was distilled under a diminished pressure. The oily 6-methyl-2-acetyloxypyridine, $b.p_4$ 118°, was hydrolyzed by hydrochloric acid to 6-methyl-2-pyridone.

Summary

It was proved that 2-picoline 1-oxide formed 2-picolyl alcohol and 6-methyl-2-pyridone through the action of acetic anhydride. The yield of 2-picolyl alcohol was 50% of the theoretical amount. It is assumed that this reaction is the best available for the preparation of 2-picolyl alcohol.

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85. **Masahiro Torigoe:** Reaction of Cysteine with Nitrogen Mustards and their N-oxides.*

(*Iatrochemical Institute of the Pharmacological Research Foundation***)

It is a well-known assumption that nitrogen mustard reacts chemically as an alkylating agent with active hydrogen atoms of some tumor cell constituents as a first step of its mitosis-inhibiting action upon proliferating cancer cells.

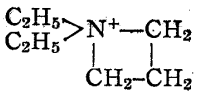
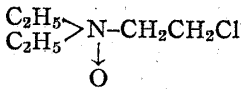
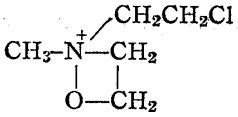
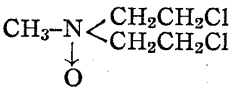
Experiments were carried out in order to compare the activities of nitrogen mustards with their N-oxides to combine with cysteine in bicarbonate buffer solution at an ordinary temperature. In the experiments, 10^{-3} mole of the compounds were added to the excess of cysteine dissolved in bicarbonate buffer and kept at 25° for a period, which was determined experimentally to be long enough for completion of the alkylating reaction; i.e. until no more increase in cysteine up-take was observed. After the reaction ceased, excess of cysteine was determined colorimetrically[1] with phosphotungstic acid[2], after treating with sodium amalgam[3] to reduce cystine, which appeared in the solution as a result of oxidation of cysteine by the N-oxide or air oxygen. The results of the experiments are summerized in Table I.

TABLE I. Cysteine Up-take of Nitrogen Mustards

	Nitrogen mustard concn. 10^{-3} Mol.	Cysteine-HCl concn. (Mole)	Time of reaction (hrs.)	Cysteine up-take (Mol. equiv.)
(I)	$CH_3-N<^{CH_2CH_2Cl}_{CH_2CH_2Cl}$	4×10^{-3}	24	2.0
(II)	$CH_3-N<^{CH_2CH_2Cl}_{CH_2CH_2OH}$	2×10^{-3}	24	0.93
(III)	$^{C_2H_5}_{C_2H_5}>N-CH_2CH_2Cl$	2×10^{-3}	72	0.94

* M. Ishidate, Y. Sakurai: Studies on Cancerocidal Substances. VII.
** Nishigahara, Kita-ku, Tokyo (鳥越政宏).

1) A. Schöberl, P. Rambacher: Biochem. Z., 295, 377(1938).
2) O. Folin, H. Trimble: J. Biol. Chem., 60, 478(1924).
3) I. M. Kolthoff, W. Strick: J. Am. Chem. Soc., 72, 1952(1950).

(IV)		4×10^{-3}	30	0
(V)		4×10^{-3}	24	0.55
(VI)		6×10^{-3}	30	1.2
(VII)		6×10^{-3}	22	1.4

The reaction mixture was buffered by excess of $NaHCO_3$ and kept at 25°.

The conclusion obtained from the data in Table I is as follows:

(1) Tertiary nitrogen mustards (I, II, III) take an almost equivalent amount of cysteine according to the number of β-chloroethyl groups in their molecule.

(2) N-oxides take less cysteine than expected, i.e. monofunctional N-oxide (V) and difunctional N-oxide (VII) take 0.55 and 1.4 mol. equivalent of cysteine, respectively.

(3) Among the intermediates (IV, VI) of the transformation reaction of N-oxides in neutral solution, (IV) never combines with cysteine in this condition but is promptly reduced to diethyl-β-hydroxyethylamine by cysteine.

These facts indicate that the dimethylene(1,2)-oximine ring has no tendency to alkylate the sulfhydryl group of cysteine but oxydizes it to a disulfide linkage. The same ring, existing in the molecule of (VI), might also be incapable of alkylating and, therefore, about one mole of cysteine up-take of (VI) should be due to the fomation of one mole of chlorhydrin (II) as an intermediate from (VI) by mere reduction with cysteine.

Analytical identification of the alkylation products of (I) and (II) was carried out. The former was isolated as a tosyl derivative or a Reineckate and the latter as a Reineckate, the analytical data of which agreed well with the calculated figures for (VIII) and (IX). Rf values of the sulfates of (VIII) and (IX), which were regenerated from the purified Reineckates in the usual manner, were determined as 0.03 and 0.22, respectively, using a mixture of phenol, butanol, and ammonia water as a developing solvent, while they appeared as 0.04 and 0.13, when developed by a mixture of butanol, acetic acid, and water. The spots were colored either by ninhydrin or by Dragendorf's reagent and their Rf values were constant as well as specific. Reaction products of (VII) with cysteine in bicarbonate buffer was also analyzed by paper chromatography using two different solvents. Two spots in each paper strip were obtained, which corresponded to those of the sulfate of (VIII) and (IX). Color intensity of the latter, however, was far weaker than that of the former. When a mixed sample of (VIII) and (IX) was developed by a butanol-acetic acid-water mixture, Rf values were somewhat varied according to the mixing ratio of the components and the Rf values of the reaction products of (VII) with cysteine approximately agreed with those of a mixture of 5γ of (VIII) and 27γ of (IX). On the contrary, if cysteine was added to the solution of the free base of (VII), which was kept previously at 37° for 5 hours, color intensity of a spot corresponding to (IX) became more dominant than the other, while deviations of Rf values were not intense. This fact implies that

most of the compound (VII) had changed into (VI) before addition of cysteine. Details are given in Table II.

TABLE II. Rf Values of the Cysteinyl Compounds

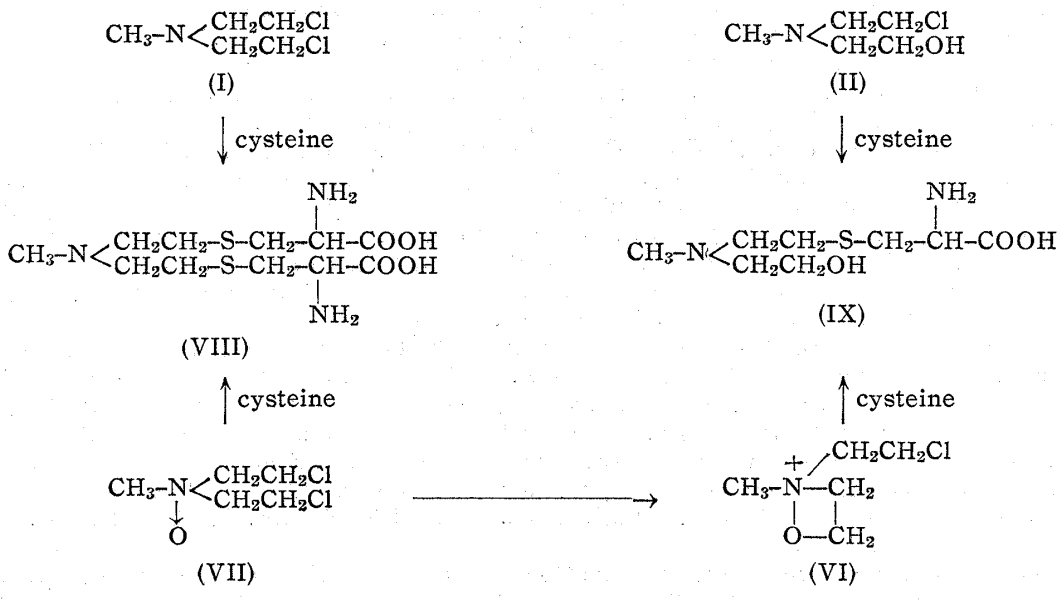
Substance		Solvent used: BuOH·AcOH·H_2O					Solvent used: BuOH·PhOH·NH_4OH			
(VIII)–H_2SO_4			0.04				0.03	0.03	0.03	0.03
(IX)–H_2SO_4			0.13				0.21	0.22	0.22	0.23
Artificial mixture of (VIII) and (IX)–H_2SO_4	lower spot	0.03	0.04	0.03	0.03	0.03				
	*upper spot	0.089	0.098	0.093	0.081	0.078				
"Reaction Product A"**	lower spot	0.03	0.03	0.03	0.02	0.03	0.02	0.02	0.02	0.02
	*upper spot	0.082	0.089	0.086	0.076	0.081	0.21	0.20	0.22	0.21
"Reaction Product B"**	lower spot	0.03	0.03				0.03	0.03	0.02	
	upper spot	0.11	0.10				0.22	0.24	0.24	

* These chromatograms were run at the same time in the same tank.
** See Experimental, 6).

The absence of free amino acids in these samples was confirmed by paper chromatogram using phenol saturated with water as a developing solvent.

It might be concluded therefore that a part of methyl-bis(β-chloroethyl)amine N-oxide (VII) is reduced directly to (I), while the rest is transformed into (VI) which is reduced in succession to (II) by cysteine. These reduction products combined with cysteine to yield (VIII) and (IX) which were identified on the paper chromatogram as mentioned above. Scheme of the reaction is shown in Fig. 1.

Fig. 1.



This N-oxide (VII) has already been reported[4] as an excellent tumor retarding agent, having high efficacy and low toxicity, and a probable aspect of its reaction mechanism *in vivo* is that the N-oxide is reduced gradually to the tertiary amine (I), which reveals its characteristic cancerocidal activity, after being taken up in animal or human tissue in the form of N-oxide. Results of this experiment with cysteine *in vitro* might be regarded as supporting this view.

4) I. Aiko, S. Owari, M. Torigoe: J. Pharm. Soc. Japan, 72, 1297(1952).

It therefore seems interesting to synthesize an N-oxide, the physicochemical properties of which are such as to increase its selective affinity to tumor tissue *in vivo*, and to be more readily reducible in a vivid proliferating tissue such as cancer than the normal ones.

Further discussions will be published in succeeding papers.

Experimental

1) Colorimetric determination of cysteine up-take—Reagent: (a) Cysteine and cystine: Cysteine-HCl was recrystallized from water and dried in air at room temp. Cystine was dissolved in 3% HCl and precipitated by addition of saturated sodium acetate solution. *Anal.* Calcd. for cystine: N, 11.66. Found: N, 11.73. (b) Phosphotungstic acid reagent: Folin and Trimble reagent.[2] (c) Acetate buffer[1]: A mixture of 10 volumes of sodium acetate solution (2 mol.) and 3 volumes of acetic acid (2 mol.).

Procedure: 2.00 cc. of the sample solution was acidified with 2*N* sulfuric acid and shaken with 1% sodium amalgam[3] for 20 mins. After the solution was transferred into a volumetric flask (50 cc.), washed out thoroughly with acetate buffer (ca. 5 cc.), phosphotungstic acid reagent (2.00 cc.) was added, and placed in a thermostat at 25° for 10 mins. The volume was then adjusted to 50 cc. and the resultant color was determined by the Beckman Model DU spectrophotometer (729 mμ).

2) Isolation of the tosyl derivative of (VIII)—A mixture of (I)-HCl (970 mg.; 5 mM.), cysteine-HCl (1.93 g.; 11 mM.), $NaHCO_3$ (3.8 g.; 45 mM.), and water (20 cc.) was incubated for 24 hrs. at 25°. A portion of the reaction mixture (9 cc.) was made alkaline with NaOH (90 mg.) and shaken vigorously with *p*-toluenesulfochloride (1.7 g.) and ether (2 cc.), with repeated addition of 2 cc. of 2*N* NaOH every 1 hour. After four hour's shaking, ether was removed, and by acidification of the aqueous layer a white powder precipitated (81%), which was reprecipitated twice from hot water by cooling. It began to sinter at 105° and decomposed at 130°. *Anal.* Calcd. for $C_{25}H_{35}O_8N_3S_4$: C, 47.39; H, 5.53; N, 6.64. Found: C, 47.19; H, 5.66; N, 6.65. The powder (4.480 mg.) was dissolved in 80% acetone (1 cc.) and titrated with 0.01*N* NaOH (indicator: phenolphthaleine). Calcd. for $C_{23}H_{33}O_4N_3S_4(COOH)_2$: 1.42 cc. Found: 1.47 cc.

3) Isolation of Reineckate of (VIII)—A mixture of (I)-HCl(2.5 mM.), cysteine-HCl(5.5 mM.), $NaHCO_3$ (22.5 mM.), and H_2O(10 cc.) was incubated at room temp.(ca. 26°) for 24 hrs. After being extracted in a continuous extractor with ether for 10 hrs., the aqueous layer (8 cc.) was acidified to Congo red and treated with warm (60°) ammonium Reineckate solution (ca. 10%). The precipitate was washed three times with water and dried (2 g.). The salt, after being dissolved in 80% acetone, was reprecipitated by addition of water and dried in air for several days. *Anal.* Calcd. for $C_{19}H_{37}O_4N_{15}S_{10}Cr_2 \cdot 2H_2O$: C, 22.82; H, 4.10; N, 21.02. Found: C, 22.57, 22.56; H, 4.24, 4.49; N, 21.16. It did not lose its water of crystallization at 130°/2 mm. Hg for 1 hour.

4) Properties of sulfate of (VIII)—The solution of the Reineckate of (VIII) (100 mg.) in 80% acetone was treated with Ag_2SO_4 (300 mg.) at 60~70° and the filtered insoluble substance was extracted with water. Excess of silver ion dissolved in the solution was precipitated by H_2S and the filtrate was evaporated to dryness in vacuum (Residue: 37 mg.). The sulfide-linkage of this compound remained intact when reduced with sodium amalgam under the conditions mentioned above, and did not liberate cysteine (nitroprusside reaction) when hydrolyzed with 1*N* HCl or 1*N* NaOH at 100° for 30 mins. (concn. of substance: ca. 10^{-3} M.).

5) Isolation of Reineckate of (IX)—The picrate (3.44 g.; 8 mM.) of (II) was converted to the corresponding hydrochloride and diluted to 32 cc. with water. After addition of cysteine-HCl (1.39 g.; 8.8 mM.) and neutralisation to Congo red, the mixture was added with $NaHCO_3$ (3.4 g.; 40 mM.) and incubated at 25° for 24 hrs. The reaction mixture was extracted continuously with ether for 8 hrs. The aqueous layer (20 cc.) was treated with ammonium Reineckate as above. Yield, 1.67 g. It was purified by the same procedure as 3) and dried in air. It began to sinter at 140~142° and decomposed at 155~158°. *Anal.* Calcd. for $C_{12}H_{25}O_3N_8S_5Cr$: C, 26.62; H, 4.62. Found: C, 27.16, 27,21; H, 4.94, 4.76.

6) Isolation of products from the reaction mixture of (VII) and cysteine—(a) A mixture of (VII)-HCl (1 mM.), cysteine-HCl (3.74 mM.), $NaHCO_3$ (13.2 mM.), and water (4 cc.) was kept at room temp. (ca. 26°) for 24 hrs. After being extracted continuously with ether for 10 hrs. the Reineckate was precipitated as described in 3) and washed three times with water. 100 mg. of the salt was converted to the corresponding sulfate as mentioned above. Yield, 46 mg. (Reaction Product A). (b) (VII)-HCl (1 mM.) in water (4 cc.) was treated with $NaHCO_3$ (1 mM.), and placed in a thermostat at 37° for 5 hrs. To the reaction mixture was then added cysteine-HCl (3.74 mM.) and $NaHCO_3$ (12.2 mM.). After being kept at room temp. (ca. 22°) for 42 hrs., the reaction product was isolated as a Reineckate. 100 mg. of the salt was converted to the corresponding sulfate. Yield, 40 mg. (Reaction Product B). (c) To ascertain the oxidizing action of (VII) on cysteine, the reaction mixture was acidified and buffered with CH_3COONa. The precipitate was treated with *p*-toluenesulfochloride by the usual means and recrystallized from 25% alcohol, m.p. 204~205°

(decomp.). No depression of melting point was observed with the authentic specimen of tosyl-cystine.

7) Paper chromatograms—Materials used: Sulfate of (VIII), sulfate of (IX), Reaction Products A and B (q.v.). Solvents used: (a) A ternary mixture from the less hydrated phase obtained from butyl alcohol (80 cc.), acetic acid (20 cc.), and water (100 cc.). (b) A mixture from less hydrated phase obtained from butyl alcohol (60 cc.), phenol (30 cc.), and 0.5% ammonia water (24 cc.). The chromatograms were run for 18 hrs. (the former solvent), or 4 hrs. (the latter solvent) at 21~22°. The paper strips were dried and sprayed with 0.2% aqueous ninhydrin solution and heated at 90~95° for 10 mins. After developing, some of the strips were divided longitudinally into two parts and one was sprayed with Dragendorf's reagent and the other, with ninhydrin. Results of the experiment are summarised in Table II.

Summary

Cysteine up-take of methyl-bis(β-chloroethyl)amine N-oxide (HN_2 N-oxide) in neutral aqueous medium was compared with those of tertiary or N-oxide-form nitrogen mustards. The results suggest that HN_2 N-oxide is reduced to HN_2 and partially to chlorhydrin of HN_2 in such a reducing medium, probably even *in vivo*.

(Received September 14, 1953)

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86. Sakahiko Owari: Transformation Reaction of Nitrogen Mustard N-Oxides in Aqeous Solution.*

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It has been reported earlier[1] that methyl-bis(β-chloroethyl)amine N-oxide (HN_2 N-oxide) tends to transform into the ring-oxide, i.e. N,N-methylchloroethyldimethylene-1,2-oximinium chloride in neutral aqueous solution, which changes successively to N,N-methyl-β-chloroethyl-O-chloroethylhydroxylamine when kept for longer periods under the same conditions. Some nitrogen mustard N-oxides, prepared in order to test their retarding effects[2] on Yoshida sarcoma, were examined in this study as to their tendencies to these transformation reactions. To determine the velocity of ring-formation, titration of liberated Cl^- and H^+ of the free base solution of the compounds at 37° was carried out.

The results are summarized in Figs. 1 and 2, the former showing the curves of the monofunctional compounds and the latter, those of the bifunctional ones.

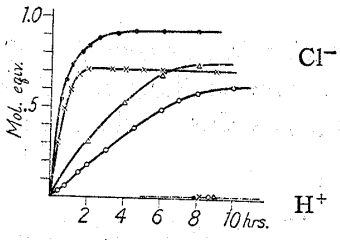

Fig. 1.
- •: Diethyl-β-chloroethylamine N-oxide
- ×: N-β-Chloroethylmorpholine N-oxide
- △: Dibenzyl-β-chloroethylamine N-oxide
- ○: Dimethyl-β-chloroethylamine N-oxide
- ——: Cl^--liberation

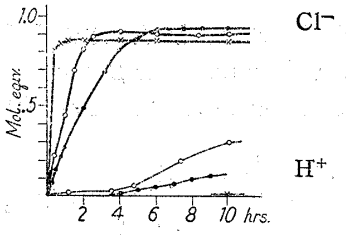

Fig. 2.
- •: N-Methyl-bis(β-chloroethyl)amine N-oxide
- ○: N-Benzyl-bis(β-chloroethyl)amine N-oxide
- ×: N-Isoamyl-bis(β-chloroethyl)amine N-oxide
- —•—•—•—: H^+-liberation

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* M. Ishidate, Y. Sakurai; Studies on Cancerocidal Substances. VIII.

** Nishigahara Kita-ku, Tokyo (尾張栄彦).

1) I. Aiko, S. Owari, M. Torigoe: J. Pharm. Soc. Japan, **72**, 1297(1952).

2) Y. Sakurai, M. Izumi: This Bulletin, **1**, 297(1953).