohol were injected into the ear vein of two respective rabbits. Two cc. samples of blood were then removed from the heart at predetermined intervals by means of a heparinized syringe. These samples were immediately shaken with petroleum ether containing non-radioactive mustard. The activity in the respective layers was then determined.

Results: The results are given in Table XVI.

Discussion: (1) In vitro mustard remained in the blood a considerable length of time; in vivo mustard disappeared very rapidly.
(2) Apparently certain organs reacted or absorbed mustard from the blood stream extremely rapidly.

(a) Work was done in collaboration with Dr. R.A. Ormsbee, Dept. of Biochemistry, Harvard Medical School.

Table XXVI

The Rate of Disappearance from Rabbit Blood at 37°C both in Vivo and Vitro (All Activities Expressed in Gammas of H-equiv.)

In Vitro Rabbit Blood 37°C		In Vivo				
		2 mg. of H injected			4 mg. of H Injected	
Reaction Time in Min.	% of H unreacted	H in Blood Gamma/cc	Residual Activity gamma/cc	Time after Injection in Min.	H in blood Gamma/cc	Residual Activity gamma/cc
1	90%			1	7.0	4.2
4	80%	0.02	2.2	4	2.0	3.8
8	55%			8	0.03	3.3
20	40%			10	0.02	3.4
40	35%	0.02	1.8	13	0.02	3.0
60	30%	0.02	1.5	20		
		0.02	1.6	40		
		0.02	1.3	80		
		0.01	1.0	160		

PART IX
CERTAIN CHEMICAL EXPERIMENTS
by F.C.H. and L.A.P.

IXa. The Mechanism of the Chemical Reaction which Produces Fixed-Mustard.

Although all available chemical evidence indicated that the fixation of mustard was by a competition factor mechanism, there was no definite proof of this point. Since the best available method of interpreting the mechanism of fixation is by comparing the competition factors of various functional groups present in proteins and other skin tissue constituents, this proof is of utmost import.

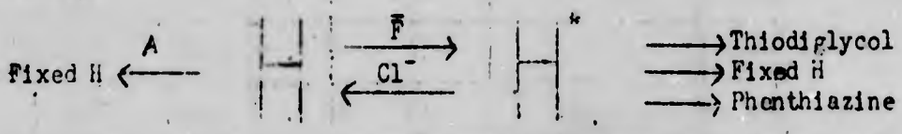
The results of V. du Vigneaud et al[14] showed that the intradermal injection of 5% solution of sodium thiosulfate into rat and rabbit immediately before a mustard application reduced the intensity of the resulting erythema. The findings of E. Ball et al[2] showed that the intraperitoneal injection of compounds with high competition factors afford partial protection against the mustard systemic effects. These data certainly tend to indicate that the fixation of mustard in skin tissue in vivo proceeded according to the competition factor mechanism.

Other evidence leading to the same conclusion has been obtained in our laboratory.

IXa-1. The Mechanism of the Fixation Reaction in Skin Tissue in Vitro.
by F.C.H. and L.A.P.

The observation that pig skin tissue brei at 4°C in an isotonic salt solution containing radioactive mustard fixed about 20% of H made available a method of determining whether or not the reaction in vitro follows a competition factor mechanism.

Let us consider the following reaction scheme and assume for the moment that there is some other reaction mechanism for fixing H (denoted by A, [i.e. as one possibility the rate determining step could conceivably be an adsorption of unactivated H on the tissue proteins]).



F denotes a competition factor path, H* is the F activated complex and arrows to the right denote aside from deactivation, the three possible modes for H* to react under the experimental conditions.

The following experiments were devised: (i) determination of percent fixed with 0.9% NaCl, (ii) percent fixed in 0.9% NaCl and 0.1M aniline, (iii) percent fixed in 0.9% NaCl and 1M $S_2O_3^=$, (iv) percent fixed in 3M NaCl, and (v) percent fixed using 100% acetone as the solvent.

To be consistent the variation in the percent fixed must follow the matrix given on the left in Table XXVII, depending on whether it is a $\bar{F}$ or an A reaction. x denotes the amount fixed in 0.9% NaCl.

Table XXVII

Data pertaining to the mechanism of fixation in pig tissue in vitro

	$\bar{F}$	A	Experimental Data Series 1	2	3	4
(i) 0.9% NaCl	x	x	18%	22%	22%	20%
(ii) 0.9% NaCl and 0.1M Aniline	<x	x	4%	6%		
(iii) 0.9% NaCl and 1M $S_2O_3^=$	<x	x			1%	1%
(iv) 3M NaCl	x	>x	25%	24%	23%	26%
(v) 100% acetone	nil	?			0%	0.2%

In (ii) and (iii), the competitors, aniline and thiosulfate ion, would compete with the tissue for H* by the $\bar{F}$ mechanism. Although both of the competitors react rapidly with H*, they do not increase the rate of formation of H* (or rate of disappearance of H), and this could not alter the amount fixed by mechanism A.

In (iv) the high Cl^- would reduce the H* concentrations by reforming H; this would not, however, by the $\bar{F}$ mechanism alter the ratio of the fraction hydrolyzing to the fraction being fixed. Since this reduction of H* reduces many-fold the rate of disappearance of H, there would be more H present in a given interval of time to react by the A mechanism.

In (v) since there would be no H* formed in an acetone media, mustard would not be fixed by the $\bar{F}$ mechanism. It is not known if any fixed-H could be formed in acetone by the hypothetical A mechanism.

Procedure: See III (a-1, g, h-2)

In order to obtain reproducible results the epidermis and upper part of the corium of pig skin were sliced on a freezing microtome set at 2.5 mu. 100 mg. portions were then taken and ground with 60-80 mesh quartz. The resulting tissue paste was placed in a centrifuge tube and 2 cc. of the appropriate solution added. To this one drop of alcohol containing 60 gamma of radioactive mustard was added. The tubes were shaken continuously for 24 hrs. at 4°C.

Any unreacted mustard (< 1%) was extracted out with petether. The solutions were then centrifuged and aliquots taken.(a) The tissues were washed three times with acetone. From the total amount of mustard sulfur in the aqueous layer and tissue, the percent of fixation was computed.

(a) The tissue substances dissolving in 0.9% NaCl and 3M NaCl and precipitated by alcohol do not appear to react appreciably with mustard under the above experimental conditions.

Discussion: (1) The data on the right side of Table XXVII show t that the heterogeneous reaction of mustard with pig skin brei in vitro was of the competition factor type.

IXa-2. Computation of the Half Life for the Mustard Fixation Reactions in Human Skin in Vivo. A Comparison of This Value with the Rate of Hydrolysis of Mustard in Physiological Solution at 37°C.

According to the competition factor mechanism, the rate of reaction of mustard in aqueous solutions was independent of the competitive substances present. Thus the rate of mustard fixation in vivo in human skin should be comparable with the rate of mustard hydrolysis and/or alkylization in physiological solution at 37°C.

Evidence supporting this view is offered by our data on the percentage of fixation, the penetration rate and rate of transport of mustard at a human skin site (see Section IVc and Vc). From this data one finds that:

(i) The penetration rate for mustard on human skin was about 1-3 gamma/min./per sq. cm.
(ii) The steady state concentration of free mustard in the skin was about 2 gamma/per sq. cm.
(iii) 12% of the mustard that penetrated became fixed.

Now if one makes the reasonable assumption that the rate of transport of mustard away from the skin site, like the fixation of mustard by the competition factor mechanism is first order in mustard, one can evaluate the fixation rate of mustard in vivo. Consider the following reaction schemes:

(p) $M \longrightarrow M_p$; r_p

(f) $M_p \longrightarrow M_f$; K_f

(t) $M_p \longrightarrow M_t$; K_t

Where M is mustard on the surface, M_p is that penetrated, M_f is that fixed, and M_t is that transported away. The K's are rate constants. r_p is the penetration rate of M.

Thus for a steady state, one has $r_p = (K_f + K_t) M_p$, but $r_p \approx 2$ gamma/per sq. cm./min. and $M \approx 2$ gamma/per sq. cm. Further,

$$\frac{K_f}{K_t} = \frac{\% \text{ Fixed}}{\% \text{ Transported}} = \frac{.12}{.88}$$

Substituting, one obtains $1/K_f = 8.5$ min.

Thus the half life for fixation is about 7 minutes and the half life for transportation of penetrated mustard away from human skin is about 1 minute.

Discussion: (1) It is of interest to note that a half life for mustard fixation of 7 min. in vivo is of the correct order for the competition factor mechanism in physiological solutions at 37°C.

(2) The data presented in section IXa-1 and IXa-2 together with that of V. du Vigneaud[1] and E.G. Ball[2] and their collaborators make the fixation of mustard in human skin in vivo by the competition factor mechanism extremely likely.

IXb. The Competition Factors of Various Substances Including Amino Acids, Blood Plasma and Plasma Proteins.

Since mustard presumably reacts with skin tissue constituents by the competition factor mechanism, a knowledge of the competition factors of various functional groups is of import. The usual method of determining competition factors is based on the rate of hydrogen ion liberation in the presence and absence of the competitor. This procedure is not applicable to numerous compounds especially weak bases and complicated molecules. However, it is possible to employ the radioactive technique to the determination of the competition factor of these substances. According to the competition factor mechanism, we have the following reaction scheme.

$$\xrightarrow{H_2O} \quad \text{Thiodiglycol}$$

$$H \longrightarrow H^* \quad \xrightarrow{\text{Competitor}} \quad \text{Derivative}$$

Let I be the initial amount of mustard present and let T be the amount of thiodiglycol formed; then the competition factor F of the competitor is given by:

$F = (I - T)/TM$ where M is the molarity of the competitor

Thus if $I = T$ (all of the mustard forming thiodiglycol) $F = 0$, and if T is 0 (no thiodiglycol formed) F is infinite.

Hence in our procedure, about 60 to 150 gamma of radioactive mustard was allowed to react completely in a 2 cc solution containing the competitor. About 100 mg. of non-radioactive thiodiglycol were added and by the procedure described in IIIj-3 and IIIm-1-i, di-p-nitrobenzoate of thiodiglycol was prepared and purified.

Thus in all cases where all of the weighable sulfur (as benzidine sulfate) is due to the carrier thiodiglycol, the radioactivity per mg. of $BzSO_4$ of the original solution is a measure of I and the radioactivity per mg. of $BzSO_4$ of the purified thiodiglycol derivative is a measure of T. The value of F for the competitor can now be computed. In order to obtain maximum accuracy, the molarity of the competitor (whenever possible) was so chosen such that $I = T$ approximately.

It should be kept in mind that the above procedure makes the basic assumption that mustard and the compounds investigated react by a competition factor mechanism. It is believed that the extensive investigation of especially Ogston, Peters et al[9] and Doering and Linstead[13] leave little doubt regarding the correctness of this assumption (a)

(a) A comprehensive presentation of the competition factor mechanism is presented in the Formal Report of Doering, Linstead and Ball.[14]

Doering and Linstead(13) have shown that our procedure leads to values about 10% too high for the competition factor. However, the investigations to be presented were undertaken with the view of obtaining relative reactivities of certain functional groups rather than precise values for competition factors of these groups.

Purpose: To determine the competition factor of certain compounds, especially certain amino acids and proteins.

Procedure: (i) The competition factors (F) on non-protein substances were determined by dissolving the appropriate amount of the compound in 2 cc of an aqueous solution (the molarity of the various solutions varied between .01M and .1M). The aqueous solutions were either water, 0.1M HCl(pH1) or 0.1M NaOH(pH 13). To this solution was now added 0.04cc of alcohol containing 60 gamma of radioactive mustard. The solution was allowed to stand 16 hrs. to insure complete reaction. 120 mg. of carrier thiodiglycol were added and the solution was now subjected to the procedure given above in the introduction.

(ii) The competition factor of pig blood plasma was determined by treating 1 cc of the plasma with .04 cc of alcohol containing 100 gamma of mustard. The solution was allowed to stand for 7 hours, 40 mg. of carrier thiodiglycol added and the plasma extracted with pet. ether to remove any unreacted mustard. The procedure is now identical with that described in the introduction except that it was necessary to re-extract the thiodiglycol from the chloroform so as to remove interfering substances extracted by the chloroform from the plasma.

(iii) The competition factors of the protein fractions of human blood plasma(b) were determined in essentially the same manner. To 2 cc of the protein solution in physiological saline (with solid fractions, 2 mg. was dissolved in 2 cc of saline) was added 150 gamma of mustard in 0.05 cc of alcohol. The solutions were allowed to stand at 4°C for 48 hrs. (1 1/2 hrs. with fibrinogen to prevent its coagulation). 120 mg. of carrier thiodiglycol were now added, followed by pet. ether extraction to remove any unreacted H. The remainder of the procedure is given in the introduction.

(b) These human protein plasma fractions were obtained from Professor E.J. Cohn of the Harvard Medical School.

Table XXVIII
Competition Factors of Various Substances

Substance	Molarity	pH	F
Methyl amine	.01	free base	390
Methyl amine hydrochloride	.1		8.5
Aniline	.01	free base	111C
Diethyl amine	.01	free base	255
Sodium Veronal	.05	9.9	260
Veronal	.025	1.3	4.5
Phenol	.05	13	75
Acetamide	.1		1.5
Imidazole	.02	free compd.	110
Imidazole	.02	13	56
Potassium acetate	.1		9

Table XXIX
Competition Factors of Amino Acids

Amino Acid	Molarity	pH	F
Lysine	.03	13	147
Histidine	.04	free compd.	67
Histidine	.03	13	71
Methionine	.027	1	510
Methionine	.04	free compd.	260
Methionine	.027	13	75
Tyrosine	.05	13	1700
Glycine	.1	free compd.	4.5
Glycine	.1	13	73
Arginine	.02	7	42
Arginine	.02	13	73
Cystine	.02	13	240
Tryptophane	.02	13	210

Table XXX

Competition Factors of Pig's Blood Plasma and Fractionated Human Blood Plasma Proteins

Fraction	Mol. wt.	Protein Concn. for determ. in % by weight	F for 1% protein soln. (10 mg./cc)	Partial Effective F in orig. Blood Plasma
I Fibrinogen	200,000	2.5	6.8	2.8
I+II Globulins	100,000	10.6	0.43	0.7
IV Globulins Lipoproteins Steroids Albumins		7.9	3.3	3.6
V Albumin	69,000	2.5	0.12	0.3
VI Residual Proteins (mostly salts)		1.5	0.85	0.3
Total F for Human Blood Plasma				7.7
Effective F for Pig Blood Plasma				15

Discussion: (1) Our value for acetate ion given in Table XXVIII is a check on our procedure.

(2) The competition factors of the amino acids were determined only at pH 1, pH 13 and at the pH of the free amino acid dissolved in water. Buffers for other pH levels could not be employed as they are competitive agents for mustard (e.g. veronal at pH 10, F = 260) or they catalyze by a competition mechanism the decomposition of mustard to thiodiglycol (e.g. borate buffer). Although pH 1 and 13 are far from biological conditions, the determination of F at these pH's enables one to postulate which types of groups are reactive in an amino acid molecule.

(3) Table XXIX shows that a free amino group has a relatively high competition factor. This is also true of the nitrogen in an imidazole ring. The presence of an H ion in the nitrogen make the reactivity of an amino group essentially negligible. The amino group in an acid amide linkage is also unreactive towards mustard.

(4) The free amino group in an amino acid seems to have an F of about 75 (histidine pH 13, F = 76; glycine pH 13, F = 73; methionine pH 13, F = 75; arginine pH 13, F = 75). The presence of an H ion on the amino nitrogen renders its F small. Thus due to zwitter ion formation, a simple amino acid has a low F when in solution as the free amino acid (e.g. glycine, F = 4.5).

(5) Histidine as the free amino acid must react with mustard on the nitrogen of the imidazole ring (F = 67). The nitrogen of the indole ring is reactive towards mustard (Tryptophane pH 13, F = 210).

Tyrosine has an F of 1700 at pH 13. Thus the phenolate ion is extremely reactive towards mustard. The tendency of mustard to react with a thioether linkage to form sulfonium salts is high (methionine pH 1, F = 510.

Thus even though the simple amino acids do not have a very high F near pH 7, the amino acids with other reactive groups do (e.g. the N of the imidizole ring of histidine, the sulfur atom of methionine, the N of the indole ring of tryptophane, and the guanido group of arginine).

(6) The effective competition factors of certain human blood plasma proteins in a 1% by wt. solution show that the relative reactivities of proteins toward mustard are by no means identical. Thus human plasma albumin on a weight basis is essentially unreactive towards mustard as compared to fibrinogen.

(7) As computed from the above values the effective competition factor of human plasma is 7.7 (12% of mustard in human plasma in vitro would form thiodiglycol). This is not too different from the experimentally determined value of 15 for pig blood plasma.

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30343

TITLE: The Mechanism of Cutaneous Injury by Mustard Gas. An Experimental Study Using Mustard Prepared with Radioactive Sulfur

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An investigation was made of the mechanism of vesication through parallel histological and chemical studies of skin exposed to mustard with radioactive sulfur. The rate at which mustard penetrates the skin, the subsequent fate of the penetrated mustard in the skin, and the significance of these chemical data in relation to the production of injury were studied. The pathology of cutaneous injury in man following exposure to mustard was compared with that occurring in animals. The correlation of fixed mustard to the severity of injury, the rate of disappearance in human skin tissue, and the mechanism of the reaction which produced fixed mustard are discussed. Details of experimental procedure are given, and the synthesis of mustard gas containing radioactive sulfur is described.

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