The halogenated alkylamines, so called nitrogen mustards (NM), investigated during the last World War as a potential warfare agent, have become useful tools in biology and medicine for their ability to increase the spontaneous mutation rate in plants and animals (1) and to palliate leukemia, lymphoma and lung carcinoma (2).

Recently, Ishidate et al. (3) have paid attention to the lower toxicity of nitrogen mustard-N-oxides (NMO) which had been prepared by Stahmann (4) in 1946 comparing to their original amines, and demonstrated that methyl-bis-\(\beta\)-chloroethylamine-N-oxide (MBAO, Nitromin) had an equal inhibitory potency to methyl-bis-\(\beta\)-chloroethylamine (MBA) on Yoshida-sarcoma.

Meanwhile, on the pharmacology of NM, many studies had been done, as seen in the review of Phillips (5), but no pharmacological research has yet been made about NMO.

The purpose of the present paper is to determine the pharmacological properties of NMO, especially of Nitromin, and of the correlation with their original amines.

**MATERIALS AND METHODS**

**Samples**

**Nitrogen mustards (NM):**
- Diethyl-\(\beta\)-chloroethylamine HCl (DBA)
- Methyl-bis-\(\beta\)-chloroethylamine HCl (MBA) [Takeda]
- Tris-\(\beta\)-chloroethylamine HCl (TBA) [Nihon Yakukagaku]

**Nitrogen mustard-N-oxides (NMO):**
- Dimethyl-\(\beta\)-chloroethylamine-N-oxide HCl (DMBAO)

1) This study was supported in part by a grant in aid for cancer research from Nara Prefecture (1952-1953).
2) We wish to express our thanks to Dr. Yoshio Sakurai (Instrochemical Institute of Pharmacological Research Foundation, Tokyo) for his valuable criticism and his generous supply of the samples of DBA, DMBAO and DBAO.
3) MBAO (Nitromin) was prepared and supplied by Yoshitomi Pharmaceutical Industries in Osaka, Japan.
Diethyl-β-chloroethylamine-N-oxide HCl (DBAO)
Methyl-bis-β-chloroethylamine-N-oxide HCl (MBAO, Nitromin)
Acetylcholine chloride [Roche]
Atropine sulfate [Merck]

Mice in the studies of lethal dose weighed 10-30 g and were equally divided by sex. Sterile physiological saline solutions were used as the solvent, and given subcutaneously in a volume of 0.5 ml/30 g. The values of LD₆₀ were calculated by van der Waerden's method (6).

In rabbit's blood pressure experiments, anesthesia was induced with subcutaneous urethane solution (0.8-1.2 g/kg), mercury manometer was connected to a cannula in a carotid artery.

Sino-auricular preparation was made according to Miyake's revised method (7) with male toad of 100 g body weight, being perfused with cold-blooded animal's Ringer solution. Solutions of samples were administered in a volume of 0.1 ml, and automatic movement of auricle was recorded on kymograph.

Movements of excised intestine of rabbit and guinea pig were recorded by Magnus's method.

Paperchromatography was carried out by an ascending method (4 hours) with Toyo Roshi No. 50 filter paper, and the solvent was the mixture of n-butanol, ethanol and water (8:1:1). The location of the spots was determined with Dragendorf's reagent or potassium iodide-starch solution.

RESULTS

A. Lethal dose of MBA and MBAO

TABLE 1. Death rate of mice during 15 days after subcutaneous administration of MBA

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<th>Days after administration</th>
<th>Dose (mg/kg)</th>
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<th>1.5</th>
<th>2.0</th>
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<th>3.0</th>
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<td>5</td>
<td>7</td>
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Each group: 10 mice
TABLE 2. Death rate of mice during 15 days after subcutaneous administration of MBAO

<table>
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<tr>
<th>Days after administration</th>
<th>Dose (mg/kg)</th>
<th>40</th>
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<th>100</th>
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Each group: 8 mice

The results are given in Table 1 and 2, where the death rate of mice is shown. From these results, we have thought it was preferable that the determination of lethal dose of NM and NMO should be done under the observation over 10 days; and the 10 days' LD₅₀ (by van der Waerden’s method) was:

MBA: 1.7±0.2 mg/kg, MBAO: 101.0±9.3 mg/kg.

B. Biological action of NM and NMO

1) In anesthetized rabbits of 2.0-2.5 kg body weight, no considerable alterations followed the intravenous injection of MBA and TBA of the amount from 1.0 to 2.0 mg/kg. In the case of MBAO the amount of injection was increased to 2.0-20.0 mg/kg, and yet no considerable effect was shown.

2) Both NM and NMO reduced the amplitude of contraction of the Bufo’s sino-auricular preparation, and the potency of this action was almost the same in each toad. Fig. 1 shows the action-concentrations curve of MBA and MBAO obtained from this experiment.

3) To the movement of the excised intestine of rabbit and guinea pig in Tyrode’s solution, NM and NMO showed contractive effect. This action was seen from 0.2 mg of NM and 2.0 mg of NMO (Fig. 2-4), and the concentrations rate to induce an equivalent contraction was NM:NMO = (nearly equal) 1:10.

C. Changes of pharmacological activity of NM and NMO, and the correlation with their chemical changes in neutral aqueous medium

1) Competition with atropine
Fig. 1. Action-concentrations curve of MBA and MBAO (Sino-auricular preparation)

Fig. 2. Effect of NM and NMO on the movement of the excised intestine of rabbit, and the competition with atropine.
1: MBAO 0.5mg, 2: MBAO 1.0mg, 3: MBA 0.04mg,
4: MBA 0.05mg, 5: MBAO 0.4mg, 6: Atropine sulfate 0.02mg.

Fig. 3. Changes of the cholinergic activity of MBAO and DMBAO on the excised intestine of rabbit by the incubation at 37°C, pH 7.0.
A: MBAO 3.3mg, B: DMBAO 3.3mg, A, B: π shows incubation time (min).
The contractive effect of NM and NMO on the excised intestine of rabbit was competed by the previous dealing of 0.02mg of atropine sulfate (Fig. 2).

2) Chemical interaction with several amino acids

Weisberger et al. (8) showed that the administration of L-cysteine HCl to animals prior to the injection of MBA modified the leukopenia induced by MBA. Iwata et al. (9) recognized also the same effect in MBAO. Bergmann (10) showed that NM had been liable to combine with the imidazole ring of histidine.

![Fig. 4. Changes of the cholinergic activity of MBAO on the excised intestine of rabbit by the incubation at 37°C, pH 7.0, and changes of the location of the spot on the paper chromatogram (4 hours) by the incubation in the same condition. Schemata of the paper chromatogram show the intensity of the colour by an indicator, potassium iodide-starch solution. A: MBAO ×100, B: MBAO ×60, (): incubated for 1 hour.](image-url)
With these amino acids (i.e., 0.5-0.7 mg/ml of L-cysteine or L-histidine HCl), and with 0.5 mg/ml of DL-methionine, incubated NM and NMO for 1-2 hours at 37°C, pH 7.0; then the response of 1.0 mg of NM and 10.0 mg of NMO to the excised intestine of guinea pig has disappeared. In the case of L-histidine, the proper relaxing action of this amino acid on the excised intestine has vanished simultaneously.

3) The correlation between pharmacological activity and chemical change in neutral aqueous medium

The cholinergic activity of 0.2 mg of MBAO on the sino-auricular preparation was changed by the incubation at 37°C, pH 7.0: namely its activity kept on increasing for 1 hour. We demonstrated this fact on the excised intestine of rabbit, too, and in the same condition, other NM and NMO changed their cholinergic activities just as MBAO did (Fig. 2-4).

About MBAO, we carried out paper chromatography with butanol-ethanol-water (8:1:1), Rf value of MBAO was 0.7, but the new spot indicating Rf 0.2 appeared after its solution has been kept for 24 hours (pH 7.0, room temperature 14°-16°C), and the spot indicating Rf 0.2 became more vivid by potassium iodide-starch solution as the time elapses. And the same phenomenon was shown by the incubation of MBAO for 1-2 hours at 37°C, pH 7.0.

According to Aiko et al. (11), the spot located at Rf 0.2 is N-β-chloroethyl-dimethylene-1, 2-oxaaminé N-methochloride (I) which is derived from MBAO by the cyclization in alkaline aqueous medium (Fig. 5).

Fig. 4 shows the relation between the cholinergic activity by Magnus's method and the appearance of I (Fig. 5) by paper chromatography (i.e., in the condition 37°C, pH 7.0), using the same samples.

DISCUSSION

In the determination of the lethal dose of MBA and MBAO, we recognized that so-called “delayed lethal syndrome” disappeared on the 11th day after the adminis-
tration of samples. The results of the present study indicate that it is preferable that the determination of lethal dose of polyfunctional NM and NMO should be done under the observation over 10 days.

As clarified by Bergmann (12) and Swain (13), in a neutral or alkaline aqueous solution, MBA underwent intramolecular transformation to form a highly reactive ethylene imonium ion (II) (Fig. 5). According to Hunt and Philips (14) the cholinergic activity of MBA is attributed to its transformation in vivo into II. As regards MBAO, Aiko et al. (11) demonstrated that, by paperchromatography with butanol-ethanol-water (8:1:1), the spot of MBAO was located at Rf 0.7 and it changed to Rf 0.2 after its solution had been kept for 7 hours (pH 8.0), and that the substance having Rf 0.2 was N-β-chloroethyl-dimethylene-1,2-oxaimine N-methochloride (I) (Fig. 5).

There is a remarkable agreement between the changes of cholinergic activity of NM and NMO which the results obtained here shows, and the chemical changes of these samples. Hence it may be concluded that the cholinergic activity of NM and NMO is due to the cyclic quaternary amines which have been derived from these compounds by the intramolecular cyclization.

In the analysis of the concentrations rate of NM and NMO to induce the contraction in the same degree upon the excised intestine of rabbit and guinea pig, the results seem to be essentially similar to those described by Yoshida (15) for the rate of minimal effective dose of MBA and MBAO against Yoshida-sarcoma. Seeing this phenomenon, both cytotoxic and cholinergic activities seem as if they could be due to the changes of NM and NMO in an aqueous solution. However, we recognized that the monofunctional NMO have shown the cholinergic activity in the same degree as polyfunctional NMO (Fig. 3). On the other hand, Ishidate et al. (16) have explained that MBAO should turn into MBA in vivo by reduction (Fig. 5), because the reaction of I is always monofunctional, and there is no cancerostatic activity in monofunctional NM; consequently, it is less possible for MBAO, which has the cancerostatic activity, to transform I in vivo as in neutral aqueous solution. Hence, the relationship between the cancerostatic and cholinergic action can not be immediately concluded as NMO concerned.

**SUMMARY**

1. It is preferable that the determination of lethal dose of NM and NMO should be done under the observation over 10 days; and the 10 days' LD_{50} (administered subcutaneously in mouse) was — 
   
   MBA: 1.7±0.2 mg/kg, MBAO: 101.0±9.3 mg/kg.
2. Upon the blood pressure of rabbit, NM and NMO show no considerable effect.
3. NM and NMO reduce the amplitude of contraction of the Bufo's sino-auricular preparation.
4. NM and NMO show a contractive effect on the excised intestine of rabbit and guinea pig; and this effect is competed by the previous dealing of atropine. By the
incubation with L-cysteine, L-histidine and DL-methionine for 1-2 hours at 37°C, pH 7.0, this effect disappear.

5. The cholinergic activity of NM and NMO is changed by the incubation at 37°C, pH 7.0, namely its activity kept on increasing for 1 hour.

6. Correlations exist between the cholinergic action of MBAO and N-β-chloroethyl-dimethylene-1, 2-oxaimine N-methochloride, which is derived from MBAO by the cyclization in neutral or alkaline aqueous medium.

REFERENCES

2) NABARRO, J.D.N.: J. Pharm. Pharmacol. 2, 865 (1950)
6) VAN DER WAERDEN, BL.: Arch. exp. Pathol. Pharmacol. 195, 339 (1940)