

76. Morizo Ishidate, Yoshio Sakurai, and Kozo Maruyama : Studies on Cancerocidal Substances. XVII.* Reaction of Nitrogen Mustard N-Oxides and Amino Acid.

(Iatrochemical Institute of Pharmacological Research Foundation**)

It is well-known that the characteristic effect of methyl-bis(2-chloroethyl)amine (HN_2) on mitotic cell is due to its alkylating reactivity on nucleophilic centers of cell constituents. It has also been believed that the cross-linking reactivity by two or more functional groups of a molecule is indispensable for an anti-cancer activity of the compounds of this series. Although the N-oxide was found to esterify a carboxyl group only monofunctionally yielding N,N,O-trisubstituted hydroxylamine derivative,¹⁾ it exhibited no alkylation activity on amino or mercapto group as long as its N-oxide structure was maintained. It is therefore our opinion that the N-oxide of this series would exhibit its anti-cancer activity on animals after being previously reduced *in vivo* to the corresponding bifunctional tertiary amine.

In this paper reactions of methyl-bis(β -chloroethyl)amine N-oxide ($\text{HN}_2\text{-O}$) with aniline and L-alanine are discussed. Among the tarry products obtained on standing the two reagents in a hydrogen carbonate solution, HN_2 , 1-methyl-4-phenylpiperazine, 1,4-dimethyl-1,4-bis(2-chloroethyl)piperazinium dichloride, and 2-methyl-2-(2-chloroethyl)-1,2-oxazetidinium chloride were identified as picrate or picrylsulfonate. As expected, a reaction product maintaining the N-oxide structure, such as 1-methyl-4-phenylpiperazine N-oxide, was not obtained and it was confirmed also in this case that deoxygenation of the N-oxide preceded alkylation of the amino group.

When $\text{HN}_2\text{-O}$ was dissolved in a hydrogen carbonate buffer solution and kept at 25°, an oxidation reaction of potassium iodide by this solution weakened gradually and disappeared after a few days. The decrease in the intensity of this reaction occurred most promptly with L-leucine, and isovaleraldehyde was isolated as 2,4-dinitrophenylhydrazone from this solution. The residual solution had an odor of isovaleric acid when it was acidified and the presence of ammonia was detected by Nessler's reagent. The oxidative decomposition of amino acid by $\text{HN}_2\text{-O}$ should not therefore be overlooked when its mode of action *in vivo* is discussed.

In a second experiment, decrease of primary amino and free carboxyl groups of alanine was quantitatively measured when it was incubated at 25° with $\text{HN}_2\text{-O}$, HN_2 , or some other related compounds in hydrogen carbonate-buffered solution.

However, it should be noted that the decrease of primary amino group caused by the N-oxides was due, as mentioned before, to both oxidation and alkylation and, furthermore, a part of the reagent was also consumed by the concurrently occurring esterification of amino acid, either in the case of N-oxides or tertiary amines.

Fig. 1 shows the rate of decrease of primary amino group determined by the micro-Van Slyke method.

2,2-Diethyl-1,2-oxazetidinium chloride (V) (a ring oxide) was expected to have no alkylating activity on amino group because it had no reactive chlorine, but it consumed 0.5 mol. equiv. of amino group within 6 hours, which was regarded as a result of oxidation. Such a ring oxide could be expected to be produced easily from $\text{HN}_2\text{-O}$

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

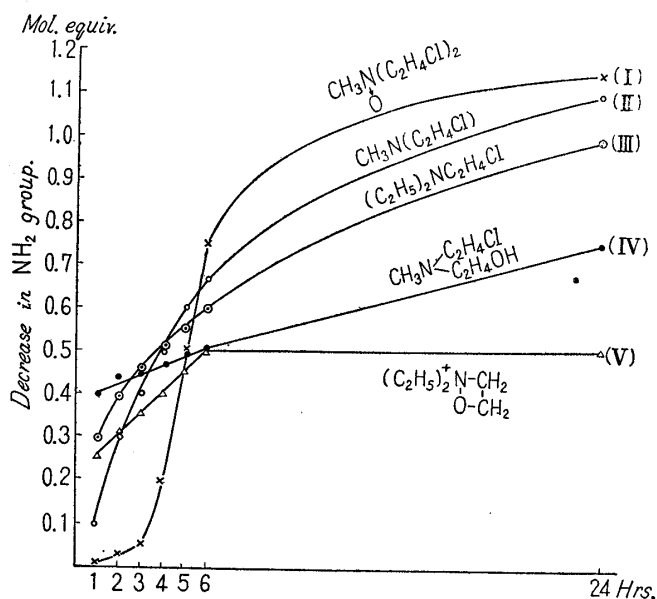


Fig. 1.
Rate of Alkylation of L-Alanine

when it is kept in a neutral aqueous solution and therefore a considerable part of amino-decrease by $\text{HN}_2\text{-O}$ might also be due to oxidative reaction. If its oxidation rate could be presumed to be not so different from that of (V), an approximate rate of alkylation would be about 0.6 mol. equiv. A delay in the ascent of curve (I) at the beginning could be understood as the time necessary to transform $\text{HN}_2\text{-O}$ to the ring oxide which is more oxidative than the original compound.

Bergmann²⁾ reported on the alkylating rate of the non-N-oxide derivatives of nitrogen mustard on the amino group of amino acids, but he did not refer to N-oxide derivatives. According to the result of our experiment, it was found that the reaction between $\text{HN}_2\text{-O}$ and amino acid was very complicated and could not be compared simply with that of the non-N-oxide derivatives, but these reactions were deemed very important in connection with reactions of $\text{HN}_2\text{-O}$ *in vivo*.

Curve (III) ascended straight to the theoretical end-value of amino-decrease as seen in Fig. 1 and this fact shows that this monofunctional compound had little tendency to esterify amino acid under this condition.

A determination of esterification rate of L-alanine by $\text{HN}_2\text{-O}$ or HN_2 was tried by means of a high frequency titration. The values of titration are given in Fig. 2. From these facts, it was approximately calculated that about 0.6 mol. equiv. of $\text{HN}_2\text{-O}$ esterified carboxyl group of amino acid on one hand, while 1.15 mol. equiv. reacted with amino group in a hydrogen carbonate solution as described above. The rest of the reagent spent itself by hydrolysis. In the case of HN_2 , it was found by similar experiment that it combined with 1.1 mol. equiv. of primary amino group and 0.47 mol. equiv. of carboxyl group of the amino acid, while 0.43 mol. equiv. of the reagent was inactivated by a mere hydrolysis in the same solution. In order to determine the esterification rate of $\text{HN}_2\text{-O}$ in non-reductive medium, in which the esterified product would be exclusively of N,N,O-trisubstituted hydroxylamine type, a reaction rate between HN_2 and benzoic acid in a hydrogen carbonate buffer solution was traced and shown in Fig. 3. As the esterification rates, shown in both Figs. 2 and 3, reached an approximately equal value within 5 hours, a chief part of esterification of $\text{HN}_2\text{-O}$ on L-alanine was supposed to be completed before the reduction occurred.

2) M. Bergmann, *et al.*: J. Org. Chem., **11**, 559(1946).

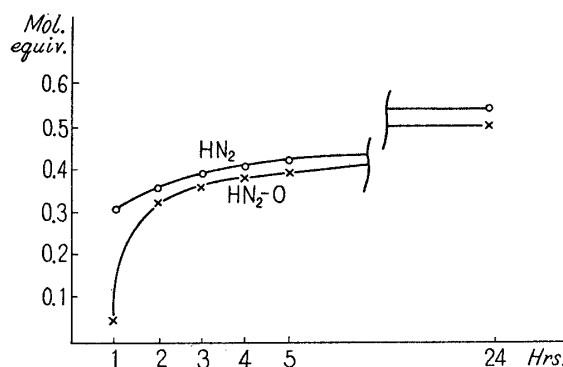
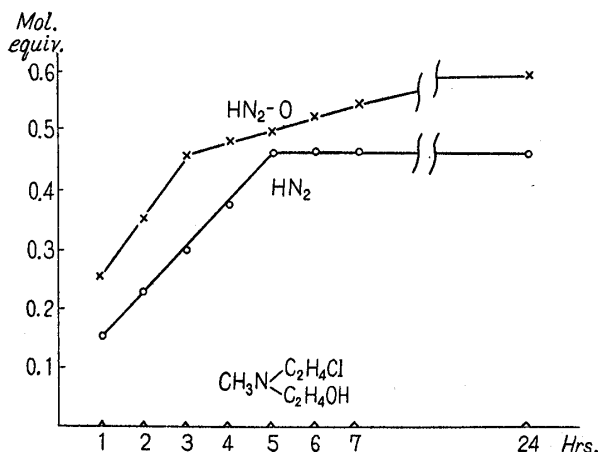
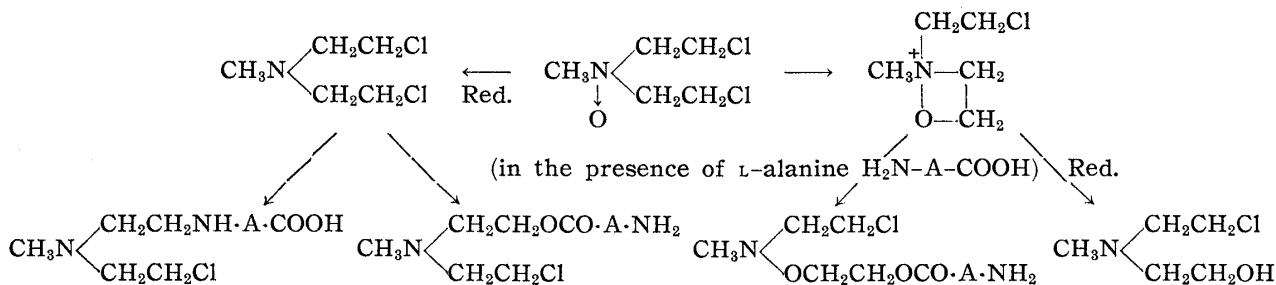


Fig. 2. Rate of Esterification of L-Alanine Fig. 3. Rate of Esterification of Benzoic Acid

The whole reaction is summarized as follows :



An attempt to determine exactly the esterification rate of glycerophosphate under similar condition was not successful by any means and only an approximate rate of decrease of the free acid residue is shown in Fig. 4, in which the rate of esterification included the rate of a concurrent hydrolysis of the reagent as described in the experimental part of this paper.

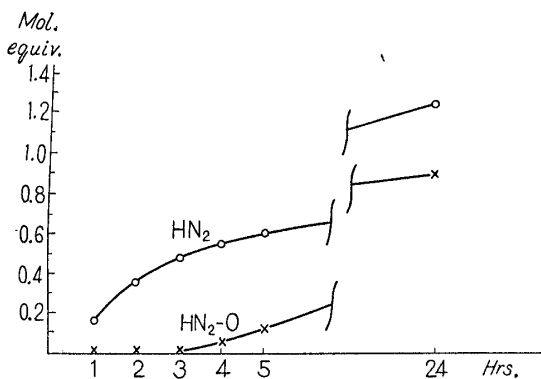


Fig. 4. Rate of Esterification of Glycerophosphate

On comparing the height of the end value at 24th hour in Fig. 4 with the data shown in the afore-cited figures, it did not seem to be exclusively due to a mere hydrolysis and it was supposed that phosphoric acid residue was also esterified by these reagents under such a mild condition.

In conclusion, it is suggested that HN₂-O does not alkylate amino group unless it is preliminarily reduced. Although its biological effect depended on the action of HN₂ which was expected to be formed by reduction of HN₂-O, the preliminary process of reductive activation of this N-oxide *in vivo* might give to this reagent a favorable factor to have a more selective action on tumor than HN₂.

Torigoe reported that HN_2 or $\text{HN}_2\text{-O}$ reacted preferentially with sulfhydryl group of cysteine, if this amino acid existed in excess in a solution. However, it should be somewhat different from an ordinary therapeutic condition. From our results, it is supposed that the reaction of the compound was oriented to the plural reactive centers of the acceptant *in vivo*. This rate of distribution should be different among the reagents according to their chemical constitutions and that might be also a factor which determined the biological effectiveness of the compounds.

It is also an interesting question whether the oxidative reaction of $\text{HN}_2\text{-O}$ on amino acid plays any part in its biological activity and an experiment is now being continued on this subject.

The authors are very grateful to Dr. Y. Kamura for his technical advice on high-frequency titration and also to Mr. D. Ohata for microanalysis.

Experimental

Reaction of $\text{HN}_2\text{-O}$ with Aniline—Aniline (0.04 mole), $\text{HN}_2\text{-O}$ hydrochloride (0.02 mole), and NaHCO_3 (10 g.) were dissolved in water (100 cc.) and the mixture was shaken at 25° for 4 hrs. The ether extract of the mixture was concentrated to a small volume and added with an ethereal solution of picric acid. A lemon-yellow picrate melting at 133° was obtained which showed no depression of m.p. with an authentic sample of $\text{HN}_2\text{-picrate}$. The aqueous mother liquor was acidified to pH 5 and added with a solution of sodium picrylsulfonate.

The first crop of precipitate which fell instantly was orange yellow and recrystallized from warm H_2O , m.p. 199° . It showed no oxidative reaction against KI-starch solution and Beilstein's halogen test was also negative. The analytical data agreed well with those of 1-methyl-4-phenylpiperazine picrylsulfonate. *Anal.* Calcd. for $\text{C}_{17}\text{H}_{19}\text{O}_9\text{N}_5\text{S}$: C, 43.49; H, 4.05; N, 14.92 Found: C, 43.18; H, 4.29; N, 14.65.

The second crop of picrylsulfonate precipitated gradually. It was also a lemon-yellow crystals after recrystallization from H_2O , m.p. 174° . It showed a strong oxidative reaction against KI-starch solution and also a strong positive reaction for halogen. It showed no m.p. depression when mixed with the authentic picrylsulfonate of the ring oxide of $\text{HN}_2\text{-O}$. *Anal.* Calcd. for $\text{C}_{11}\text{H}_{13}\text{O}_{10}\text{N}_4\text{ClS}$: C, 30.80; H, 3.03; N, 13.06. Found: C, 31.08; H, 3.01; N, 13.33.

Even when aniline was treated with $\text{HN}_2\text{-O}$ for 24 hrs. instead of 4 hrs., nothing but the aforementioned products was obtained.

Reaction between $\text{HN}_2\text{-O}$ and L-Leucine—L-Leucine (4.8 millimole) and $\text{HN}_2\text{-O}$ hydrochloride (2.4 millimole) were dissolved in a satd. NaHCO_3 solution (45 cc.) and incubated at 25° for 24 hrs. A portion of the solution was then made strongly alkaline, it had an odor of NH_3 which was also confirmed by Nessler's reagent. The rest was acidified with dil. HCl and distilled. The distillate had an odor of isovaleric acid and the neutralized distillate was redistilled. Isovaleryl aldehyde was separated from this distillate as its 2,4-dinitrophenylhydrazone, m.p. 123° , which was identified by admixture with the authentic sample.

Determination of Amino Decrease of L-Alanine by $\text{HN}_2\text{-O}$ or Related Compounds—Hydrochloride of $\text{HN}_2\text{-O}$ or other related reagents (1.6 millimole), L-alanine (6.4 millimole), and NaOH (1.6 millimole) were dissolved in a satd. NaHCO_3 solution (12 cc.) and the mixture (pH 8.4) was kept at 25° . An aliquot (usually 1 cc.) of the solution was then taken out at definite time intervals and quantity of the primary amino group was determined by micro-Van Slyke method. A difference with a blank test was defined as the $\text{HN}_2\text{-decrease}$.

Determination of Esterification Rate by High-Frequency Titration—Hydrochloride of the amine (0.8 millimole), L-alanine (3.2 millimole), and NaHCO_3 (4.8 millimole) were dissolved in H_2O (6 cc.) and the mixture was incubated at 25° . A portion (0.1 cc.) of the solution was taken out at certain time intervals and diluted to 200 cc. with distilled water. A diluent was added with 0.1N NaOH (0.5 cc.) and sodium salt of alanine was titrated with 0.01N HCl in a high-frequency titration apparatus.* When a similar titration was carried out without the addition of alanine, only a slant line corresponding to NaHCO_3 alone appeared.

Reaction of $\text{HN}_2\text{-O}$ and HN_2 with Benzoic Acid—Hydrochloride of the amine (2 millimole) and sodium benzoate (8 millimole) were dissolved in a saturated solution of NaHCO_3 (15 cc.) and incubated at 25° . One cc. of the solution was taken out successively at intervals and added with 1 cc. of 5% HCl. Benzoic acid, which was extracted with ether, was dissolved in EtOH and titrated with 0.1N

* The analysis was carried out after the procedure reported by Y. Kamura (Kagaku no Ryoiki, 9, (6), 36(1955)) using the apparatus made by Towa Dempa K.K., Tokyo (IK-1 Type).

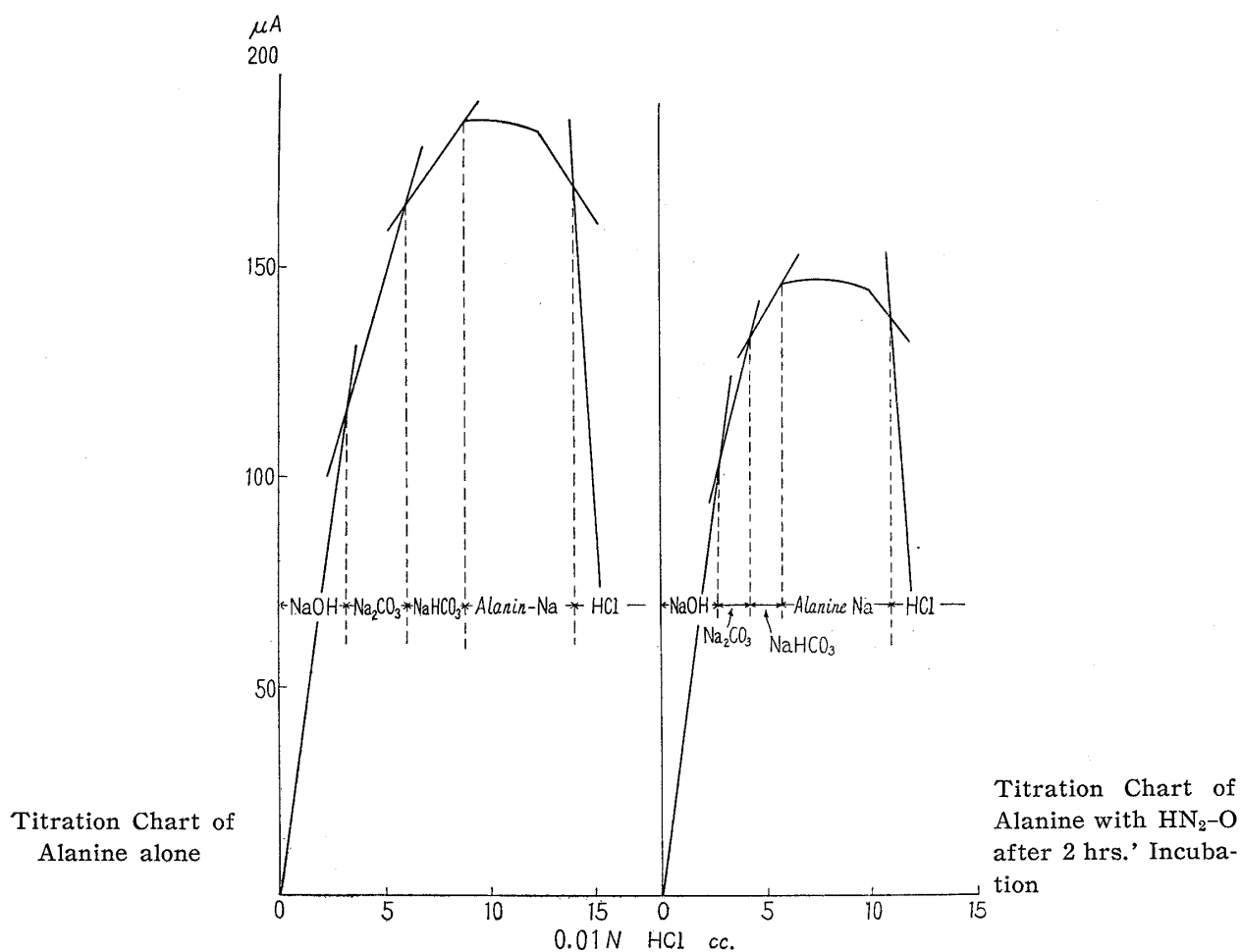


Fig. 5.

NaOH (phenolphthalein).

Reaction of $\text{HN}_2\text{-O}$ and HN_2 with Glycerophosphoric Acid—Hydrochloride of the amine (0.25 millimole), NaOH (0.25 millimole), and sodium glycerophosphate (1.0 millimole) were dissolved in H_2O (25 cc.) and incubated at 25° . One cc. of the solution was taken out successively at intervals and the sodium salt was titrated with 0.01N HCl (methyl orange). Decrease in consumption of 0.01N HCl corresponded to the total rate of esterification and hydrolysis of the amine. Reaction of the solution was pH 7.1 at the beginning and changed to pH 6.8 after 24 hrs.

Summary

Rates of alkylation, oxidation, and esterification of N-methyl-bis(2-chloroethyl)amine N-oxide ($\text{HN}_2\text{-O}$) on amino and carboxyl groups of L-alanine in a hydrogen carbonate buffer solution were discussed. The data were compared with those of the related compounds and the results suggested that the fate and reaction of $\text{HN}_2\text{-O}$ *in vivo* might be somewhat different from those of HN_2 .

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