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Experimental Anticancer Studies. XXXVI.[1] The Effect of 2-(2-Hydroxyphenyliminomethyl)-4-*n*-hexylphenol on Ribonucleic Acid Synthesis and Energy Metabolism in *Bacillus subtilis*[2]

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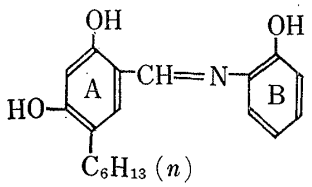
In order to obtain some information on the action mechanism of 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (II) as a possible anticancer agent a series of experiments were performed, in which the influence of II on macromolecular synthesis and energy metabolism was examined employing *B. subtilis in vitro*.

As the results, net synthesis of RNA was fairly inhibited by II at the level of 10 μM, though DNA and protein syntheses were less affected under the same conditions. Both respiration and anaerobic glycolysis of the bacterial cell were suppressed at the concentration of 33.3 μM or more of II. Then, it might be postulated that depression of RNA synthesis in one hand and suppression of respiration and anaerobic glycolysis in the other are responsible for the inhibition of the bacterial growth.

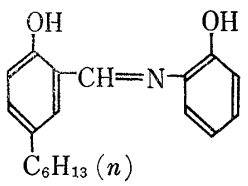
Additionally, experimental data suggest that metal chelating ability of II might be contributing to the antibacterial action, because the inhibition of bacterial growth by II could be almost completely reversed by either Fe^{2+}, or Fe^{3+}, or Mn^{2+}.

In previous works[4] it was demonstrated that 4-*n*-hexyl-6-(2-hydroxyphenyliminomethyl) resorcinol (I) exhibited anticancer effect against Ehrlich carcinoma (ascites form and subcutaneous form), Sarcoma 180 and Yoshida ascites sarcoma in animals. The results of a series of anticancer experiments with compounds having structural relationship with I led to the conclusion that in molecule I, hexyl radical, azomethine (–CH=N–), which binds A and B rings, and *ortho*–substituted hydroxy radicals were contributive to its biological activity.[4]

These experiments also showed that closely related compound to I, 2-(2-hydroxyphenyliminomethyl)-4-*n*-hexylphenol (II), was effective to suppress the growth of Ehrlich ascites carcinoma in mice and of ascites hepatoma AH 13 in rats.[5]



I



II

The mode of action of these compounds is, however, still obscure. Then, in order to obtain some information on the action mechanism of such Schiff bases as possible anticancer agents, a series of *in vitro* experiments were performed employing bacterial cell system.

The present paper deals with the study in which the influence of II on macromolecular synthesis and energy metabolism in *Bacillus subtilis* was examined.

1) Part XXXV: Y. Tani, R. Koshiura, S. Koshimura, K. Tsuyama, H. Takeuchi, and Y. Nitta, *Ann. Rep. Cancer Inst. Kanazawa* (*Japanese text*), **1**, 215 (1967).

2) Presented at the 88th Meeting of the Pharmaceutical Society of Japan, Tokyo, April 1968. This work was supported in part by a Grant-in-Aid from Scientific Research Expenditure of the Ministry of Education, Japan.

3) Location: *Takara-machi, 13-1, Kanazawa.*

4) *a*) T. Ujiie, *Ann. Rep. Cancer Inst. Kanazawa* (*Japanese text*), **1**, 109 (1967); *b*) T. Ujiie, *Chem. Pharm. Bull.* (Tokyo), **16**, 165 (1968).

5) Personal communication from Dr. Hiroshi Satoh of Sasaki Institute, Tokyo.

Materials and Methods

Chemicals——II and 5-*n*-hexylsalicylaldehyde (III) were prepared by the method described in previous paper.[4b] II; mp 111—112°. λ_{max}^{EtOH} mμ (ε): 271 (11270), 355 (12590). III; bp 131—133°(3 mmHg). Because of little solubility in water and little stability in solvent containing water, immediately before use, II was dissolved in ethanol, and its solution was added to culture medium. Mitomycin C was purchased from Sankyo Co., Ltd., actionomycin D was kindly supplied from Nippon Merck-Banyu Co., Ltd. Calf thymus deoxyribonucleic acid (DNA) (mol. wt., $3—4\times10^6$) was indebted to Prof. Kameyama of this Institute. Yeast highly polymerized ribonucleic acid (RNA) was purchased from Wako Pure Chemical Industries, Ltd.

Bacterial Strain and Medium——*B. subtilis* 749 used throughout this work, was kindly supplied from Dr. Taketo of school of Medicine of this University. *Staphylococcus aureus* Terashima, *Escherchia coli* B and *Mycobacterium tuberculosis* $H_{37}R_v$ were also kindly supplied from Dr. Fukuyama of this Institute. *Streptococcus hemolyticus* Su used was a stock strain of this laboratory. The culture medium used for most of the works was consisted of 2.5 g of $(NH_4)_2HPO_4$, 1.5 g of KH_2PO_4, 5.0 g of NaCl, 3.0 g of sodium glutamate, 3.0 g of glucose and 0.1 g of $MgSO_4\cdot7H_2O$ in 1000 ml of dist. water (adjusted to pH 7.2 with 1 N NaOH).

Cultivation of the Bacteria and Preparation of Cell Suspension——The cells of *B. subtilis* were inoculated into the glucose-salts medium, and incubated under shaking at 37° for 8 hr. The bacterial growth was followed by observing absorbance at 660 mμ, using Erma electrophotometer Model 4. In the exponential phase, the cells were harvested by centrifugation and resuspended in fresh medium to give the value of A.=0.30 at 660 mμ (approximately 5×10^8 cells per ml). The viable cell count assay was performed by the usual method. This suspension was divided into some portions, to each of which a certain amount of chemicals to be tested was added. Each portion was incubated at 37° under mechanical shaking and periodic determinations of the growth rate was made turbidmetrically.

Determination of Nucleic Acids and Protein——Nucleic acids and protein analyses were carried out on the cells obtained from 40 ml of the culture above-mentioned. The harvested cells were washed twice with 10 ml of cold 10^{-2} M Tris-buffer containing 10^{-2} M NaCl (pH 7.4), and once with 10 ml of cold 5% perchloric acid. The insoluble part was heated with 5.0 ml of 5% perchloric acid at 90° for 15 min. The supernatant by centrifugation was submitted to analyses of nucleic acids. DNA and RNA were determined by the Burton[6] and orcinol method[7], respectively. The residual part was dissolved in 3.0 ml of 1 N NaOH, and was analysed for protein by the Folin-Lowry method.[8]

Warburg's Technique——1) Respiration: The bacterial cells in exponential phase were harvested by centrifugation, and resuspended, after washing with 10^{-2} M Tris-saline (pH 7.4), in the buffer solution to contain 1.2×10^{10} cells per ml (dry weight, 72.2 mg). 0.5 ml of the cell suspension and 1.0 ml of 2×10^{-2} M glucose in Tris-saline were pipetted into main chamber of manometer flask equipped with sub-chamber, in which previously 0.2 ml of 10% KOH was added, and then the flask was incubated at 37° under shaking. 0.5 ml of chemical to be tested was tipped into the main compartment from the vessel side-arm at zero time.

2) Anaerobic glycolysis——2.4×10^{10} of the cells (dry weight, 113 mg) were suspended in 0.5 ml of Krebs-Ringer bicarbonate solution (pH 7.4), and to this suspension were added 1.0 ml of 2×10^{-2} M glucose. Incubation was performed in the atomosphere of 95% of N_2 and 5% of CO_2.

Preparation of Cu Complex (1:1) of II——To 25 ml of methanolic solution of 0.6 g of II was added gradually 25 ml of hot methanolic solution of 0.5 g of $CuSO\cdot5H_2O$. Instantly, there occurred precipitation of yellowish green powders. The precipitate resulted was collected on a filter, washed with hot methanol, with water, and dried. Yield, 0.5 g. mp >300°(from benzene). *Anal.* Calcd. for $C_{19}H_{21}O_2N\cdot Cu$: C, 63.59; H, 5.86; N, 3.86; Cu, 17.75. Found: C, 63.44; H, 6.13; N, 4.06; Cu, 18.15. $\lambda_{max}^{C_6H_6}$ mμ (ε): 380 (7360), 430 (8730).

Complex Formation of II with Several Metallic Ions——To 9.9 ml of 10^{-4} M ethanolic solution of II, 0.1 ml of 10^{-2}M aqueous solution of metallic salt [$CuSO_4\cdot5H_2O$, $Co(OAc)_2\cdot4H_2O$, $ZnCl_2$, $MnCl_2\cdot4H_2O$, $AgNO_3$, $FeSO_4\cdot7H_2O$, $FeCl_3\cdot6H_2O$, $Ni(NO_3)_2\cdot6H_2O$, $CaCl_2$, $MgCl_2\cdot6H_2O$, $Ba(NO_3)_2$, and $Pb(OAc)_2\cdot2H_2O$] was added, and absorption of the mixed solution was examined spectrometrically over the range of 320—500 mμ.

Measurement of Difference Spectra——Measurements were made in 10^{-2}M Tris-buffer containing 30% ethanol (pH 7.2). The solution containing II and nucleic acid or II, nucleic acid and metallic salt was prepared, with reference to solution containing only II or II and metallic salt. Difference spectra were obtained by estimation, using 1 cm light path, in Hitachi spectrophotometer, Model EPU.

6) K. Burton, *Biochem. J.*, **62**, 315 (1956).
7) A.H. Brown, *Arch. Biochem.*, **11**, 269 (1946).
8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Results and Discussion

Effect of II on the Growth of Various Kinds of Bacteria

As shown in Table I, II was found to be inhibitory to *Str. hemolyticus* at lower concentration of 0.34 μg/ml, while did not inhibit the growth of *Myco. tuberculosis* $H_{37}R_v$ and *E. coli* even at the concentration of 100 μg/ml. Since the growth of *B. subtilis* and *Staph. aureus* were completely inhibited by II at moderate concentration (12.5 μg/ml) on the other hand,

Table I. Action of II on Several Bacteria

Bacteria[a]	MIC[b] (μg/ml)[c]
Staph. aureus Terashima	12.5
E. coli B	> 100
Str. hemolyticus Su	0.34
B. subtilis 749	12.5
Myco. tuberculosis $H_{37}R_v$	> 100

a) Used Todd-Hewitt's broth for bacteria other than *M. tuberculosis*, in which case Sauton's medium was employed.
b) Minimum growth inhibitory concentration: Bacetrial growth was macroscopically observed after 2 days incubation at 37°, while in the case of *M. tuberculosis* after one-month incubation.
c) Original ethanolic solution (1%) of II was diluted with the medium to give the concentration of 100 μg/ml and followed by serial two fold dilution. To each dilution tube an aliquot of cell culture was added.

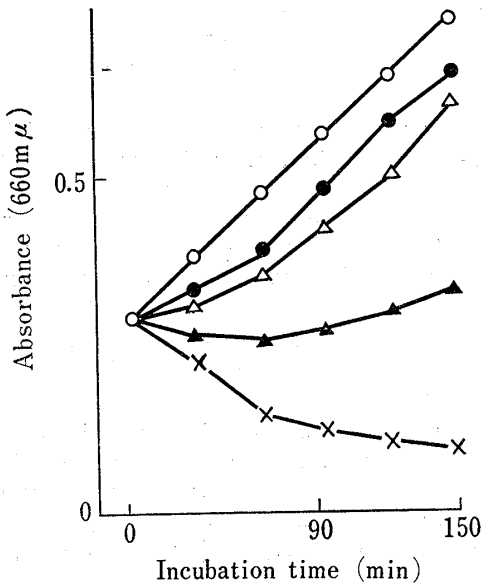


Fig. 1. Effect of II on the Growth of *B. subtilis*

○—○ control ●—● 1.0 μg/ml of II
△—△ 2.0 μg/ml of II ▲—▲ 4.0 μg/ml of II
×—× 8.0 μg/ml of II

the former was conveniently employed throughout the experiments.

In order to examine more closely the effect of II on the growth of *B. subtilis*, further experiments were carried out. The bacterial cells in exponential growth were harvested by centrifugation, suspended in glucose–salts medium, and incubated under shaking at 37°. The growth rate of the cells was followed by measuring the changes in absorbance (660 mμ) at 30 min intervals. As shown in Fig. 1, the growth curves on exposing the cells to different concentration of II indicated that the exposure of 4.0 μg of II to cells (approximately 5×10^8 cells/ml) resulted in markedly inhibiting the bacterial cell growth, whereas only slight growth retardation was observed with 1.0 or 2.0 μg of II. Prominent decrease of turbidity at 660 mμ was observed in 8.0 μg/ml concentration of II. Further incubation of this cell suspension, after making a ten-fold dilution with fresh medium, did not give rise to increase of optical density.

Effect of II on DNA, RNA and Protein Syntheses in Growing Cells

To determine whether the antibacterial activity of II depends on the blocking of macromolecular synthesis in growing cells, the influence of II on nucleic acids and protein syntheses was investigated, as compared with those of mitomycin C and actinomycin D. The results are presented in Table II. As is seen from this table it may be noticed that net synthesis of RNA was fairly affected by the presence of II in concentration of 2.0 μg/ml, as well as in case of 0.13 μg/ml dose level of actinomycin D.

Actinomycin D has been seen to inhibit DNA dependent RNA polymerase (2.7.7.6.) activity by means of binding to primer DNA.[9] However, in this line of work the activity of RNA polymerase was little affected with II,[10] when it was tested along with actinomycin D as an inhibitor for RNA synthesis.

In addition, the results of difference spectra measurements indicated that *in vitro* interactions of II with nucleic acids were almost negligible.

In contrast, the influence of II on net synthesis of DNA and protein was found to be less than that in case of RNA under the same conditions.

Table II. Effect of II on Nucleic Acids and Protein Syntheses in *B. subtilis*

Chemical	Concentration μg/ml	Turbidity A. 660 mμ		DNA Burton: A. 600 mμ		RNA Orcinol: A. 660 mμ		Protein Lowry: A. 600 mμ	
		60[a]	120	60	120	60	120	60	120
II	1.0	0.49	0.59	—[b]	—	—	—	—	—
	2.0	0.34	0.47	0.087	0.154	0.340	0.476	0.327	0.480
	4.0	0.25	0.30	0.057	0.050	0.127	0.394	0.260	0.407
Actinomycin D	0.06	0.42	0.46	0.101	0.156	0.411	1.114	0.296	0.685
	0.125	0.34	0.36	0.076	0.119	0.186	0.722	0.224	0.396
	0.25	0.30	0.30	0.046	0.113	0.137	0.435	0.056	0.281
Mitomycin C	0.5	0.44	0.55	0.050	0.071	0.930	1.200	0.444	0.646
	1.0	0.37	0.44	t[c]	0.030	0.830	0.720	0.372	0.533
	2.0	0.30	0.30	t	t	0.334	0.231	0.234	0.410
Control		0.49	0.69	0.135	0.198	0.980	1.360	0.510	0.760

a) incubation time *b*) not examined *c*) trace amounts

Recently, Kirita[11] in this Laboratory found that bis (2-hydroxy-3,5-dibromophenylazo)-4-*n*-propylphloroglucinol (IV), an anticancerous azo compound,[12] was effective to inhibit the cell growth of *Streptococcus faecalis*, and that the inhibition by IV was recovered by the addition of folinic acid, but not by folic acid into the culture medium. He assumed that IV might act on some metabolic sites participating the C_1-unit transfer reactions. From resemblance of chemical structure of II with that of IV, it was supposed that the same is true with II.

Effect of II on Energy Metabolism of *B. subtilis*

To investigate whether II may influence energy metabolism or not, determinations of respiration and anaerobic glycolysis were manometrically performed by standard Warburg's technique. As is seen in Table III, at the concentration of 10 μg/ml or more II clearly disturbed both respiration and anaerobic glycolysis of the cell in above stated environment. Inhibition of these reactions will cause the diminution of the level of adenosine triphosphate in the cell, which in turn supposedly results in the decrease of net RNA synthesis in *B. subtilis*. These results seem to be analogous to the data presented by Laszlo, *et al.*,[13] who observed an inhibition of respiration and glycolysis of human leukemic leukocytes in the presence of actinomycin D, and indicated that the inhibitory effects on respiration and on RNA synthesis could not be dissociated from one another.

9) I.H. Goldberg, M. Rabinovitz, and E. Reich, *Proc. Natl. Acad. Sci. U.S.*, **48**, 2094 (1962); J. Hurwitz, J.S. Furth, M. Maloney, and M. Alexander, *ibid.*, **48**, 1222 (1962).
10) Unpublished datum.
11) T. Kirita, *Kanazawa Kekken Nempo*, **24**, 191 (1967).
12) R. Hirata, *Japan. J. Exp. Med.*, **27**, 99 (1957).
13) J. Laszlo, D.S. Miller, K.S. McCarty, and P. Hochstein, *Science*, **151**, 1007 (1966).

Table III. Effect of II on Respiration and Anaerobic Glycolysis in *B. subtilis*

Chemical[a]	Concentration μg/ml	Respiration (Q_{O_2}) time (min)		Anaerobic glycolysis ($Q^{N_2}_{CO_2}$) time (min)	
		60	120	60	120
II	5.0	3.22	2.00	—[b]	—
	10.0	1.10	0.78	2.15	1.62
	20.0	0.78	0.44	1.63	1.14
Actinomycin D	0.3	1.89	1.26	—	—
	0.6	1.73	1.17	—	—
Mitomycin C	2.5	3.84	3.31	—	—
	10.0	2.86	1.98	—	—
Control	.[c]	3.54	2.60	2.72	2.39
	.	4.98	3.60	—	—

a) II and actinomycin D in buffer solution containing ethanol in 5% were added to the cell suspensions.
b) not examined c) Added 5% ethanol.

Influence of Possible Products Derived from II on the Growth of *B. subtilis*

Chatterjee, *et al.*[14] indicated the lability of some Schiff bases including N-salicylidene aniline, N-benzylidene-*o*-hydroxyaniline and so on in the presence of water. In their spectrometrical studies, these Schiff bases were hydrolysed in a matter of minutes in 50% water–dioxane and almost instantly in acidified conditions. In the case of II, no significant change in its absorption spectrum of ethanolic solution was observed even after a few days. There occurred, however, rapid spectral changes in 30% alcoholic solution of II as shown in Fig. 2. These changes may be due to hydrolysis of II to yield III and *o*-aminophenol (V).

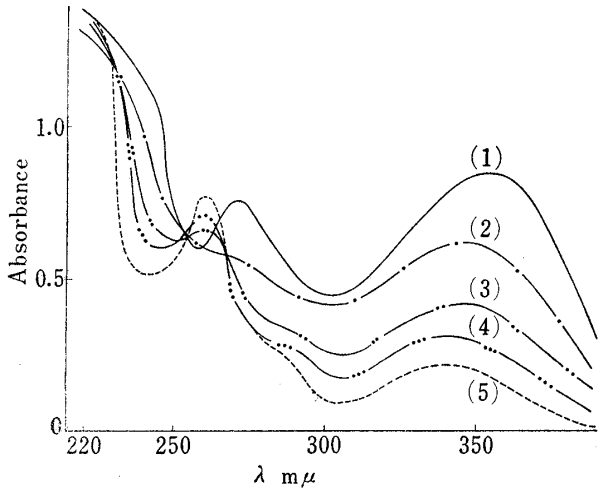


Fig. 2. Changes in Absorption Spectra of II in Ethanol (1), in 10^{-2}M Tris–Buffer Containing 30% Ethanol (pH 7.2), (2—5); (2) at 0 Time, (3) at 30 min, (4) at 60 min, and (5) at 2 Days after Mixing

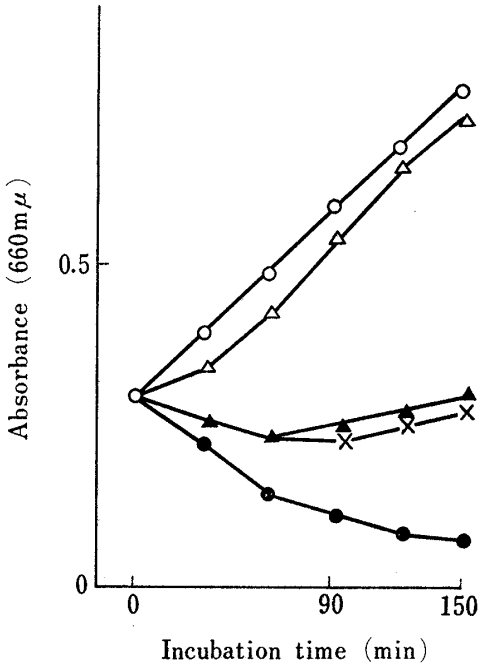


Fig. 3. Comparative Effect of II, III, and V on the Growth of *B. subtilis*

II (●—●, 2.7×10^{-5}M), III (▲—▲, 4×10^{-5}M), V (△—△, 8×10^{-5}M), and III plus V (×—×), control (○—○)

14) K.K. Chatterjee, N. Farrier, and B.E. Douglas, *J. Am. Chem. Soc.*, **85**, 2919 (1963).

Because of instability of II in the presence of water, alcoholic solution of II was directly added into culture media to give appropriate concentrations. While, the results of comparative antibacterial experiments with II, III and V suggested that the activity was not always ascribed to its hydrolysates. But, it could not deny the possibility that biological effect of II might be partly due to the hydrolysates (Fig. 3).

Influence of Various Metallic Ions on Growth Inhibition of *B. subtilis* by II

Pfeiffer, *et al.*[15] demonstrated the metal complex forming activity of N-salicylidene-*o*-aminophenol (VI) by way of isolation of complex (1:1) with metallic ions (Cu^{2+} and Ni^{2+}). II which has VI moiety in its structure was shown to bind similarly with cupric ion to give water insoluble complex (1:1). The results of spectrometrical studies revealed that II had an interaction to form metal–complexes with metallic ions including Cu^{2+}, Co^{2+}, Ni^{2+}, Ag^{1+}, Zn^{2+}, Pb^{2+}, Fe^{2+}, and Fe^{3+} in alcoholic solutions, but Mg^{2+}, Mn^{2+}, Ca^{2+}, and Ba^{2+} were not reactable with II under the same conditions (Fig. 4).

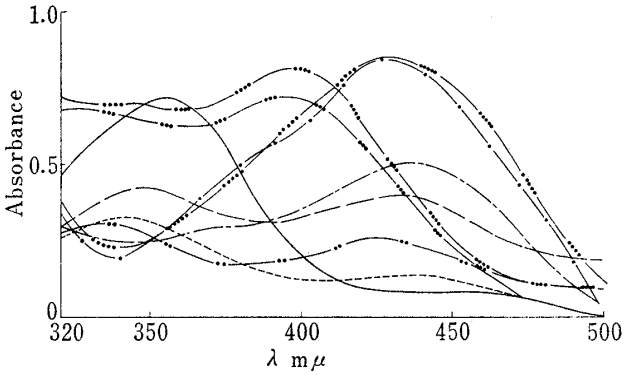


Fig. 4. Complex Formation of II (——) with Cu^{2+} (—·—), Ni^{2+} (—··—), Fe^{2+} (—···—), Fe^{3+} (——), Co^{2+} (——), Ag^{1+} (— —), Pb^{2+} (— - —), and Zn^{2+} (------) in Ethanol

concentration: II, metallic salt (10^{-4} M)

These data seemed of interest in suggesting that the mechanism of metallic competition might be involved in biological activity of II. Therefore, several metallic ions were tested for their ability to reverse the inhibitory effect of II on the growth of *B. subtilis*.

As shown in Fig. 5, the inhibition of multiplication of bacterial cells by II was markedly restored by the addition of 10^{-4} M of Mn^{2+} or Fe^{2+} salt, or of 10^{-3} M of Fe^{3+} salt in final concentration. While, Mg^{2+}, Ca^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+} ions in each even in the concentration of 10^{-3} M had no influence on the growth-inhibitory effect of II.

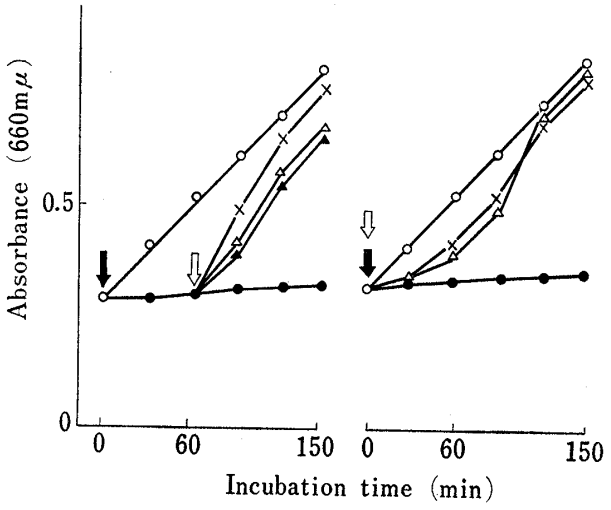


Fig. 5. Influence of Metallic Ions (Mn^{2+}, Fe^{2+}, Fe^{3+}) on II Inhibition of *B. subtilis*

addition of II (⇩), and of metallic salts (⇩)
control (○—○), II, 4 μg/ml (●—●), Fe^{2+}, 10^{-4} M (×—×), Mn^{2+}, 10^{-4} M (△—△), Fe^{3+}, 10^{-3} M (▲—▲)

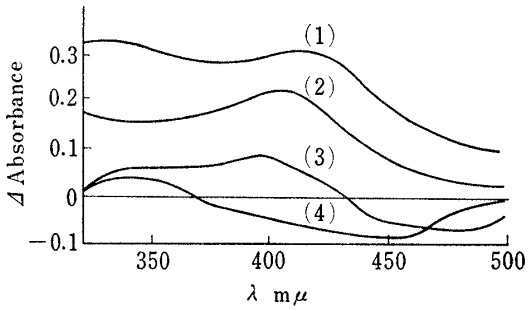


Fig. 6. Difference Spectra of II with Nucleic Acids in the Presence of Metallic Ions

II (8.5×10^{-5} M) in 30% ethanol–tris buffer (pH 7.2)
(1) II, Fe^{3+} (10^{-4} M), DNA (0.03%)
(2) II, Fe^{3+} (10^{-4} M), DNA (0.003%)
(3) II, Fe^{3+} (10^{-4} M), RNA (0.03%)
(4) II, Co^{2+} (10^{-4}), RNA (0.03%)

15) P. Pfeiffer, T. Hesse, H. Pfitzner, W. Scholl, and H. Thielert, *J. Prakt. Chem.*, **149**, 216 (1937).

Spectrometrical studies on the interaction between II and nucleic acids gave a negative results in despite of presence or absence of Mg^{2+} ions, but there was observed some interaction between nucleic acids and II in the presence of Fe^{3+} or Co^{2+} ion as indicated in Fig. 6.

Anyway, the data that the cell growth inhibition by II was reversed by the addition of Mn^{2+} or Fe^{2+} or Fe^{3+} to culture medium indicate that metal complex forming activity of II might be contributing to the antibacterial events.

Although complete data to elucidate the mechanism of action of II against *B. subtilis* were not in hand, it may, at least, be said that depression of RNA synthesis in one hand and suppression of respiration and anaerobic glycolysis in the other are responsible for the inhibition of growth of *B. subtilis*. Furthermore, experimental results so far obtained support that metal chelating activity of II also might be contributing to the inhibition mechanism.

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