UDC 576.8.095.3 : 547.864

100. Tchan Gi Bak and Itiro Yoshioka : The Effect of Phenazine Derivatives on the Respiration of Microorganisms.

(*School of Pharmacy, Osaka University*[*1])

A variety of phenazine derivatives have been identified among microbial products and many of them were shown to be strongly bacteriostatic.[1~5] Many other phenazine derivatives synthesized in this laboratory have also been found to inhibit the growth of bacteria[6,7] and fungi.[8] Little is, however, known of the mechanism by which these compounds inhibit the growth of microorganisms. The present paper reports that phenazine derivatives are capable of inhibiting the respiration of certain microorganisms.

Materials and Methods

Compounds——The phenazine derivatives were synthesized in this laboratory as described previously,[9] except for griseolutein B which was isolated from culture broth of *Streptomyces griseoluteus* and kindly supplied from Dr. S. Nakamura of the Institute of Applied Microbiology, University of Tokyo. These compounds were dissolved in 50% propylene glycol for the use in biochemical experiments.

Organisms——All the strain employed, except for *Xanthomonas oryzae*, were obtained from the collection at the Institute for Microbial Disease, Osaka University. A strain of *X. oryzae* was given by Dr. S. Matsunaka of the National Institute of Agricultural Sciences, Tokyo. *Escherichia coli* B, *Pseudomonas fluorescens* A3-12, *Proteus vulgaris* KS and *Staphylococcus aureus* 209P were grown at 30° for 18 hr. on an agar medium containing 1% peptone, 1% meat extract, 0.2% NaCl, and 0.5% yeast extract (pH 7.4). *Saccharomyces cerevisiae* was cultured at 30° for 18 hr. on the same medium enforced with 0.5% glucose. *X. oryzae* was grown at 30° for 48 hr. on an agar medium containing 2% sucrose, 0.5% peptone, 0.2% yeast extract, 0.2% K_2HPO_4, 0.1% $MgCl_2$, 0.2% sodium glutamate and 0.005% $FeSO_4 \cdot 7H_2O$ (pH 7.4). *Streptococcus faecalis* R was grown at 30° for 18 hr. on an agar medium containing 1% yeast extract, 1% Difco tryptone and 0.5% glucose (pH 7.4). The cells harvested from media were washed three times with distilled water and suspended in distilled water (usually 1 to 2 mg. dry weight per cc.).

Respirometry——The rate of oxygen uptake was measured at 37° in a Warburg manometer using air as the gas phase. The reaction mixture consisted of 1.0 cc. of cell suspension (1 to 2 mg. dry weight), 0.6 cc. of 0.1*M* phosphate buffer (pH 7.4), 0.2 cc. of 0.5*M* substrate (glucose, malate or succinate), and 0.2 cc. of 50% propylene glycol or solution of phenazine derivative (in 50% propylene glycol). In the center well was placed 0.1 cc. of 20% KOH. After equilibration, the reaction was started by mixing the contents of the main chamber and the side arm. While the cell suspension and phosphate buffer were always placed in the main chamber, the other components of reaction mixture were placed either in the main chamber or in the side arm according to purposes. The oxygen uptake was measured for 1 hr. at 10 min. intervals. The per cent inhibition (H) was calculated from the equation :

$$H = (1 - v_i/v) \times 100$$

where v and v_i are oxygen uptake (μl.) per hr. in the absence and presence of inhibitor, respectively.

*1 Hotarugaike, Toyonaka, Osaka-fu (朴 昌基, 吉岡一郎).

1) L.H. Frank, R.D. DeMoss : J. Bacteriol., **77**, 776 (1959).
2) H. Umezawa, S. Hayano, K. Maeda, Y. Okami : J. Antibiotics (Japan). Ser. A, **4**, 34 (1951).
3) A.J. Kluyver : J. Bacteriol., **72**, 406 (1956).
4) S. Nakamura : Chem. Pharm. Bull.(Tokyo), **6**, 539, 543, 547 (1958).
5) M.P. Starr, W. Blau, G. Cosens : Biochem. Z., **333**, 328 (1960).
6) I. Yosioka, A. Zaizen : Chem. Pharm. Bull.(Tokyo), **6**, 688 (1958).
7) I. Yosioka, S. Uehara : Yakugaku Zasshi, **78**, 351 (1958).
8) I. Yosioka, T. Tanaka : *Ibid.*, **78**, 353 (1958).
9) I. Yosioka, Y. Kidani : *Ibid.*, **72**, 1301 (1952).

Results

Effect of 1-Phenazinol on Microbial Respiration——Table I shows the results of experiments in which the effect of $10^{-3}M$ (final concentration) of 1-phenazinol on the oxygen uptake by various organisms was examined. In these experiments, glucose was

Table I. Effect of 1-Phenazinol on the Glucose Oxidation of Various Microorganisms

Microorganisms	Dry weight of cell in reaction mixture	Oxygen uptake (μl./hr.) 1-phenazinol (final $10^{-3}M$)		Percent inhibition
	(mg.)	−	+	
Escherichia coil B	1.0	119.0	69.0	42
Pseudomonas fluorescens A3-12	2.0	88.5	79.5	10
Proteus vulgaris KS	0.5	162.0	153.8	5
Xanthomonas oryzae	1.2	69.0	26.9	61
Staphylococcus aureus 209P	1.8	159.0	180.0	−13
Streptococcus feacalis R	2.4	42.3	41.0	3
Saccharomyces cerevisiae	1.6	179.0	73.5	59

Reaction conditions: 1-Phenazinol and glucose simultaneously added to reaction mixture.

used as the respiratory substrate and the inhibitor was added to the cell suspension simultaneously with the substrate. It will be seen that the respiration of *E. coli*, *X. oryzae* and yeast was rather strongly inhibited, whereas that of *Ps. fluorescens*, *Pr. vulgaris*, *Staph. aureus* and *Strep. faecalis* was only slightly affected or not inhibited at all. The reasons for such differences in sensitivity to 1-phenazinol are not yet clear. Similar inhibition was also observed in the endogenous respiration of *E. coli*.

Effect of Preincubation——Fig. 1 shows that the respiration of *E. coli* with glucose as substrate proceeded linearly with time for at least 60 minutes and that the addition of $5\times10^{-4}M$ of 1-phenazinol simultaneously with the substrate caused at 37% inhibition. The inhibited respiration was also linear with time for at least 60 minutes. Fig. 1 also shows that the preincubation of cells with the inhibitor for 60 minutes prior to the initiation of reaction by adding glucose resulted in more pronounced inhibition (57%).

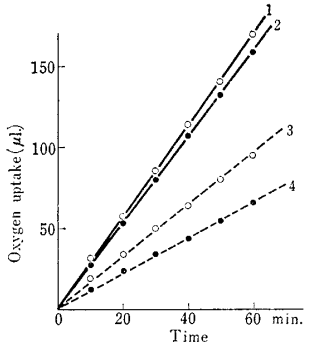


Fig. 1. Effect of 1-Phenazinol on Respiration of *E. coli* (1.5 mg./vessel) with Glucose as Substrate

1, 2: control
3, 4: experiments in the presence of $5\times10^{-4}M$ 1-phenazinol
o---o: without preincubation
•----•: preincubated for 60 min.

The inhibition of glucose oxidation by 1-phenazinol was shown to with increasing preincubation time up to 60 minutes (inhibitions were 36, 46 and 50% for preincubation time 0, 30 and 60 minutes, respectively).

Similar increase in inhibition of glucose oxidation by preincubating the cells with 1-phenazinol was also observed with yeast and *Staph. aureus*. It is of special interest that *Staph. aureus* became significantly sensitive to the inhibitor after preincubation, 26% inhibited by $10^{-3}M$ 1-phenazinol after 60 minutes preincubation, although its respiration was not inhibited at all (actually slightly accelerated) when the inhibitor was added at time zero without preincubation (see Table I).

Elimination of Preincubation Effect by Glucose——Although the incubation of *E. coli* cells with 1-phenazinol caused a progressive inactivation of the respiratory activity, the oxygen uptake observed when both glucose and the inhibitor were added simultaneously proceeded linearly for at least 60 minutes (see Fig. 1). This suggests that glucose protects the cells against the progressive inactivation. It was in fact found that the presence of $10^{-3}M$ glucose during the preincubation of cells with 1-phenazinol almost completely eliminated the inhibition increasing effect of preincubation.

Effects of Metal Ions——1-Phenazinol can form chelates with various metal ions.[10] It seemed, therefore, of interest to examine the effects of metal ions on the respiratory inhibition by 1-phenazinol. Various metal ions (final concentration, $2\times10^{-4}M$) were incubated with *E. coli* cells at 37° for 60 minutes both in the presence and absence of 1-phenazinol (final concentration $10^{-4}M$) and then the oxygen uptake was measured by adding glucose as substrate. As recorded in Table II, none of the metal ions tested had no significant effect on the respiratory rate when they were added to the cells in the absence of 1-phenazinol. However, when the metal ions were preincubated with the cells in the presence of the inhibitor, it was found that the inhibition was more or less decreased. Zn^{2+} and Fe^{2+} were especially effective in decreasing the inhibition.

Table II. Effect of Metals to the Inhibition of Glucose Oxidation by 1-Phenazinol

Added metal (final conc. $2\times10^{-4}M$)	Oxygen uptake (μl./hr.) 1-phenazinol (final conc. $10^{-4}M$)		Percent inhibition
	−	+	
None	94.0	36.7	61
$FeSO_4$	90.0	66.0	27
$FeCl_3$	94.0	47.0	50
None	102.0	36.0	65
Na_2MoO_4	100.0	32.0	68
None	81.0	32.4	60
$MgSO_4$	81.0	37.3	54
$MnCl_2$	73.0	45.0	42
None	97.0	37.0	62
$CaCl_2$	102.0	68.4	33
$ZnSO_4$	93.0	79.0	15
None	90.0	37.8	58
$Al_2(SO_4)_3$	92.0	45.0	51

Reaction conditions: Metal and 1-phenazinol simultaneously added to bacterial cell suspension. Then after preincubated.

Effect of Substrate Concentration——The inhibition by 1-phenazinol and 1-methoxyphenazine of the respiration with glucose as substrate was not affected by changing the substrate concentration from $5\times10^{-3}M$ to $10^{-1}M$ (Table III). It is evident from these data that the inhibition is not competitive with respect to the substrate concentration.

Table III. Effect of Glucose Concentration on the Inhibition by 1-Phenazinol and 1-Methoxyphenazine

Inhibitor concentration (M)	Glucose concentration (M)	Percent inhibition
1-Phenazinol 10^{-3}	5×10^{-2}	38
	10^{-2}	49
	5×10^{-3}	43
1-Methoxyphenazine 2×10^{-3}	5×10^{-2}	20
	10^{-2}	19
	5×10^{-3}	25

Reaction conditions: without preincubation

Effect of Various Phenazine Derivatives——In Fig. 2 are summarized the effects of various phenazine derivatives on the respiration of *E. coli* using glucose, malate or succinate as substrate. In these experiments, the cells were preincubated with the inhibitor at 37° for 60 minutes prior to the addition of substrates. The percent inhibition (H) was plotted against the negative logarithm of inhibitor concentration (pI, in terms of moles per liter). As will be seen, all the phenazine derivatives more or less inhibited the respiration. Among the inhibitors tested, 1-phenazinol, griseolutein B, 2-methoxyphenazine, etc. were most powerful inhibitors. With all the substrates used, 1-phenazinol was more toxic than 2-methoxyphenazine. In experiments which are not shown in Fig. 2, it was found that the respiration of *E. coli* with glucose as substrate is less sensitive to dihydroxyphenazines as compared to 1-phenazinol. Thus, 1,6-phenazinediol and 1,7-phenazinediol at $10^{-4}M$ inhibited the respiration by 25 and 27%, respectively, while the same concentration of 1-phenazinol caused an inhibition of 58%. It will also be seen from Fig. 2 that the respiratory inhibition caused by phenazine derivatives differs considerably according to the substrate used. In general the respiration with malate as substrate was most sensitive to phenazine derivatives; $10^{-3}M$ of griseolutein B and 2-methoxyphenazine completely inhibiting the malate oxidation. It will further be noticed that the slope of inhibition curves in the H-pI graphs differs

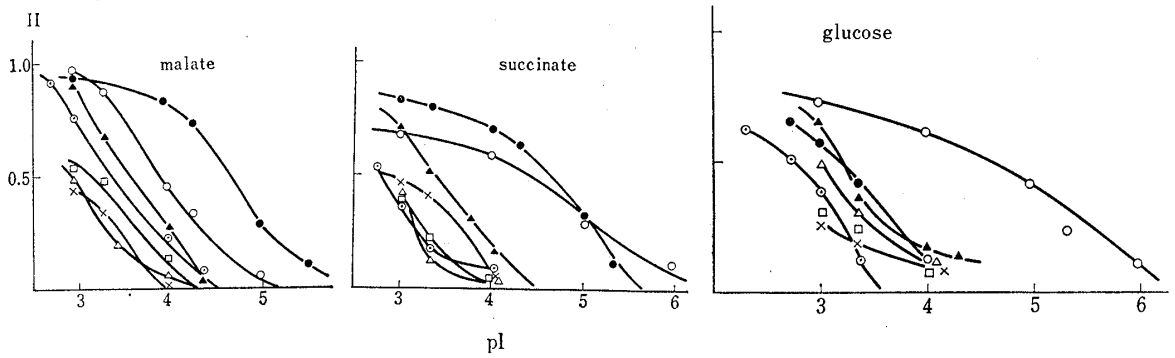


Fig. 2. H-pI Curve of Phenazine Derivatives for Respiration of *E. coli*

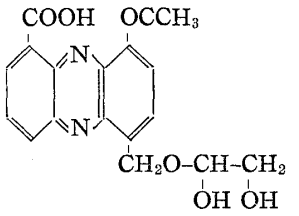
● griseolutein B △ 1-phenazinecarboxylic acid ○ 1-phenazinol
▲ 2-methoxyphenazine □ phenazine ⊙ 1-methoxyphenazine
× 2-phenazinol

considerably according to the inhibitor and substrate used. Since phenazine, 1-phenazinecarboxylic acid, 2-phenazinol and 1-phenazinol produced crystals at concentrations higher than $10^{-3}M$, it was not possible to examine the effects of these compounds at higher concentrations than $10^{-3}M$.

Discussion

The data presented in this paper indicate that phenazine and several of its derivatives exert inhibitory actions on the respiration of *E. coli* and certain other microorganisms. It seems that this inhibitory activity may, at least partly, account for the bacteriostatic actions of this class of compounds. It is, however, probable that the mechanism of respiratory inhibition may be different among the individual compounds. 1-Phenazinol and 1-phenazinecarboxylic acid have been known to form chelates with a variety of metals[10] and griseolutein B appears also to be a chelating agent. It is, therefore, likely that these compounds inhibit the respiration by forming chelates with

10) Y. Kidani: This Bulletin, **7**, 74 (1959).



griseolutein B[4]

a metal or metals which are essential for the respiratory mechanisms.[11] The fact that the inhibition by 1-phenazinol is decreased by the presence of metal ions such as Zn^{2+} and Fe^{2+} (Table II) may be explained by preferential chelation of the inhibitor with the added metal ions rather than the respiratory metals. The stronger inhibition by 1-phenazinol than by 2-phenazinol, a poorer chelating agent, may also be related to this mechanism of inhibition. The inhibition mechanism by phenazine and methoxyphenazines is not yet clear. It might be suggested that these compounds also form metal chelates, probably in similar manners to 2,2′-bipyridine. It is further possible that the ability of chelation of phenazine derivatives is strongly influenced by the position and kind of substituent groups.

The fact that preincubation of the cells with inhibitors is required for the maximal inhibition may be explained by the time required for the inhibitors to reach the active site of respiratory mechanism. This effect of preincubation, however, can be abolished by the presence of glucose in the incubation mixture. This finding, together with the fact that the inhibition caused by a phenazine derivative varies with the respiratory substrates used, is difficult to explain by the present investigations in which intact cells were used. Experiments with cell-free systems are desired to elucidate these problems as well as the non-competitive nature of the inhibition with respect to substrate concentration.

The authors wish to express their thanks to Dr. R. Sato of the Institute for Protein Research, Osaka University, for his guidance and encouragements during the course of this investigation.

Summary

The respiration of *Escherichia coli* and some other microorganisms was found to be more or less inhibited by phenazine and its derivatives tested. 1-phenazinol, 2-methoxyphenazine and griseolutein B were most inhibitory. The inhibition varied with the respiratory substrates used and it was strongest when malate was used as compared with glucose and succinate. The inhibition was not competitive with substrate concentration. In order to attain maximal inhibition, it was necessary to preincubate the cells with the inhibitors for a certain period of time. The inhibition mechanisms were briefly discussed.

(Received August 31, 1962)

11) K. Tamari, J. Kaji : J. Biochem. (Tokyo), **41**. 143 (1954).