17. **Michimasa Izumi**: Studies on Cancerocidal Substances. XII.[1] Reaction of N-Bis(β-chloroethyl)amino Acids and their N-Oxides in an Aqueous Solution.

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The intramolecular cyclization of nitrogen mustards in an aqueous solution was first studied by Bergmann,[2] Rydon,[3] and Ross[4] and an ethylene immonium ion thus formed is accepted at present as an intermediate in the alkylating reaction of these compounds. On the chemical reaction of N-oxide derivatives of nitorgen mustards in an aqueous solution, Aiko, Owari, and Torigoe[5] once described their experiments and showed that the chemical behavior of these compounds *in vitro* was different from that of the corresponding tertiary nitorgen mustards, since it tended to form a four-membered cyclic intermediate, 1,2-dimethylene-oxaimonium ion, under the similar conditions.

The author reported on the synthesis and biological action of N-bis(β-chloroethyl)-amino acids[6] and their N-oxides[1] in preceding papers. Among these compounds, N-bis(β-chloroethyl)alanine was found to have a characteristic behavior either in its toxicity on animals or in the tumor spectrum, on which a close investigation has been continued and the report will be published in the near future. In this paper, the reaction in aqueous solution of N-bis(β-chloroethyl)glycine (I) and its N-oxide (IV), N-bis(β-chloroethyl)alanine (II) and its N-oxide (V), and N-bis(β-chloroethyl)taurine (III) and its N-oxide (VI), including methyl-bis(β-chloroethyl)amine (VII) and its N-oxide (VIII) as control substances, is discussed. These compounds have an amphoteric character and are readily soluble in water. The pH values of 0.5% aqueous solution of the hydrochloride of (I), (II), (VII), (IV), (V), and (VIII) were measured to be 2.0, 2.0, 3.4, 2.0, 2.0 and 2.8, respectively, and the solutions of (III) and (VI) showed pH 3.6 and 2.6 in the same concentration.

To study the stability of the cyclic intermediate of these compounds in an incubated acid solution, liberation rates of H^+ and Cl^- were successively determined by titration and the results are shown in Figs. 1 and 2.

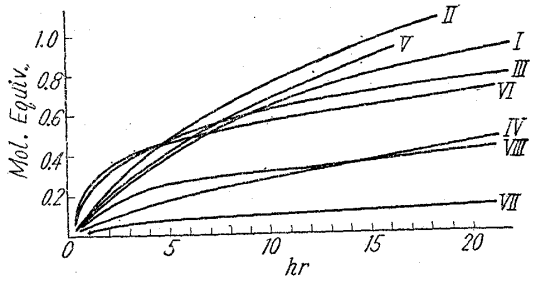

Fig. 1. Cl^- Liberation

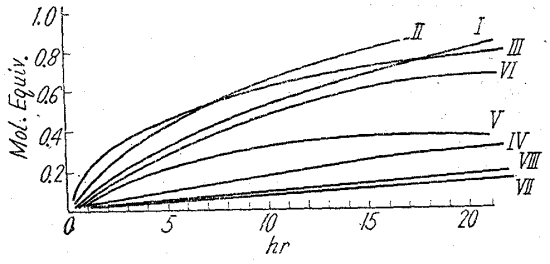

Fig. 2. H^+ Liberation

To make the reaction of the solution closer to the physiological state, the titration of Cl^- was repeated in a bicarbonate-buffered solution using the same samples. However,

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1) Part XI: This Bulletin, **2**, 279(1954).
2) M. Bergmann: J. Org. Chem., **11**, 518(1946).
3) H. N. Rydon: J. Chem. Soc., **1947**, 513.
4) W. C. Ross: *Ibid.*, **1950**, 3056.
5) I. Aiko, S. Owari, M. Torigoe: J. Pharm. Soc. Japan, **72**, 1297(1952).
6) M. Izumi: This Bulletin, **2**, 275(1954).

since direct titration of H^+ in this buffered solution was difficult, a rate of H^+ liberation at pH 8.4 was calculated from the total amount of alkali which was dropwise and continuously added into the solution of the amine to maintain it constantly at pH 8.4 from the beginning to the end, without a buffering substance.

The graphical illustrations of the titrations are shown in Figs. 3, 4, 5, and 6.

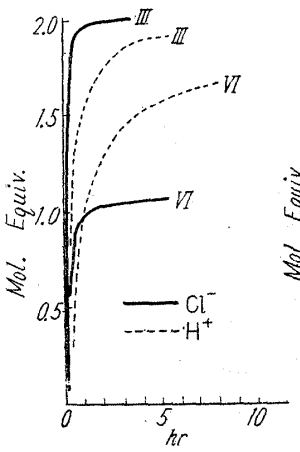


Fig. 3.

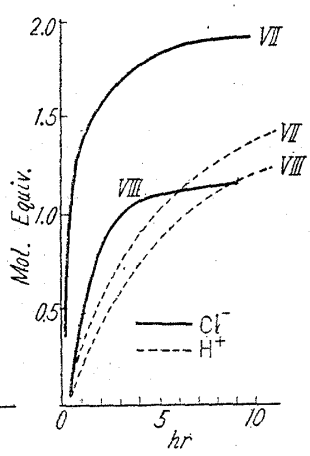


Fig. 4.

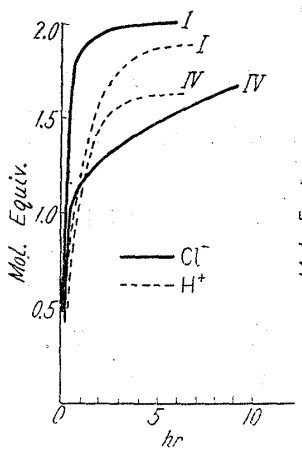


Fig. 5.

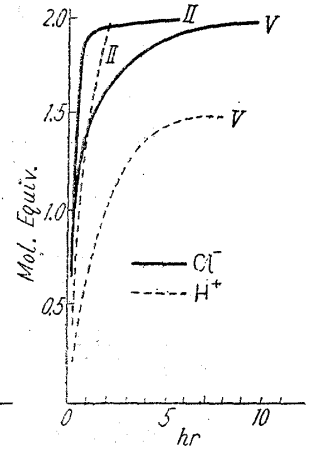
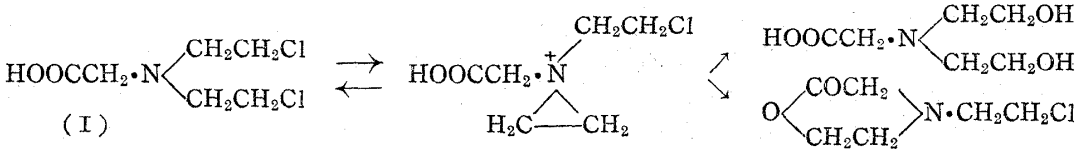


Fig. 6.

It was concluded from these results that N-bis(β-chloroethyl)amino acids also tended to form ethylene-imonium ion in an aqueous solution, because Cl^- liberation always preceded H^+ liberation under these conditions. The precedence, however, was much slighter and the velocity of liberations of both H^+ and Cl^- was more rapid than in the case of (VII). This fact was supposed to demonstrate an easier cyclization of the amines and a relative instability of the cyclic intermediates, which was readily subjected to hydrolysis.

Ross reported that the cyclic intermediate of nitrogen mustard reacts easily with carboxylic anion yielding an ester, and it was also confirmed in this paper that N-bis-(β-chloroethyl)glycine yielded an intramolecular ester, viz., N-β-chloroethylmorpholone, accompanied with N-bis(β-hydroxyethyl)glycine, when dissolved in a bicarbonate buffer and kept at a room temperature.

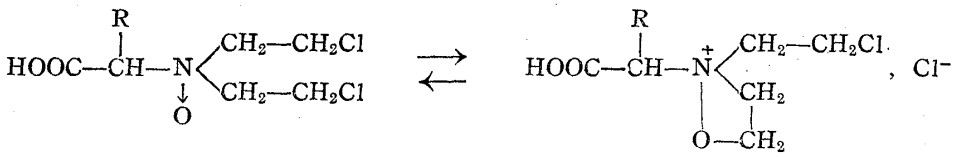


In the case of N-oxide of N-bis(β-chloroethyl)amino acid, a difference between rates of Cl^- and H^+ liberations was also observed and it could be concluded that 1,2-dimethyleneoxaimonium ion (oximonium ion) was formed from this type of N-oxide in a dilute aqueous solution at least at the beginning of the reaction. However, their comparatively prompt H^+ liberation seemed to point out that the intermediate ions of these N-oxides were less stable and more easily subjected to hydrolysis than that of (VIII). The titration values are illustrated in Figs. 1, 2, 3, and 4.

Although it is still unknown at present whether or not a redox potential of the N-oxide has any direct relation to its biological activity, half-wave reduction potentials of (IV) and (V) were determined at pH 3.5 by polarography and their values were found to be more negative than that of (VIII) as shown in Table I.

If the compounds were dissolved in a bicarbonate buffer and kept standing at a room temperature for 30 minutes before their polarograms were taken, one more reduction potential was recorded at about −0.4 v., which could be analogized from the case of

(VIII)[7] to be the corresponding potential to 1,2-dimethyleneoxaimonium ion (oximonium ion) of the N-oxide.



(IV) R=H or (V) R=CH_3-

TABLE I. Half-Wave Reduction Potentials of the N-Oxides (Polarograph)

Compounds	$E_{1/2}$ vs. N. C. E. (V)*	
	Original N-oxide	Ring oxide**
IV	−1.013	−0.421
V	−0.949	−0.443
VIII	−0.78	−0.20

* pH 3.5 : Biphthalate, HCl buffer.
** Cyclic intermediate formed in a neutral aqueous solution (oximonium ion).

Next, a gradual decrease of thiosulfate up-take of these compounds was studied, when they were dissolved in a bicarbonate buffer and incubated at 37° for some period. Beginning from an instance of dissolution, aliquots were pipetted out at a certain interval of time and added with an excess of thiosulfate solution. After ten minutes' incubation at 25°, uncombined thiosulfate was titrated and the so-called ten minute-thiosulfate titre of the compound was obtained, which was accepted at present as corresponding to the biological activity of nitrogen mustards (Fig. 7).

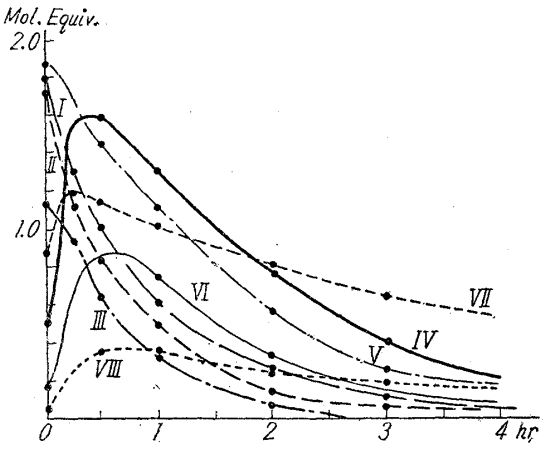


Fig. 7

Regarding the non-N-oxide compounds, it was demonstrated in the graph that the initial up-take of thiosulfate of (I), (II), and (III) surpassed that of (VII), but the declination of curves of (I), (II), and (III) was steeper than that of (VII). A maximum point was observed alone on the curve of (VII) at about the fifteenth minute of incubation, which might correspond to the time necessary to form a cyclic intermediate in the solution. In short, the facts were thought to correspond to the rapid cyclization and hydrolysis of (I), (II), and (III) on the one hand, and to their unequivocal but not lasting biological effect upon the Yoshida sarcoma, on the other.

Since a thiosulfate consumption of the N-oxide of nitrogen mustards was due in part to its oxidation, the curves of (IV), (V), (VI), and (VIII) should not be simply interpreted. The existence of maximum points in these cases, except in (V), was also believed to point out the gradual formation of the four-membered cyclic intermediates in the solutions. The steeper declination of their curves than that of (VIII) coincided with the conclusion derived from H^+- and Cl^--titrations.

The author is indebted to Messrs. I. Aiko, K. Sawatari, Y. Suenobu, and K. Tsubone for their cooperation in this study.

7) I. Aiko : This Bulletin, **1**, 335(1953).
8) M. Torigoe : This Bulletin, **1**, 349(1953).

Experimental

1) **Determination of Cl^- and H^+ Liberation in Acid Solution**—An aqueous solution of the sample (0.02 m*M*/cc.) was incubated at 37° and a portion of the solution was successively taken out at 1-hr. intervals. Cl^- and H^+ liberated were titrated with 0.1 *N* $AgNO_3$ (bromophenol blue) and 0.1 *N* NaOH (phenolphthalein), respectively.

2) **Determination of Cl^- Liberation in $NaHCO_3$ Buffer**—An aqueous solution of the sample (0.02 m*M*/cc.) was first added with 2 mol. equiv. of NaOH, except in the cases of (III) and (VI) in which 1 mol. equiv. of the alkali is enough. The solution was then added with 0.08 m*M*/cc. $NaHCO_3$ and incubated at 37°. Titration of Cl^- was the same as that in an acid solution.

3) **Determination of H^+ Liberation in pH 8.4 Solution**—The test solution (0.02 m*M*/cc.) was added with 3 drops of a mixed indicator (a mixture of 1 part of 0.1% cresol red sodium salt and 3 parts of 0.1% thymol blue sodium salt). The solution was continuously titrated with 0.1 *N* NaOH at short intervals to maintain its color equal to that of a control solution, which was prepared at the same time by adding the same indicator to the Kolthoff's buffer (pH 8.4). Total consumption of NaOH was estimated to be approximately equivalent to H^+ liberation at pH 8.4.

4) **Determination of Thiosulfate Up-take of the Incubated Solution**—The test solution was prepared and incubated as in (2). An aliquot (5 cc.) was taken out at certain intervals and added with 10 cc. of 0.02 *N* $Na_2S_2O_3$. After 10 mins.' incubation at 25°, it was titrated with 0.02 *N* I_2.

5) **Reaction of N-Bis(β-chloroethyl)glycine in $NaHCO_3$ Buffer Solution**—Five g. of (I) was dissolved in 100 cc. sat. $NaHCO_3$ solution and 100 cc. benzene was added over it. It was kept at 37° and shaken vigorously every 20 mins. for 4 hrs. Then the benzene layer was removed and evaporated. The residue was dissolved in ether and an acetone solution of picric acid was added. The picrate melted at 157~158°(from acetone), undepressed by admixture with the authentic sample of N-β-chloroethylmorpholone picrate.[6] The aq. layer, separated from benzene, was acidified to pH 3 with HCl and evaporated *in vacuo* to a small quantity. NaCl was removed by filtration and the same quantity of EtOH was poured into the filtrate. An oily substance separated here was removed and the residual solution was again evaporated to dryness. The residue was then dissolved in a small quantity of 80% EtOH and kept standing in a cool place. Separated crystals were recrystallized from 80% EtOH to m.p. 190°, undepressed by admixture with synthesized[6] N-bis(β-hydroxyethyl)glycine hydrochloride.

Summary

A cyclization of β-chloroethyl group of N-bis(β-chloroethyl)amino acids and their N-oxides in a dilute aqueous solution was discussed in this paper. The intermediates formed in the solution, viz., ethyleneimonium ions from the tertiary amines or 1,2-dimethyleneoxaimonium ions (oximonium ions) from the N-oxides, were found to be less stable and more readily subjected to hydrolysis than that of methyl-bis(β-chloroethyl)amine and its N-oxide.

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