[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

CHEMICAL REACTIONS OF MUSTARD GAS AND RELATED COMPOUNDS.[1] II. THE REACTION OF MUSTARD GAS WITH CARBOXYL GROUPS AND WITH THE AMINO GROUPS OF AMINO ACIDS AND PEPTIDES

STANFORD MOORE, WILLIAM H. STEIN, AND JOSEPH S. FRUTON[2]

Received March 22, 1946

Biochemical studies on the reactions of mustard gas (H) have been based upon the reasonable assumption that the physiological effects of this agent are the consequence of chemical processes which H initiates by its reaction with body constituents. In the previous paper of this series (1), data were presented on the chemical reactions of H with water. The experiments described in the present communication concern the reactions of H with carboxyl groups and with the amino groups of amino acids and peptides.

The reaction of H with carboxyl groups. The reaction of H with carboxyl groups is of particular interest, since there is evidence that carboxyl groups are involved when H reacts with proteins both *in vitro* and *in vivo*. Other investigators have studied in detail the kinetics of the reactions of H with organic acids (2, 3). The experiments reported below, which were performed primarily from the preparative standpoint, show that in aqueous solution at *p*H 8, H reacts readily with sodium salts of carboxylic acids to form esters of thiodiglycol.

Measurement has been made of the amounts of organic esters of thiodiglycol present at the end of 24 hours when H is shaken with aqueous solutions of the sodium salts of a number of organic acids. The results reported in Table I indicate that, under the experimental conditions employed, about half of the initial H appears in the form of organic esters. The products of the reaction of H with the sodium salts of acetic, hippuric, salicylic, and diethylbarbituric acids have been prepared and found to be disubstituted derivates of thiodiglycol. The presence of monosubstituted derivatives is not excluded.

The reaction of H with substances such as acetate, hippurate, citrate, and succinate lends support to the belief that H is capable of reacting with some of the β-carboxyl groups of aspartic acid or the γ-carboxyl groups of glutamic acid which might exist in an uncombined state in proteins. Moreover, the reaction with simple organic acids, such as succinic, suggests that *in vivo* H may react with important cellular metabolites of this type. The reaction of H with stearate is of interest in view of the fact that Reichstein and Goldschmidt (4) isolated bis(β-hydroxyethyl)sulfoxide from adrenals. These authors expressed the view that this compound occurred naturally in the form of esters of higher fatty acids.

[1] This work was done in whole under Contract No. OEMsr-313 between The Rockefeller Institute for Medical Research and the Office of Scientific Research and Development, which assumes no responsibility for the accuracy of the statements contained herein. The experiments were performed during the period January 1942–August 1944.

[2] Present address, Yale University, New Haven, Connecticut.

Finally, the reactions with acetate, citrate, and veronal are of some practical consequence and point to the inadvisability of using substances of this type as buffers in experiments in which H is employed.

To determine whether the extent of the reaction of H with carboxyl groups of substituted amino acids is markedly influenced by structural differences between the various compounds bearing the carboxyl group, three acids of different

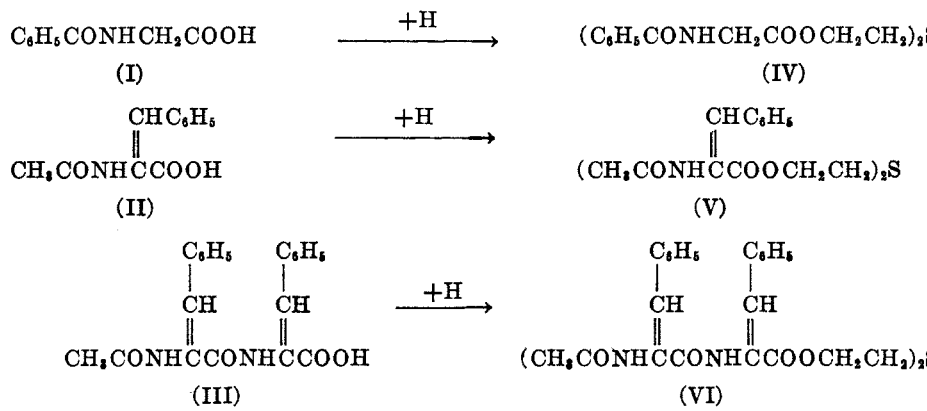
TABLE I

REACTION OF H WITH ORGANIC ACIDS

H (4 *mM*) was shaken at 20–25° for about 24 hours with an aqueous solution (25 cc.) of the reactant. Individual variations in this procedure, which were sometimes necessary, are given in the experimental section. The extent of esterification was determined in the manner described in the experimental section.

REACTANT	AMOUNT OF REACTANT, *mM*	H RECOVERED AS ESTER, %
Sodium acetate	16	40
Sodium acetate	60	80
Sodium stearate	16	50
Trisodium citrate	8	60
Disodium succinate	8	60
Sodium diethylbarbiturate (Veronal-Na)	16	60
Sodium hippurate	16	40
Sodium salicylate	16	45

structure were compared. These acids were hippuric acid (I), acetyldehydrophenylalanine (II), and acetyldehydrophenylalanyldehydrophenylalanine (III).



It was found that all three carboxylic acids combine with H to almost the same extent at *p*H 8 to form saponifiable esters of thiodiglycol. Under the conditions described in the experimental section, 37% of the available chloroethyl groups of H reacted with hippuric acid (I), while 35% combined with acid (II), and about 28% with acid (III). However, variations in the reactivities of widely differing organic acids have been observed (3).

The reaction of H with amino groups. In solutions buffered with $NaHCO_3$, H may react to a measurable extent with the amino groups of glycine, alanine, lysine, glycylglycine, and benzoyllysineamide (Table II). In these experiments, samples of H were shaken with aqueous solutions of the amino acids or peptides at room temperature and the decrease in NH_2-N determined by the Van Slyke nitrous acid method. In acidic solutions (absence of $NaHCO_3$) no reaction with the amino groups of glycine or glycylglycine was observed.

TABLE II

REACTION OF H WITH AMINO GROUPS OF AMINO ACIDS AND PEPTIDES

COMPOSITION OF AQUEOUS SOLUTION (VOLUME 25 CC.)			H ADDED, *mM*.	DECREASE IN NH_2-N, M.EQUIV.	DECREASE IN NH_2-N PER *mM* OF H, M.EQUIV.
Glycine	16	*mM*	4.0	1.3	0.32
$NaHCO_3$	8	*mM*			
Alanine	8	*mM*	4.0	1.0	0.25
$NaHCO_3$	12	*mM*			
Lysine 2 HCl (Neutr. with NaOH)	8	*mM*	4.0	1.8 ($\alpha + \epsilon$)	0.45
$NaHCO_3$	12	*mM*			
Glycylglycine	16	*mM*	4.0	2.5	0.62
$NaHCO_3$	8	*mM*			
Glycine (Without $NaHCO_3$)	16	*mM*	4.0	0.0	0.0
Glycylglycine (Without $NaHCO_3$)	18	*mM*	4.0	0.0	0.0
Benzoyllysineamide	0.32	*mM*[a]	0.19	0.03	0.15
$NaHCO_3$	0.8	*mM*			

[a] Volume of solution was 1 cc.

In the reaction of H with amino groups, either secondary amines or thiazanes might result (5, 6). It has not been established which of these two structures is formed in the reactions reported in Table II.

The experiment with benzoyllysineamide (Table II) is more nearly analogous to the possible reaction of H with the amino groups in proteins than are the other examples. The lysine residue in benzoyllysineamide is bound in a manner similar to that in which lysine occurs in proteins; the ϵ-amino group in this structure is free whereas the α-amino group and the carboxyl group are blocked. It will be noted that, under similar conditions, the extent of the reaction of H with the ϵ-amino group of benzoyllysineamide is lower than that with α-amino groups.

The above experiments, carried out under mild conditions of *p*H and temperature, may be regarded as evidence that the amino groups of proteins, peptides,

and amino acids are to be considered among the groups which H may attack in biological systems.

The reaction of H with pyridine, nicotinamide, and nicotinic acid. H reacts readily with the pyridine nitrogen of these three compounds to form pyridinium compounds. The products of the reaction of H with pyridine and nicotinamide have been isolated in good yield (*cf.*, experimental section). Analytical data have been obtained to show that, under the experimental conditions employed, the reaction between H and nicotinic acid is nearly quantitative, 95% of the chloroethyl groups of H having reacted to form pyridinium compounds.

EXPERIMENTAL

Reaction of H with sodium acetate. The reaction mixture was made up in the manner indicated in Table I. After 24 hours shaking, no unreacted H remained. The resulting aqueous suspension of diacetylthiodiglycol was made alkaline with $NaHCO_3$ and extracted with ether. From the ether extract, diacetylthiodiglycol was obtained as a Cl-free oil in a yield of 40% (determined by saponification equivalent). This compound has been prepared previously (7, 8). The diacetate (500 mg.) was transformed into the crystalline sulfilimine by treatment in aqueous solution with 700 mg. of chloramine-T; yield 70%. The sulfilimine was recrystallized from benzene; m.p. 116–117.5°.

Anal. Calc'd for $C_{15}H_{21}NO_6S_2$: C, 48.0; H, 5.6; N, 3.8.
Found: C, 48.2; H, 5.8; N, 3.6.

In a control experiment no diacetate was formed when thiodiglycol (4 *mM*), acetic acid (8 *mM*), sodium acetate (8 *mM*), and NaCl (8 *mM*) in 25 cc. of water were kept at room temperature for three days.

Reaction of H with sodium stearate. The reaction was carried out with an aqueous suspension of sodium stearate. The extent of esterification (Table I) was determined by saponification of an ether extract of the alkaline reaction mixture.

Reaction of H with trisodium citrate and disodium succinate. The reactions were carried out in the manner described for the reaction with sodium acetate, except that the saponification equivalents were determined directly on the aqueous solution with an approximate correction for the presence of sulfonium chlorides. The esters obtained in these experiments are acidic esters, and are not extractable from neutral or alkaline solution with ether.

Reaction of H with sodium diethylbarbiturate (veronal-Na). The yield of ester (60%) given in Table I is based on the weight of products extractable by ether from alkaline solution. The ether extract was concentrated *in vacuo* and, upon addition of petroleum ether, the diveronal derivative of thiodiglycol crystallized. It was recrystallized from alcohol; m.p. 148–149°.

Anal. Calc'd for $C_{20}H_{30}N_4O_6S$: C, 52.9; H, 6.6; N, 12.3.
Found: C, 53.0; H, 6.6; N, 12.3.

Reaction of H with sodium hippurate. The reaction was carried out in 50 cc. of 60% acetone to ensure complete reaction of H. On removal of the acetone *in vacuo*, the dihippurylthiodiglycol crystallized; yield 650 mg., 37%. It was recrystallized from alcohol; m.p. 119°.

Anal. Calc'd for $C_{22}H_{24}N_2O_6S$: C, 59.4; H, 5.4; N, 6.4.
Found, C, 59.4; H, 5.4; N, 6.3.

In the absence of acetone, the ester separates during the reaction as an oily solid contaminated with unreacted H.

Reaction of H with sodium salicylate. The reaction mixture (Table I) was made alkaline with $NaHCO_3$ and extracted with ether. The yield of ester given in Table I is based upon the weight of the material found in the ether extract. The crude disalicylthiodiglycol obtained on removal of the ether was crystallized from alcohol; m.p. 74–75°.

Anal. Calc'd for $C_{18}H_{18}O_6S$: C, 59.7; H, 5.0.
Found: C, 59.7; H, 5.3.

Extent of reaction of H with hippuric acid (I), acetyldehydrophenylalanine (II), and acetyldehydrophenylalanyldehydrophenylalanine (III). H (4 *mM*) was shaken for 48 hours at about 25° with 12 *mM* of the sodium salts of I, II, and III in the presence of 12 *mM* of $NaHCO_3$. The total volume was 25 cc. In each case, a solid separated during the reaction. Hydrochloric acid was added and the CO_2 removed *in vacuo*. Absolute alcohol (50 cc.) was added to give a clear solution. The solution was neutralized to phenolphthalein, and 5.0 cc. of *N* NaOH were added. After the solution had stood at room temperature for 30 minutes, back titration with 0.1 *N* HCl gave the amount of ester saponified. In the case of the reaction with III, the titration was not completely satisfactory due to the yellow color of the solution.

Reaction of H with pyridine. H (2.55 cc., 20.0 *mM*) was added to 100 cc. of water containing 6.32 g. (80.0 *mM*) of pyridine. The mixture was shaken at room temperature for 24 hours and then evaporated to dryness *in vacuo*. The residue was washed with acetone and dissolved in absolute ethanol. Upon the addition of dry ether, crystallization of bis(β-pyridiniumethyl)sulfide dichloride occurred; yield 5.25 g. (83% of theory). The compound was recrystallized twice from an absolute ethanol-ether mixture and dried over P_2O_5 *in vacuo* at room temperature to constant weight. The substance is very hygroscopic.

Anal. Calc'd for $C_{14}H_{18}Cl_2N_2S$: C, 53.0; H, 5.7; N, 8.8; Cl^-, 22.35.
Found: C, 52.6; H, 5.9; N, 8.7; Cl^-, 22.2.

To convert the dichloride to the dipicrylsulfonate, 6.34 g. was dissolved in 200 cc. of water and a solution of 1.46 g. of picrylsulfonic acid in 10 cc. of water was added with stirring. The precipitate which separated was filtered off and discarded. A solution of 13.15 g. of picrylsulfonic acid in 90 cc. of water was then added with stirring. After standing at 4° for 4 hours, the product was collected and washed with water. The yield was 14.49 g. After recrystallization from a solution of 90% methylcellosolve it melted at 216–218°.

Anal. Calc'd for $C_{14}H_{18}N_2S \cdot 2\ C_6H_2N_3O_9S$: C, 37.6; H, 2.7; N, 13.5; S, 11.6.
Found: C, 37.5; H, 2.6; N, 13.5; S, 11.7.

Reaction of H with nicotinamide. A reaction mixture containing 7.3 g. (60 *mM*) of nicotinamide, 5.05 g. (60 *mM*) of $NaHCO_3$, and 1.9 cc. (15 *mM*) of H in 150 cc. water was shaken for 20 hours at room temperature. The solution was concentrated to dryness under reduced pressure and the residue was extracted with 200 cc. of hot absolute ethanol. The undissolved portion was taken up in 10 cc. of water, acidified with HCl and the resulting solution was evaporated under reduced pressure. The residue was evaporated once with absolute ethanol and once with absolute methanol to remove the last traces of water. The residue was then extracted with 150 cc. of boiling absolute methanol and filtered. Upon cooling the filtrate overnight at 4°, pink crystals of the dichloride of the nicotinamide derivative were obtained. The compound was recrystallized from absolute methanol and dried to constant weight in air; yield 0.8 g.; m.p. 151–153°.

Anal. Calc'd for $C_{16}H_{20}Cl_2N_4O_2S \cdot H_2O$: C, 45.6; H, 5.25; N, 13.3; Cl^-, 16.8; H_2O, 4.3.
Found: C, 45.6; H, 5.3; N, 13.3; Cl^-, 16.9; H_2O, 4.6.

A second but less pure crop was obtained by working up the methanolic mother liquors; yield 0.7 g.; m.p. 144–148°.

The reaction of H with nicotinic acid. H (0.5 cc., 4 *mM*) was shaken with 20 cc. of a solution of 16 *mM* of nicotinic acid (sodium salt) neutralized to phenolphthalein. After 20 hours, the liberated acid was titrated and found to be 0.4 *mM* (theory for complete hydrolysis of H, 8 *mM*). Thus, 95% of the chloroethyl groups had reacted to form "onium" compounds. In order to decide whether these "onium" compounds were of the sulfonium or the ammonium type, the solution was heated for 1 hour at 100°. The slight increase in acidity after heating indicated that not more than 1% of the "onium" compounds could be sulfonium salts.

The authors wish to express their indebtedness to the late Dr. Max Bergmann for the constant advice and encouragement which he gave in the course of this research. Thanks are due also to Miss Rosalind E. Joseph for assistance in the

conduct of these experiments and to Mr. Stephen M. Nagy, who performed the microanalyses reported in this paper.

NEW YORK, N. Y.

REFERENCES

(1) STEIN, MOORE, AND BERGMANN, *J. Org. Chem.* (Paper I of this series).
(2) OGSTON, (1941).[a]
(3) DOERING AND LINSTEAD (1942).[b]
(4) REICHSTEIN AND GOLDSCHMIDT, *Helv. chim. Acta*, **19**, 401 (1936).
(5) CASHMORE AND MCCOMBIE, *J. Chem. Soc.*, **123**, 2884 (1923).
(6) CLARKE, *J. Chem. Soc.*, **121**, 2137 (1922).
(7) HELFRICH AND REID, *J. Am. Chem. Soc.*, **42**, 1208 (1920).
(8) CLAYTON AND REID, *J. Am. Chem. Soc.*, **64**, 908 (1942).

---

[a] Unpublished data obtained in Great Britain.
[b] Unpublished data obtained in the United States.