UDC 547.864 : 576.852.2.095.18

138. Itiro Yosioka* and Akiko Zaizen**: Studies on Phenazines. XX.[1] Antibacterial Activity of Phenazine Derivatives against *Mycobacterium smegmatis* and the Effect of Copper.

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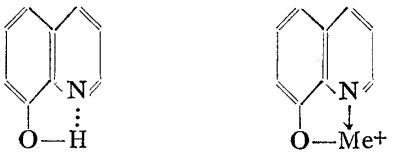
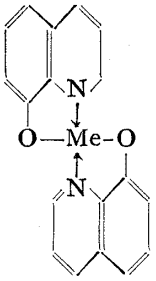
The close relationship existing between metal chelates of organic compound and their antibacterial activity has been pointed out by Albert and Rubbo[2] in connection with 8-hydroxyquinoline (I). 8-Hydroxyquinoline itself is capable of inhibiting the growth of bacteria and fungi, but the effect is potentiated by the addition of a minute quantity of iron or copper. The afore-mentioned workers made detailed study of this phenomenon using *Staphylococcus aureus*. Substitution of the hydroxyl in positions other than 8 in quinoline results in the loss of inhibitory action and effect of the heavy metals also vanishes. Albert and others found that 1 : 1 metal chelate of 8-hydroxyquinoline (II) was toxic while that of 2 : 1 was ineffective.



(I) (II)

or



In 1952, Erlenmeyer and others[3] also observed the intensifying action of copper with numerous compounds having structures analogous to 8-hydroxyquinoline against tubercle bacilli. According to their hypothetical explanation, the compound with chelating ability binds with residual valency of requisite metal contained in an enzyme by chelate formation and this makes the metal labile. Addition of an excess of copper at this stage will result in substitution of copper for the metal bound in the enzyme and, as a result, the enzyme will be inactivated and inhibition of bacterial growth is increased.

In 1953, Okazaki and others[4] also observed intensification of antifungal activity by the application of copper with 8-hydroxyquinoline against Trichophyton and other fungi.

A certain number of phenazine derivatives have been synthesized in the writers' laboratory and some of these possessed structure similar to that of 8-hydroxyquinoline. Therefore, their growth inhibitory activity and effect of copper on their action were examined with *Staphylococcus aureus*[5] and *Trichophyton rubrum*.[6] It was thereby found that copper effected intensification of such activity, as was found by Albert and others, when a hydroxyl is present in 1(α)-position in the case of *Staph. aureus* but not in the case of *Trichophyton*.

In the present series of work, growth inhibitory action of 14 kinds of phenazine derivatives

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1) Part XIX: This Bulletin, **7**, in press (1959).
2) A. Albert, *et al.*: Brit. J. Exptl. Pathol., **28**, 69 (1947); S. D. Rubbo, A. Albert, M. I. Gibson: *Ibid.*, **31**, 4125 (1950).
3) E. Sorkin, W. Roth, H. Erlenmeyer: Helv. Chim. Acta, **35**, 1736 (1952).
4) K. Okazaki, A. Homma: Yakugaku Zasshi, **73**, 818 (1953).
5) I. Yosioka, S. Uehara: *Ibid.*, **78**, 351 (1958).
6) I. Yosioka, T. Tanaka: *Ibid.*, **78**, 353 (1958).

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and effect of copper on this action were examined in some detail using *Mycobacterium smegmatis* by shake culture method.[7]

Materials and Method

Materials: Fourteen kinds of phenazine derivatives were selected from those synthesized earlier.

Method: A strain of *Mycobacterium smegmatis*, preserved in the National Institute of Health, Tokyo, was used for shake culture. The medium used was a Sauton medium added with 1% of Tween 80.[7]

Inoculation and Culture: Each chemical was dissolved in ethylene glycol and serially diluted with the medium. Another series was prepared by adding $2\times10^{-4}M$ of Cu^{2+} and the two series were compared. Bacterial pad of *Myco. smegmatis* grown on the Sauton medium for 48 hrs. was collected by platinum loop, ground with crystal beads, and diluted with sterilized distilled water. Bacterial solution was prepared by adding Tween 80 to the final concentration of 1%.

This bacterial solution was added to each of the sample solutions while measuring the optical density with the Coleman photometer so as to make solutions with optical density of 0.05~0.07, which corresponds to 0.6~0.8 mg. of dried bacteria. This mixture was incubated at 37° for 9~11 hrs. with shaking and optical density of the solution was determined every 2~3 hrs., plotting the growth curve. After 9 hrs. (log phase, ca. 2 generations), the growth of bacteria was represented by percentage with growth of the control as 100 (cf. Table I).

Figs. 1 to 6 are the graphs obtained by plotting the values hereby obtained on the ordinate, as a percentage of probit measure, and concentration of the chemical on the abscissa. From these graphs, ID_{50} (50% inhibition dose) is obtained and these values are listed in Table II.

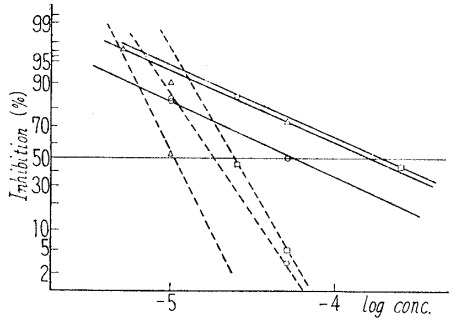

Fig. 1.

○——○ without Cu
○---○ with M/5000 Cu } 1-Hydroxyphenazine

□——□ without Cu
□---□ with M/5000 Cu } 1,6-Dihydroxyphenazine

△——△ without Cu
△---△ with M/5000 Cu } 1,6-Dihydroxyphenazine 5,10-dioxide

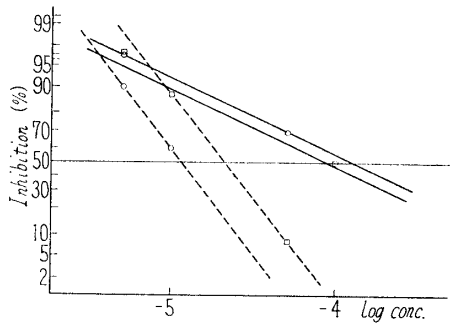

Fig. 2.

○——○ without Cu
○---○ with M/5000 Cu } 1-Hydroxy-6-methylphenazine

□——□ without Cu
□---□ with M/5000 Cu } 1-Hydroxy-8-methylphenazine

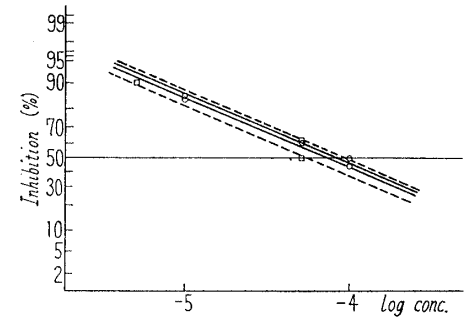

Fig. 3.

○——○ without Cu
○---○ with M/5000 Cu } Phenazine 5-oxide

□——□ without Cu
□---□ with M/5000 Cu } Phenazine 5,10-dioxide

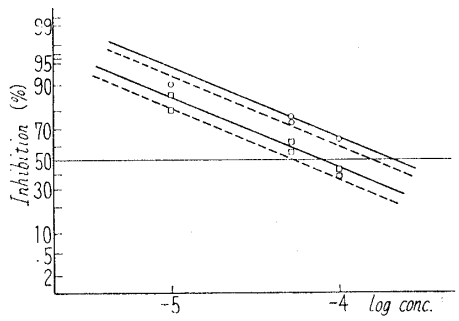

Fig. 4.

○——○ without Cu
○---○ with M/5000 Cu } 1,7-Dihydroxyphenazine

□——□ without Cu
□---□ with M/5000 Cu } 1,9-Dihydroxyphenazine

7) T. Aoyagi, D. Mizuno: Nippon Saikingaku Zasshi, **11**, 629 (1956).

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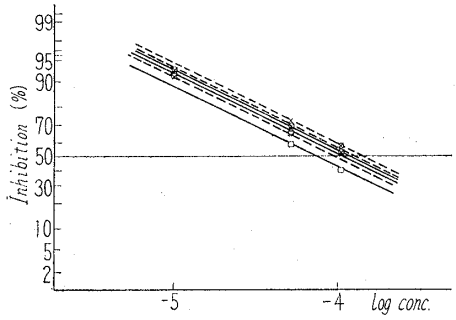

Fig. 5.

○——○ without Cu
○---○ with *M*/5000 Cu } Phenazine-1-carboxylic acid

□——□ without Cu
□---□ with *M*/5000 Cu } Phenazine-1-carbohydrazide

×——× without Cu
×---× with *M*/5000 Cu } 1-Chlorophenazine

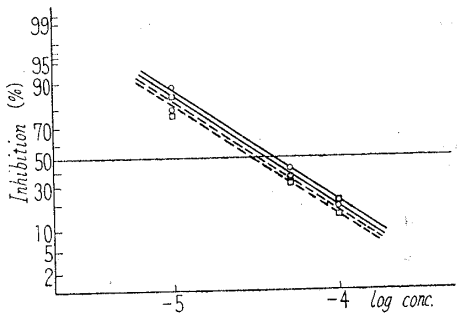

Fig. 6.

○——○ without Cu
○---○ with *M*/5000 Cu } 1-Methoxyphenazine

□——□ without Cu
□---□ with *M*/5000 Cu } 2-Methoxyphenazine

TABLE I. Growth of *Myco. smegmatis* in various Concentrations of Phenazine Derivatives

Compound	Dilution series (*M*)	Without Cu		With Cu^{2+}	
		Optical density	%	Optical density	%
Control		0.31		0.31	
1-Hydroxy-8-methylphenazine	10^{-4}	0.16	51	—	—
	5×10^{-5}	—	—	0.03	10
	10^{-5}	0.27	87	—	—
	5×10^{-6}	0.28	90	0.3	97
Phenazine 5,10-dioxide	5×10^{-5}	0.19	61	0.15	48
	10^{-5}	0.26	84	0.26	84
	5×10^{-6}	—	—	0·28	90
1,6-Dihydroxyphenazine	2.5×10^{-4}	0.14	45	—	—
	5×10^{-5}	—	—	0.04	13
	2.5×10^{-5}	0.26	84	0.14	45
1,6-Dihydroxyphenazine 5,10-dioxide	5×10^{-5}	0.23	74	—	—
	10^{-5}	0.28	90	0.16	51
	5×10^{-6}	—	—	0.3	97
1,7-Dihydroxyphenazine	10^{-4}	0.2	65	—	—
	5×10^{-5}	0.24	77	0.23	74
	10^{-5}	—	—	0.28	90
Control		0.32		0.32	
1-Methoxyphenazine	10^{-4}	0.07	22	0.06	16
	5×10^{-5}	0.11	34	0.11	33
	10^{-5}	0.27	84	0.24	75
Control		0.68		0.68	
1-Hydroxy-6-methylphenazine	5×10^{-5}	0.54	68	—	—
	10^{-5}	—	—	0.46	58
	5×10^{-6}	0.67	95	0.64	90
Control		0.30		0.30	
Phenazine 5-oxide	10^{-4}	0.13	43	0.15	50
	5×10^{-5}	0.19	63	0.19	63
	10^{-5}	0.26	87	0.26	87
Phenazine-1-carboxylic acid	10^{-4}	0.16	53	0.17	57
	5×10^{-5}	0.20	66	0.21	70
	10^{-5}	0.28	93	0.28	93
1-Chlorophenazine	10^{-4}	0.16	53	0.17	55
	5×10^{-5}	0.21	70	0.22	73
	10^{-5}	0.27	91	0.28	93

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Phenazine-1-carbohydrazide	10^{-4}	0.12	40	—	—
	5×10^{-5}	0.18	60	0.2	65
	10^{-5}	—	—	0.28	93
Control		0.36		0.36	
1-Hydroxyphenazine	5×10^{-5}	0.18	50	0.01	3
	10^{-5}	0.3	84	0.3	83
Control		0.37		0.37	
1,9-Dihydroxyphenazine	10^{-4}	0.17	46	0.15	40
	5×10^{-5}	0.23	62	0.22	60
	10^{-5}	0.32	86	0.31	81
2-Methoxyphenazine	10^{-4}	0.08	22	0.07	19
	5×10^{-5}	0.16	43	0.13	35
	10^{-5}	0.33	89	0.29	78

TABLE II. ID_{50} of Phenazine Derivatives against *Mycobacterium smegmatis*

Compound	50% Inhibtion dose (M)	
	Without Cu	With Cu^{2+}
Phenazine 5-oxide	8×10^{-5}	8×10^{-5}
Phenazine 5,10-dioxide	10^{-4}	5×10^{-5}
1-Hydroxyphenazine	5×10^{-5}	1.7×10^{-5}
1,6-Dihydroxyphenazine	1.7×10^{-4}	2.5×10^{-5}
1-Hydroxy-6-methylphenazine	1.2×10^{-4}	1.2×10^{-5}
1-Hydroxy-8-methylphenazine	10^{-4}	2×10^{-5}
1,7-Dihydroxyphenazine	2.2×10^{-4}	1.7×10^{-4}
1,9-Dihydroxyphenazine	9×10^{-5}	6×10^{-5}
1,6-Dihydroxyphenazine 5,10-dioxide (Iodinine)	2.5×10^{-4}	10^{-5}
1-Methoxyphenazine	4.4×10^{-5}	3.4×10^{-5}
2-Methoxyphenazine	3.8×10^{-5}	2.8×10^{-5}
Phenazine-1-carboxylic acid	1.2×10^{-4}	1.3×10^{-4}
1-Chlorophenazine	1.1×10^{-4}	1.2×10^{-4}
Phenazine-1-carbohydrazide	7.5×10^{-5}	10^{-4}

Discussion

Treffers[8] reported that the concentration-inhibition curve, obtained by plotting the probit measure of percentage inhibition of bacterial growth by antibiotics on the ordinate, became linear and showed that a different slope of this straight line meant difference in the action mechanism of a chemical against the bacteria. Figs. 1 and 2 are concentration-inhibition curves affected by the addition of $2\times10^{-4}M$ of Cu^{2+}, while those in Figs. 3~6 are unaffected. In any of these cases, the straight line of phenazine derivatives is in parallel. When addition of copper affects inhibition, the slope of the curve differs in each case but to the same degree. Even if the slope of the straight line of conentration-inhibition curve was the same, action mechanism would not necessarily be the same. Neverthless, in the present case, all the derivatives have the same phenazine ring as the parent structure and the same slope probably indicates the same action mechanism. The change in the slope by intensification of potency with addition of coppper is thought to be due to the potency of copper chelate of phenazine derivative. In fact, all the compounds whose activity was intensified by copper possessed a hydroxyl in 1(α)-position of the phenazine ring and are those with possibility of chelate formation. It is therefore considered possible that the inhibitory action mechanism of the phenazine derivatives themselves and that of their copper chelates are different. In some cases, intensification of activity by copper was not observed even on the formation of copper chelate,

8) H. P. Treffers: J. Bacteriol., **72**, 108 (1956).

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as in 1,7-dihydroxy- and 1,9-dihydroxy-phenazines. In the case of 1,7-dihydroxyphenazine, the pH of the medium for the formation of stable chelate is extremely to the alkaline side[9] and even if a chelate is formed at around neutral region, it must be very labile. In the case of 1,9-dihydroxyphenazine, the chelate, even if formed, is extremely unstable due to steric hindrance. Phenazine-1-carboxylic acid and its hydrazide are considered to form a chelate but such a chelate formed must be of a different nature and there is no intensification of their activity by copper ion.

According to Albert and others, blocking of the hydroxyl in 8-position of 8-hydroxylquinoline or its transfer to other positions to stop chelate formation results in the loss of antibacterial activity. In the case of phenazine derivatives, however, absence of a hydroxyl in 1-position does not result in the disappearance of antibacterial activity. Comparison of LD_{50} of the 14 kinds of compound tested shows that the strongest antibacterial activity was present in compounds not capable of forming chelates, like 1-methoxy- and 2-methoxy-phenazines. This fact indicates that either the antibacterial activity of phenazine derivatives is independent of chelate formation, differing from 8-hydroxyquinoline, or the chelate formation is only partly responsible for such action. When the potency is intensified by addition of copper, it should be safe to conclude that, as in the case on 8-hydroxyquinoline, a formation of copper chelate has taken place and that this chelate effected strong inhibitory activity.

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Summary

Inhibitory activity of 14 kinds of phenazine derivatives on the growth of *Mycobaeterium smegmatis* and effect of copper on this activity were examined by shake culture. It was thereby found that the activity of compounds was affected by copper when a hydroxyl was present in 1-position of the phenazine ring and this was considered to be due to formation of a copper chelate. The growth inhibitory activity of the phenazine derivatives themselves and their copper chelates was found to be different from the slope of straight line in concentration-inhibition curves.

From the LD_{50} of phenazine derivatives, the strongest inhibitory activity was found in compounds having no ability to form a chelate, like 1- and 2-methoxyphenazine.

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9) Y. Kidani: Unpublished data.

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