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78. Hiroshi Imamura: Studies on Carcinostatic Substances. XXVIII.*1 Activation of the Derivatives of 2-Chloroethylamine with Latent Activity.

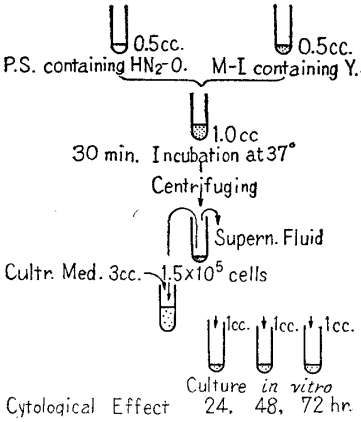
(*Iatrochemical Institute of Pharmacological Research Foundation**2)

The preceding report[1] alluded to the activation of N-methyl-bis(2-chloroethyl)amine N-oxide (HN_2-O) by the *in vitro*-cultured Yoshida sarcoma cells. Further details of the activation of this and related compounds are presented in this paper.

Experimental and Results

The test compound was serially diluted with physiological saline solution and 0.5 cc. each of the diluted solution was mixed with 0.5 cc. of the Yoshida sarcoma-cell suspension in the Medium-I (M-I) at a certain cell population (cell no./cc.) and then incubated at 37° for 30 min. The cells were centrifuged at 2,000 r.p.m., washed with saline solution, and about 1.5×10^5 cells from its residue were transferred into 3 cc. of the culture medium. It was divided equally into 3 portions and each was incubated at 37° for 72 hr. The same procedures were repeated with all the diluted solutions.

The morphological observation was carried out 24, 48, and 72 hr. after incubation. The cell population during the contact period varied from 2×10^5 to 2×10^8 cells/cc. and concentration of the compound from 1 to 10^{-4} m.*M.* An example of the procedure and results obtained with HN_2-O are demonstrated in Fig. 1.



HN_2-O (m.M. final) \ Cells/cc. (final)	Yoshida Sarcoma Cells 2×10^5	1×10^6	5×10^6	1×10^7	1×10^8
1	+				
5×10^{-1}	+				
2.5×10^{-1}	+				
1×10^{-1}	−	+	+	+	+
5×10^{-2}	−	+	+	+	+
2.5×10^{-2}	−	+	+	+	+
1×10^{-2}	−	−	+	+	+
5×10^{-3}	−	−	+	+	+
2.5×10^{-3}	−	−	+	+	+
1×10^{-3}	−	−	−	+	+
5×10^{-4}	−	−	−	−	+
2.5×10^{-4}	−	−	−	−	−
1×10^{-4}	−	−	−	−	−

Fig. 1. Shifting of MEC of HN_2-O with Increase of Tumor Cell Population

Y. S. : Yoshida sarcoma

P. S. : Physiological saline solution (0.85% NaCl)

M-I : Horse serum (inactivated) 96%
Physiological saline soln. 4%
containing
K-Penicillin G 200 units/cc.
Dihydrostreptomycin sulfate 200 γ/cc.

Culture medium
Horse serum (inactivated) 48%
Earle's solution 50%
Physiological saline soln. 2%
containing
K-Penicillin G 100 units/cc.
Dihydrostreptomycin sulfate 100 γ/cc.

The minimum effective concentration (MEC) was greatly lowered as the cell population increased, as demonstrated in Fig. 2.

It has been well-known that HN_2-O is easily reduced to N-methyl-bis(2-chloroethyl)amine (HN_2) by chemical reducing agents under mild conditions,[2] but the above fact seemed to indicate that the reduction took place in this contact solution chiefly by the organism, because the biological

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*1 This paper constitutes a part of series entitled "Studies on Carcinostatic Substances" by M. Ishidate and Y. Sakurai.

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1) Part XXVII. M. Ishidate, *et al.* : This Bulletin, 8, 444(1960).

2) M. Torigoe : *Ibid.*, **1**, 349(1953).

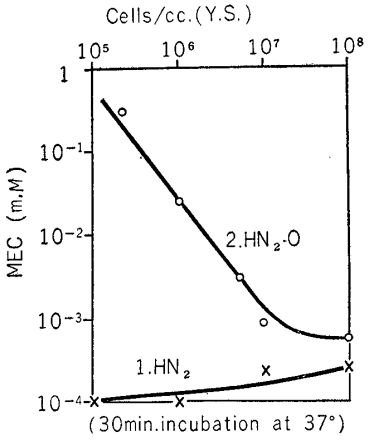


Fig. 2.
Shifting of MEC with Increase of Tumor Cell Number

activity of HN_2-O, due to its reduction product, appeared to be dependent on the cell population. On the contrary, it was confirmed that activity of the essentially active substance like HN_2 or No. 533 could not be changed greatly by altering the cell population, as seen in Table II.

The supernatants of the contact solutions in the above experiment were determined qualitatively and quantitatively after incubation with the Yoshida sarcoma cells (10^8 cells/cc.) to see if they actually contained the reduced product of HN_2-O, viz. HN_2.

In fact, HN_2 was clearly detected by paper chromatography (solvent, BuOH:EtOH:H_2O = 8:1:1, coloring agent, Dragendorff, Rf 0.38). For the quantitative determination of the same lot of solution, the supernatant was serially diluted and incubated with a small number of Yoshida sarcoma cells (7.5×10^4 cells/cc.), with which population it had been confirmed that the effect of HN_2-O remained unchanged and never appeared. From the maximum dilution rate, at which the slightest observable morphological aberration of the cell could be checked, the concentration of HN_2 in the initial supernatant could be calculated, because MEC of HN_2 against the Yoshida sarcoma cells *in vitro* had been precisely determined to be 2.5×10^{-4} m.*M*.[3]

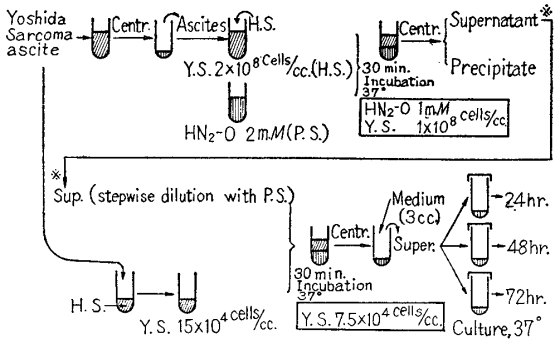


Fig. 3.

It was also proved that the supernatant solution of HN_2-O, obtained after contact with tumor cells other than the Yoshida sarcoma, for instance, a rat ascites hepatoma AH 7974 (a hepatoma resistant against HN_2-O or HN_2) or two kinds of HN_2-O-resistant strains of Yoshida sarcoma (RA and RC), also contained HN_2 and exhibited the effect against the *in vitro*-cultured Yoshida sarcoma at a small population (7.5×10^4 cells/cc.), as shown in Table I.

As shown in Table I, the rate of reduction, viz. reducing efficiency, increased as the concentration of the test compound was reduced and it is very interesting that, if the population of the cells and concentration of the reagent were fixed at an arbitrary value, the concentration of the reduced product in the supernatant was found to be similar in all of the above-stated four kinds of tumors. It could therefore be concluded that resistance of the tumors to anti-tumor action of the N-oxide was independent of their reducing activity against the N-oxide.

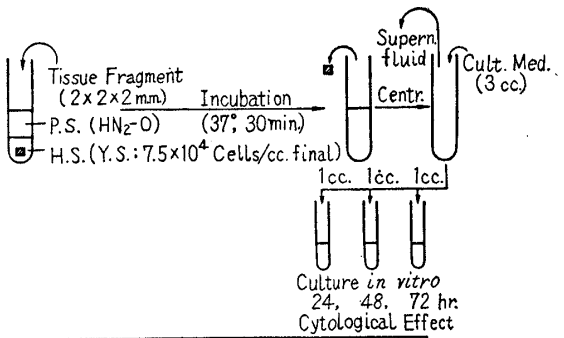
Furthermore, this biological reducing activity was found not exclusively in the tumors but in the various benign tissues such as heart muscle, spleen, kidney and liver, the experimental data of which are shown in Fig. 4.

3) M. Ishidate, *et al.* : *Ibid.*, **7**, 873(1959).

Table I. Concentration of HN_2 in Medium liberated from HN_2-O by Tumor Cells

Tumor (MEC of HN_2, m.M)	Contact condition: Cells/cc.	Contact condition: HN_2-O (m.M)	Contact condition: Incubation time at 37° (min.)	Result: MEC (dilution ratio)	Result: Concn. of HN_2 (m.M, equiv.)	Result: Rate of reduction (HN_2/HN_2-O($\times 100$))
Yoshida Sarcoma (2.5×10^{-4})	10^8	1	30	100	2.5×10^{-2}	2.5
		10^{-1}		40	1×10^{-2}	10
		10^{-2}		10	2.5×10^{-3}	25
	10^6	1		10	2.5×10^{-3}	0.25
		10^{-1}		(−)		
	10^8	1	15	100	2.5×10^{-2}	2.5
			30	100	2.5×10^{-2}	2.5
			60	100	2.5×10^{-2}	2.5
			120	100	2.5×10^{-2}	2.5
			180	100	2.5×10^{-2}	2.5
AH 7974 (2.5×10^{-3})	10^8	10^{-2}	30	10	2.5×10^{-3}	25
Y. S. (R. A.) (1×10^{-2})		10^{-1}		40	1×10^{-2}	10
Y. S. (R. C.) (2.5×10^{-3})				40	1×10^{-2}	10

AH 7974 : HN_2-resistant strain of rat ascites hepatoma.
R. A., R. C. : HN_2-resistant forms of Yoshida sarcoma induced by prolonged contact with HN_2.



HN_2-O (m.M) \ Fragment	Normal Rat Tissue: Liver	Kidney	Spleen	Heart	Control
1×10^{-1}	+	+	+	+	−
5×10^{-2}	+	+	+	+	−
2.5×10^{-2}	+	+	+	+	−
1×10^{-2}	−	−	−	−	−
5×10^{-3}	−	−	−	−	−
2.5×10^{-3}	−	−	−	−	−
1×10^{-3}	−	−	−	−	−
5×10^{-4}	−	−	−	−	−
2.5×10^{-4}	−	−	−	−	−
1×10^{-4}	−	−	−	−	−

Fig. 4.
Activation of HN_2-O by Normal Rat Tissues

In the present work, some N-oxide derivatives other than HN_2-O were also tested as to the mode of their activation by using a similar procedure, results of which are listed in Table II.

Discussion

Out of the N-oxides here presented, Nos. 534, 183, and 366 showed a similar mode of activation curve as HN_2-O, but Nos. 534 and 206 behaved differently. In these cases, MEC began to decrease only after the cell population was increased over 10^7 cells/cc.

Generally, the fate of N-oxides in the contact solution was assumed to be reduction to a tertiary amine by the organism as one possible reaction, and the other, the automatic transformation reaction, which converts the reagent to the essentially inactive intermediates as shown in the following formula :

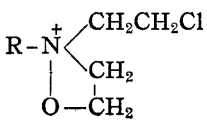


Table II-a.

Compound No.	Formula	MEC (m.M)[a]	$E_{1/2}$[c] vs. S. C. E. (pH 3.53)
24	$CH_3-N(CH_2CH_2Cl)_2$	2.5×10^{-4}	
533	$(CH_3)_2CH-N(CH_2CH_2Cl)_2$	1×10^{-3}	
27	$CH_3-N(CH_2CH_2Cl)_2 \rightarrow O$	—[b]	−0.74
464	$CH_3-O-(CH_2)_2-N(CH_2CH_2Cl)_2 \rightarrow O$	—	
183	$C_6H_5-CH_2-N(CH_2CH_2Cl)_2 \rightarrow O$	—	-0.59_4
336	$CH_3-N(CH_2CHClCH_3)_2 \rightarrow O$	—	-0.64_0
534	$(CH_3)_2CH-N(CH_2CH_2Cl)_2 \rightarrow O$	—	−0.62
206	cyclo. $C_6H_{11}-N(CH_2CH_2Cl)_2 \rightarrow O$	—	−0.48
524	$(CH_2)_3-\overset{+}{N}(CH_2CH_2Cl)_2$, O^-	1×10^{-2}	-0.48_8
587	$ClCH-CH_2-\overset{+}{N}(CH_2CH_2Cl)-CH_2-CH_2-O-CH_3$	1×10^{-3}	-0.46_0
558	$C_2H_5O-\overset{+}{N}(CH_2CH_2Cl)_2$, CH_3	2.5×10^{-3}	-0.63_0
481	$(CH_2)_3$, $O=P-N(CH_2CH_2Cl)_2$, NH	—	

a) MEC by the routine screening procedure (contact time, 48 hr.).
b) — No effect.
c) Quoted from the experimental data by I. Aiko (This Bulletin., **1**, 355(1953)) and M. Torigoe (unpublished).

Table II-b.

Compound No.	Tumor[a]	Contact time (min.)	MEC (m.M) 1×10^5 cells/cc.	1×10^6 cells/cc.	1×10^7 cells/cc.	1×10^8 cells/cc.
24	YS	30	1×10^{-4}	1×10^{-4}	2.5×10^{-4}	2.5×10^{-4}
533	〃	30	2.5×10^{-3}	2.5×10^{-3}	2.5×10^{-3}	2.5×10^{-3}
27	AH 7974	30	5×10^{-1}	1×10^{-1}	1×10^{-2}	1×10^{-2}
	RA	30	—	—	—	5×10^{-2}
	RC	30	—	—	2.5×10^{-1}	1×10^{-2}
464	YS	30	5×10^{-1}	1×10^{-1}	1×10^{-2}	2.5×10^{-3}
183	〃	120	5×10^{-1}	5×10^{-2}	2.5×10^{-3}	1×10^{-3}
336	〃	30	—	1	2.5×10^{-2}	1×10^{-2}
534	〃	30	1×10^{-1}	2.5×10^{-1}	2.5×10^{-1}	1×10^{-1}
	〃	120	1×10^{-1}	2.5×10^{-1}	1×10^{-1}	2.5×10^{-2}
206	〃	30	2.5×10^{-1}	2.5×10^{-1}	2.5×10^{-1}	2.5×10^{-2}
	〃	120	2.5×10^{-1}	2.5×10^{-1}	1×10^{-1}	1×10^{-2}
524	〃	120	1	5×10^{-2}	2.5×10^{-3}	1×10^{-3}
587	〃	120	2.5×10^{-2}	5×10^{-3}	1×10^{-3}	5×10^{-4}
558	〃	60	1×10^{-2}	1×10^{-2}	2.5×10^{-2}	5×10^{-2}
481	〃	120	—	—	—	—

a) YS : Yoshida sarcoma.
AH 7974 : Ascites hepatoma of rat.
RA, RC : Resistant strains of Yoshida sarcoma.

Both reactions were always competing with each other but, if the automatic transformation (inactivation) was more rapid than the biological reduction, it might be that the concentration of the reduced tertiary amine could hardly be accumulated over a certain value during the period of contact with the cells of comparatively small population. In such a situation, it is easily anticipated that the activation curve would appear as seen in Table II. In fact, the analytical data of both compounds affirmed the extremely rapid Cl^- liberation, due to the above-mentioned transformation, in a neutral aqueous solution, the titration data of which are shown in Table III.

Table III. Rate of Cl^- Liberation in $NaHCO_3$ Buffer Solution

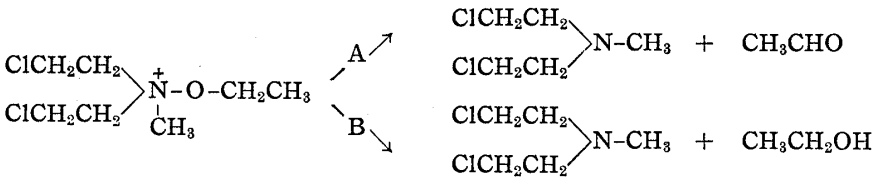
Incubation time (min. at 37°)	10	30	60	120
		Liberated Cl^- (mol. equiv.)		
CH_3-N(CH_2CH_2Cl)$_2$ ↓ O	0.24	0.27	0.49	0.79
$(CH_3)_2$-CH-N(CH_2CH_2Cl)$_2$ ↓ O	0.73	1.05	1.08	1.08
cycl. C_6H_{11}-N(CH_2CH_2Cl)$_2$ ↓ O	0.79	1.04	1.08	1.08

2,2-Bis(2-chloroethyl)isoxazolidinium iodide (No. 524) and its related compound (No. 587) are typical derivatives with latent activity, prepared by Ishidate, Sakurai, and Torigoe,[4] and the two compounds showed a similar type of curve as those of the ordinary N-oxide, as shown in Table II.

In comparing these compounds with HN_2-O, the former two were found active in the routine screening with a small cell population, as shown in Table II. The reason is not yet clear but it may be due to difference either of the reduction potentials of both groups of compound as indicated by half-wave potentials in polarography or of their stability in aqueous solution.

N,N-Bis(2-chloroethyl)-O-ethylhydroxylamine methochloride (No. 588) was also prepared and proved to be a derivative of nitrogen mustard with latent activity by Torigoe.[5] This compound attracted attention becaues it behaved as if it were the originally active derivative like HN_2 or No. 533, as shown in Table II.

Recently Torigoe investigated chemical reactions of this compound closely and concluded that it decomposed readily at 37° in a neutral aqueous solution without being reduced after the following β-dehydration process (A):



Concerning the contact condition in this experimental technique, the automatic β-dehydrating decomposition (A) should of course be competing with the biological reduction (B), but, if the former reaction is sufficiently rapid and predominates the latter, it might look as if it were originally active in itself in this activation experiment. Assuming that No. 558 was rapidly activated by itself without biological activation, the curve in Table II is well understood.

A relationship between contact time and MEC, the latter of which is regarded to

4) M. Ishidate, *et al.*: Proc. Japan. Cancer Assoc., 17th General Meeting, November, 1958, 47.
5) M. Torigoe: Unpublished data.

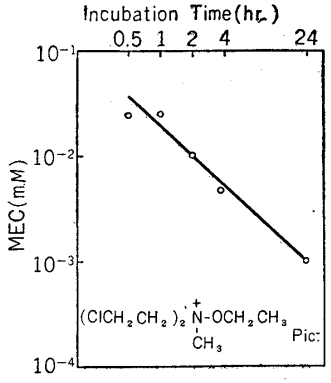


Fig. 5.
Effect of Contact Time on MEC

correspond to concentration of the active component released, is graphically demonstrated in Fig. 5.

Contrary to the concave curve obtained with HN_2–O, as reported in the preceding paper,[1] No. 558 gave a straight line. This fact supports the presumption that the automatic β–dehydration overcame the other activating reactions of this compound.

The compound No. 481 was a different kind of derivative with latent activity which had been prepared by Brock[6] and proved to be effective on rats bearing the Yoshida sarcoma. However, the present experimental method failed to display its anti-tumor activity against the *in vitro*–cultured Yoshida sarcoma cells even when the largest population (10^8 cells/cc.) was used.

In short, it was found that each of the compounds sweepingly called "compounds with latent activity" or "masked compounds" should be regarded to have an individual character in regard to the mode of activation. It is suggested that the compounds with latent activity could be generally analyzed in detail by this technique. Further investigations to find the most reasonable type of masking of alkylating agent are now under progress.

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Summary

Reductive activation of the N–oxide derivatives of 2–chloroethylamine by living cells was discussed in detail and it was found that this experimental method was useful for finding a suitable means of masking of anti-tumor agents with latent activity.

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6) N. Brock : Arzneimittel-Forsch., 8, 1(1958).