Studies on the Antibiotic Action of the Bacterial Pigment, Iodenin : II.Lysis of Bacteria by Iodinin

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II. Lysis of Bacteria by Iodinin

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INTRODUCTION

Various kinds of microorganism were found to be susceptible to growth inhibition by iodinin, 1,6-phenazinediol-5,10-di-N-oxide, at a concentration of 1 to 30 *μg/ml*, especially members of the genus *Bacillus* were sensitive to iodinin[1]. Phenazine-5-N-oxide shows a strong bacteriostatic activity against *Xanthomonas oryzae* and as to the mechanism of bacteriostatic action by phenazine-5-N-oxide, it may be described that it caused delay in reduction of the cytochrome system of *X. oryzae*[2,3]. Reactions of iodinin with *Bacillus* were investigated in respect of inhibition of bacterial growth and respiration. This report describes an approach to the elucidation of the mode of growth inhibition of *Bacillus* by iodinin.

MATERIALS AND METHODS

Organisms. Some authentic strains were served for investigation on the mechanism of antibacterial action by iodinin. *Bacillus megaterium* IAM 1030 and *Bac. megaterium* IFO 3003 were employed for iodinin-sensitive strains, and *Bac. cereus* IAM 1029 and *Escherichia coli* IFO 3806 for iodinin-insensitive strains.

The cells were collected by means of centrifugation of a 24-hr culture in nutrient broth at 3,000 or 5,500 r.p.m. for 15 minutes. Twice washed with *M*/10 phosphate buffer solution (*pH* 7.4), the cells were re-suspended in the same solution. The optical density of the intact cell suspension was adjusted to 1.0, corresponding to the dried cell weight of approximately 4.0 *mg* in a milliliter of the suspension. In order to prepare a sonicate of bacterium, a cell suspension was given a concentration of 0.5 to 1.0 *g* wet cell weight/*ml*.

Methods for measuring. Changes in the number of viable cells, amounts of protein

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leaking out of the cells after treatment with iodinin, and lactic acid, were determined by the plating method, the method of LOWRY et al.[4], and the method of BARKER and SUMMERSON[5], respectively.

Amounts of iodinin were determined spectrophotometrically by the method of TANABE and OBAYASHI[6].

Lipids of bacterial cells were extracted by the method of CARD et al.[7], and that of LANG and LUNDGREN[8].

Reduction of the cytochrome system of bacteria was observed on the differential spectrum, employing Hitachi recording spectrophotometer. Reaction mixture was prepared according to the method described in Table 1.

Table 1. Reaction mixture

	Ox.-form	Red.-form
cell suspension (1/50 O.D.=0.4—0.5)	1.9*ml*	1.9*ml*
0.1*M* Phosphate buffer (*pH* 7.4)	1.0*ml*	1.0*ml*
0.01 *M* Potassium Ferricyanide solution	0.03*ml*	—
Deionized water	0.07*ml*	0.1*ml*
$Na_2S_2O_4$	—	0.1-1.0*mg*

Oxygen uptake by intact cells was determined by the oxygen consumption recorder at 30° *C* in the same way as described by WONG et al.[9] The reaction mixture in the vessel consisted of 2.4 *ml* of the cell suspension, in *M*/10 phosphate buffer solution, at *pH* 7.0 and in 0.1 *ml* of 0.1 % glucose solution.

RESULTS AND DISCUSSION

Effect of iodinin on intact cells

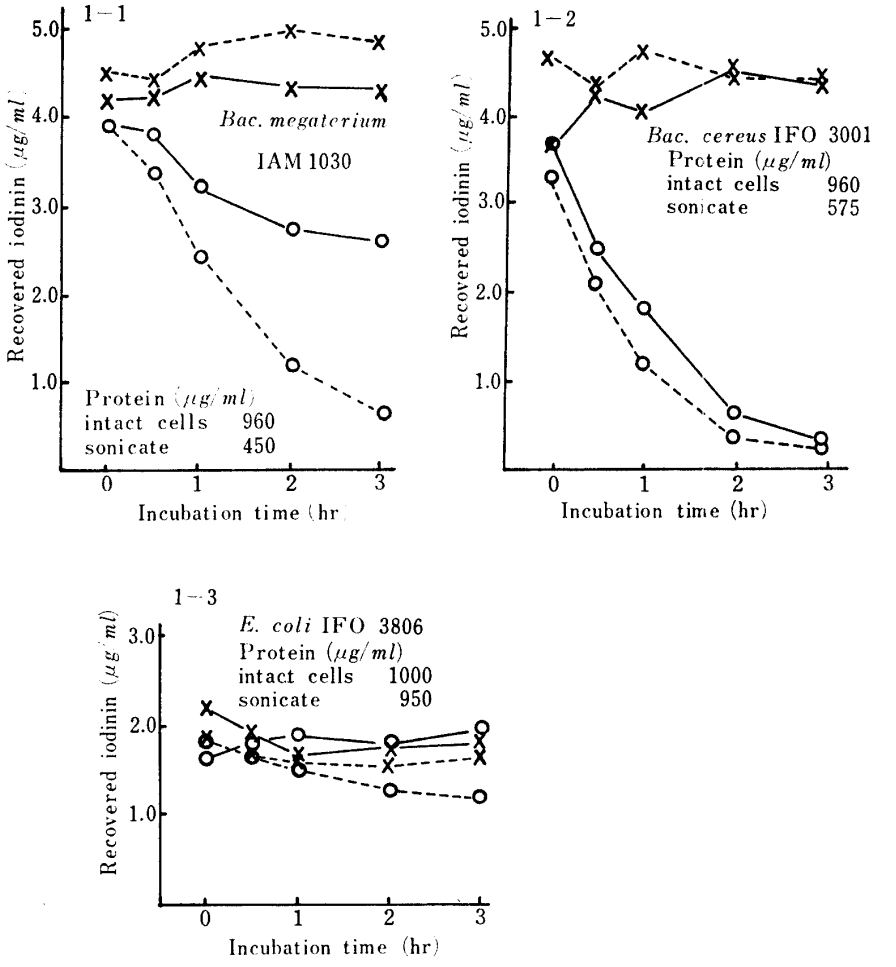
Inhibitory effect of iodinin was investigated with intact cells of iodinin-sensitive strain, *Bac. megaterium* IAM 1030. Changes in the number of viable cells and the amount of protein leaking out of cells after the addition of iodinin were determined by the plating method and the method of LOWRY et al.[4], respectively. Decrease in viable cell counts was very small and the amount of leaking protein was negligible, compared with the total protein content of the cells, as shown in Table 2. These results suggest that intact cells were hardly affected by iodinin.

Reaction with intact cells and sonicate

Reduction of iodinin was carried out with intact cells and sonicate of iodinin-sensitive strain, *Bac. megaterium* IAM 1030 and iodinin-insensitive strains, *Bac. cereus* IFO 3001 and *E. coli* IFO 3806, as shown in Fig. 1. Intact cells of two strains of *Bacillus* reduced iodinin to 1,6-phenazinediol, and *E. coli* hardly reduced iodinin. Iodinin was reduced neither by the sonicates of two strains of *Bacillus* nor by those of *E. coli*, whether glucose as substrate was in presence or not.

Table 2. Viable cell counts and protein content.

hours after addition of iodinin	viable cell counts (No./*ml*)	Protein content (Supernatant)	(Cells)
0	4.5×10^7	4.4$\mu g/ml$	960$\mu g/ml$
2	3.2×10^7	7.5$\mu g/ml$	—
control (not added) 2	4.2×10^7	5.0$\mu g/ml$	—



(no glucose) (in the presence of glucose)

intact cells ..... ○—○, ○...○

sonicate ..... ×—×, ×...×

Fig. 1. Reactions of intact cells and sonicates with iodinin.

Effect on lactic acid fermentation

Effect of iodinin on lactic acid fermentation was investigated by using the intact cells of lactic acid bacteria. The cells of *Lac. acidophilus* IFO 3831, iodinin-sensitive, fermented a great amount of added glucose into lactic acid both in the absence of iodinin and in the presence of it, as shown in Table 3. As inhibition of lactic acid fermentation was not observed, both permeation of glucose into the cell and glycolysis in the cell were considered to be unaffected by iodinin.

Table 3. Effect of iodinin on lactic actic acid fermentation.

	lactic acid produced
control	960 *μg/ml*
iod. added (10*μg/ml*)	970 *μg/ml*

Used strain : *Lac. acidophilus* IFO 3831

* Reaction mixture :

cell suspension (dr. wt. 4.9 *mg*)	0.35 *ml*
M/20 phosphate buffer (*pH* 6.8)	0.40 *ml*
6 *μM* glucose solution	0.25 *ml*
iodinin	10*μg/ml*
Total	1 *ml*

** at 30°*C*. 1 hr.

Effect of iodinin on the bacterial growth

Ten *μg/ml* of iodinin was added to the growing cultures at an optical density of approximately 0.1, 0.2, and 0.4, respectively. The time courses of multiplication of various strains after the addition of iodinin to the culture were shown in Fig. 2. A rapid fall in the optical density of the cultures of *Bac. megaterium* IAM 1030 and *Bac. megaterium* IFO 3003, showing lysis of bacterial cell, appeared at the time when iodinin was added to the growing culture, as shown in Fig. 2-1 and 2-2. Growth inhibition of *Bac. cereus* IAM 1029 by iodinin at concentrations of 10 *μg/ml* and 30 *μg/ml* was observed for several hours after the addition of iodinin to the culture, as shown in Fig. 2-3. *E. coli* IFO 3806 was not susceptible to growth inhibition by iodinin, as shown in Fig. 2-4. A great decrease in the viable cell counts of *Lac. acidophilus* IFO 3831 and *Lac. sake* 012, both sensitive to iodinin, appeared after the addition of iodinin to the culture, although an increase in the optical density of the culture was observed as shown in Fig. 3-1 and 3-2. This fact indicates that the increase of dead cells exceeds that of viable cells. Growth of *Lac. plantarum* 11, insensitive to iodinin, was not affected by iodinin. An increase in the optical density and the viable cell counts were shown in Fig. 3-3.

Effect of iodinin on the cells at various growth phases

As shown in Fig. 4, lysis of cells occurred only when iodinin was added to the culture of *Bac. megaterium* IAM 1030 at the early logarithmic growth phase, at which the optical density of the culture was 0.25 to 0.5. Therefore, it is elucidated that iodinin acts only on actively growing cells at the early stage in the time course of growth.

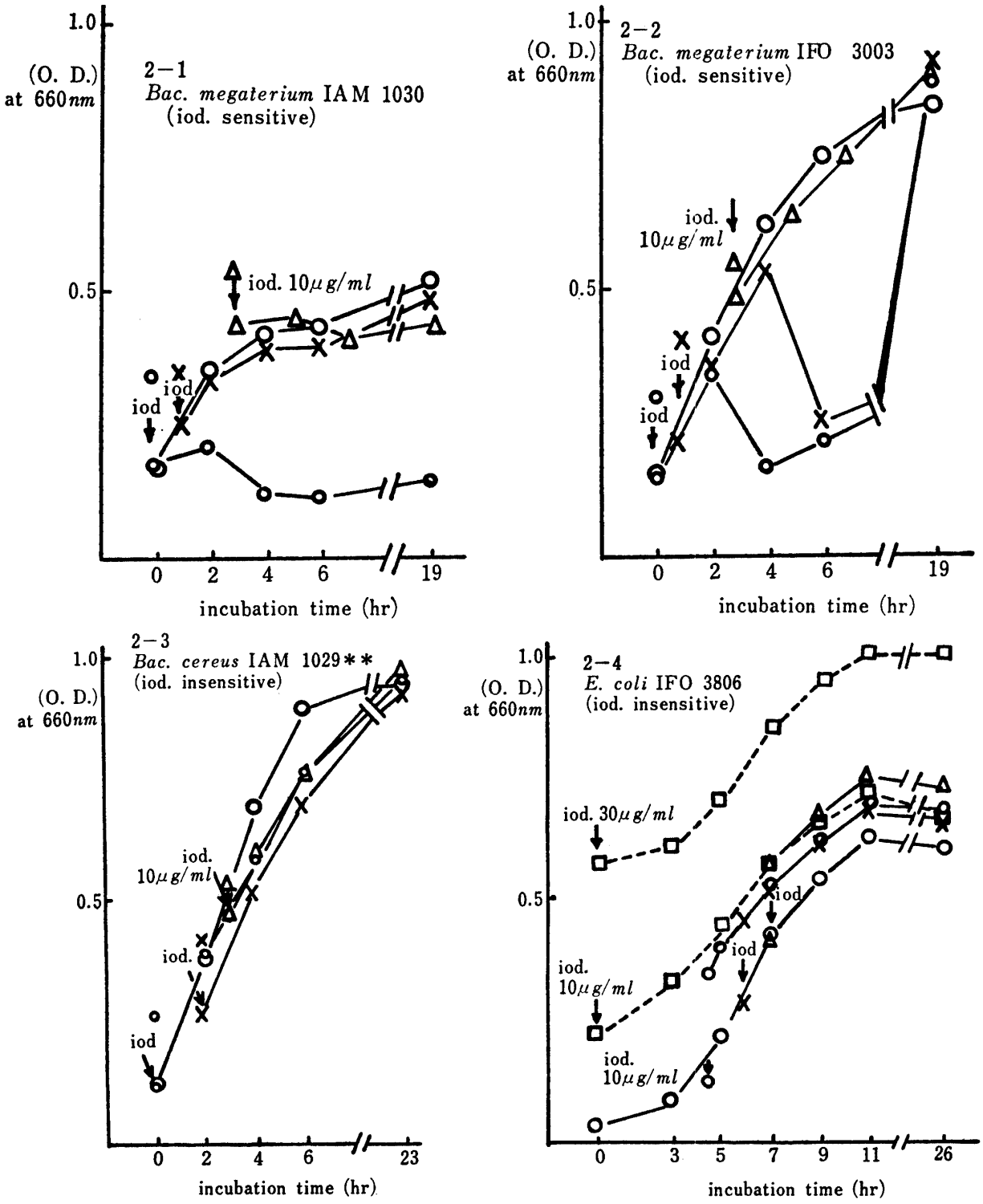


Fig. 2. Effect of iodinin on growth of bacteria in nutrient broth.

* An arrow shows the addition of iodinin.

**

iod. conc. ($\mu g/ml$)	incubation time (hr) 6	13	24
30	−	−	+
10	−	+	+
0	+	+	+

+ : growth
− : no growth

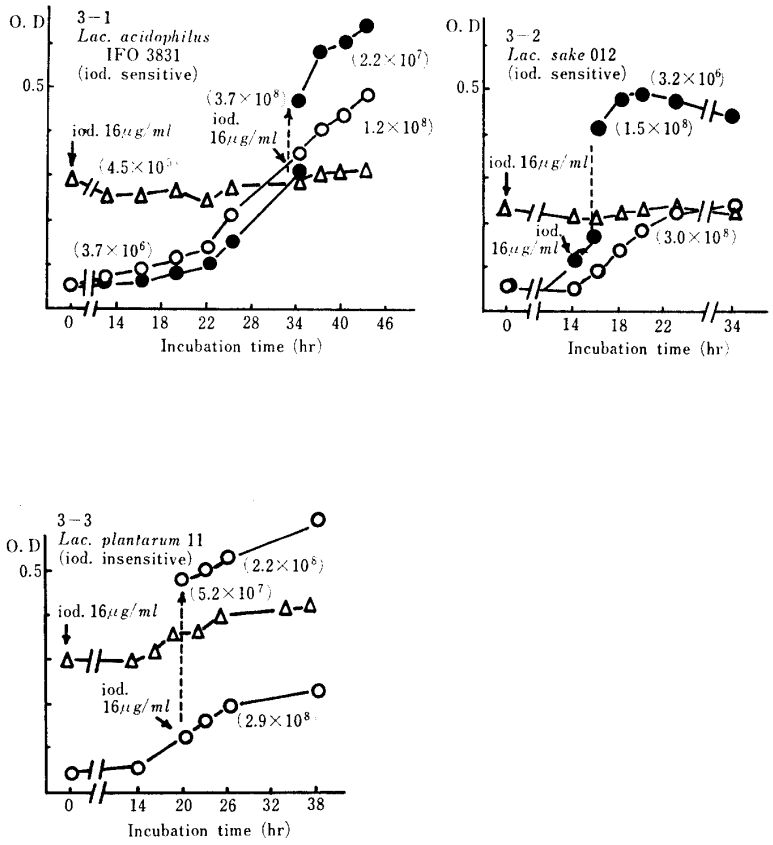


Fig. 3. Effect of iodinin on growth of lactic acid bacteria.
* A number in a parenthesis shows viable cell counts.
** An arrow shows the addition of iodinin.

When iodinin was added to the culture at an optical density of 1.0 at 660 *nm*, the turbidity of the culture increased continuously for 1 hr after the addition of iodinin to a concentration of 25 *μg/ml*, and then it stopped increasing and remained unchanged. Distortion of the cells of *Bac. megaterium* IAM 1030, exposed to iodinin, was observed under a microscope by means of Gram staining. Protoplasmic protrusions appeared 1 hr after exposure to iodinin. Further incubation led to increase in size of protrusions, and appearance of vacuolated protoplasts. The whole process goes probably quite rapidly, judging from a quick decrease in the optical density.

Bacterial lysis in hypertonic nutrient broth

In order to ascertain whether lysis of bacterial cells by iodinin is to be observed in hypertonic solution or not, iodinin was added to the growing culture of *Bac. megaterium* IAM 1030 in the nutrient broth containing sucrose at a concentration of 0.3 *M*. Lysis of bacterial cells by iodinin also appeared under the experimental conditions, as shown in Fig. 5. It seemed that both cell wall and cytoplasmic membrane of the growing cells were damaged by iodinin.

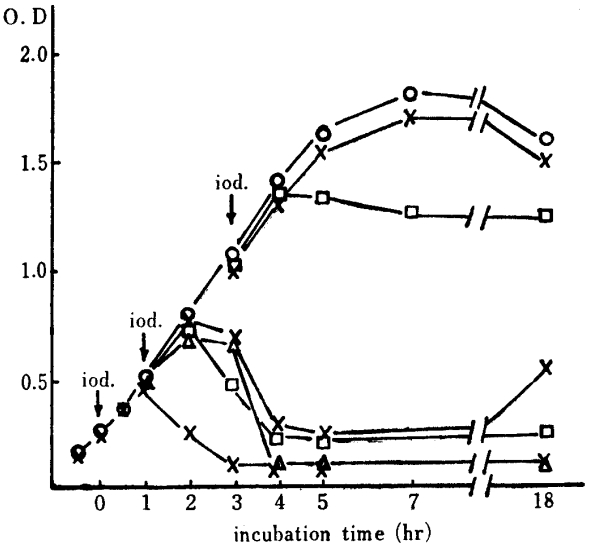


Fig. 4. Effect of iodinin on growth of *B. megaterium* IAM 1030 at various growth phases.
○: control. △: iod. 2.5$\mu g/ml$. ×: iod. 5$\mu g/ml$. □: iodinin 25$\mu g/ml$.
* An arrow shows the addition of iodinin.

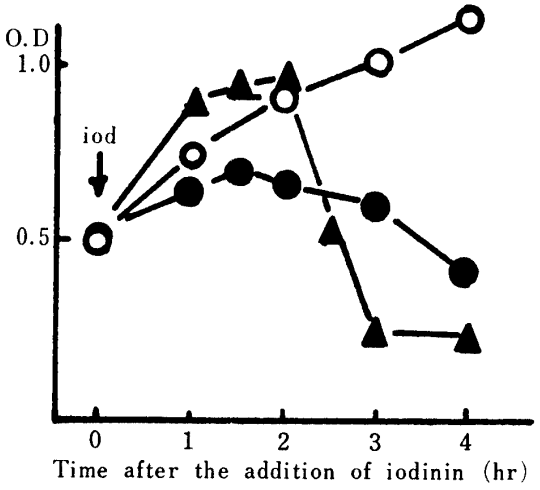


Fig. 5. Lytic action in nutrient broth containing sucrose at a concentration of 0.3 *M*.
○: control. ●: iod. 10 $\mu g/ml$.
▲: iod. 10$\mu g/ml$ (nutrient broth containing no sucrose).
* An arrow shows the addition of iodinin.

Composition of phospholipid of the cells

It was researched whether or not a change in the lipid composition of cytoplasmic membrane of bacteria is to be caused in a rapid lysis of the cells of *Bac. megaterium* IAM 1030, by iodinin. The cells were collected before, and after the addition of iodinin to the culture at the incubation time, as shown in Fig. 6 and subjected immediately to extraction of lipid by the method of CARD et al.[7], and that of LANG AND LUNDGREN[8]. Thin layer chromatogram of the extracted lipids was shown in Fig. 7. As shown in Fig. 7, there was no appreciable difference in lipid composition between the normal cells and the cells treated by iodinin. This result suggests that a lysis of bacterial cell by iodinin is not to be explained by disintegration of the membraneous lipids.

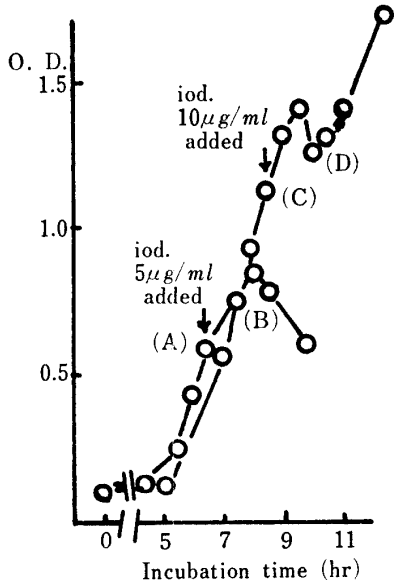


Fig. 6. Growth of *Bac. megaterium* IAM 1030 in nutrient broth.
* An arrow shows the addition of iodinin.

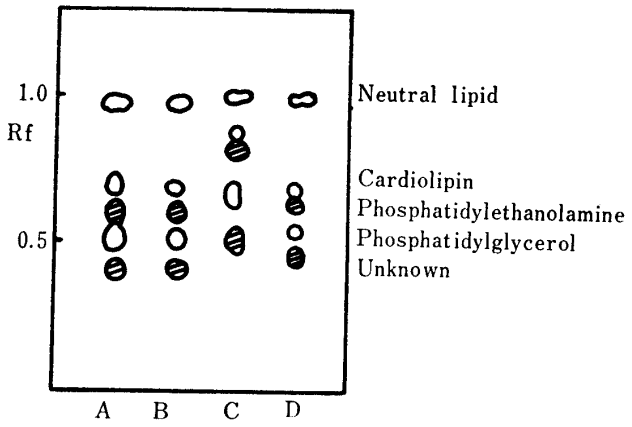


Fig. 7. Thin layer chromatogram of lipids extracted from *Bac. megaterium* IAM 1030.
* The cells employed for extraction were obtained from the culture before and after the addition of iodinin at the incubation times shown in Fig. 6 (A, B, C, and D). Spots were demonstrated with I_2 vapors, Dittmer's reagent and ninhydrin. Ninhydrin-positive components are shaded.

Lysis of bacterial cells by iodinin in the medium supplemented with yeast extract or polypepton

Lysis of the cells of *Bac. megaterium* IAM 1030 by iodinin apeared in the medium supplemented more rapidly than in the medium not supplemented, as shown in Fig. 8 and 9. In this case, iodinin was added to the culture at a very early stage of the logarithmic growth phase, at which the culture reached an optical density of 1.0 at 660 *nm*. Lysis of the cells of the other two strains, *Bac. megaterium* IFO 3003 and *Bac. cereus* IFO 3001 by iodinin was investigated in the same way, as shown in Fig. 10. Elongation of the cells was observed in both the strains in the presence of iodinin. Two hours after the addition of iodinin, a rapid lysis appeared in the culture of *Bac. megaterium* IFO 3003 as well as in that of *Bac. megaterium* IAM 1030, whereas no lysis appeared in the culture of *Bac. cereus* IFO 3003. Probably a long time is needful for iodinin to penetrate through cell membrane because of the insolubility of iodinin in water. Amounts of the pigment recovered with chloroform from the growing culture of *Bac. megaterium* IAM 1030 decreased with the time of incubation, as shown in Fig. 8-2. The spectrum of the pigment recovered indicates the presence of iodinin and 1,6-phenazinediol.

Effect of the addition of riboflavin

Growth inhibition of *Streptococcus haemolyticus* by iodinin, and natural antagonists to iodinin, naphthoquinones and anthraquinones, were presented by McILWAIN[10]. It was found that riboflavin deficiency was caused by the addition of phenazine analogues in the culture of *Lac. casei*, and the inhibitory effects were eliminated by the addition of sufficient amounts of riboflavin to the culture[11]. Varying amounts of riboflavin were supplemented to the nutrient broth, since iodinin was considered to be a structural analogue to riboflavin. As shown in Fig. 11, the growth of *Bac. megaterium* IAM 1030 in the nutrient broth was inhibited by iodinin at a concentration of 20 *μg/ml*. In the

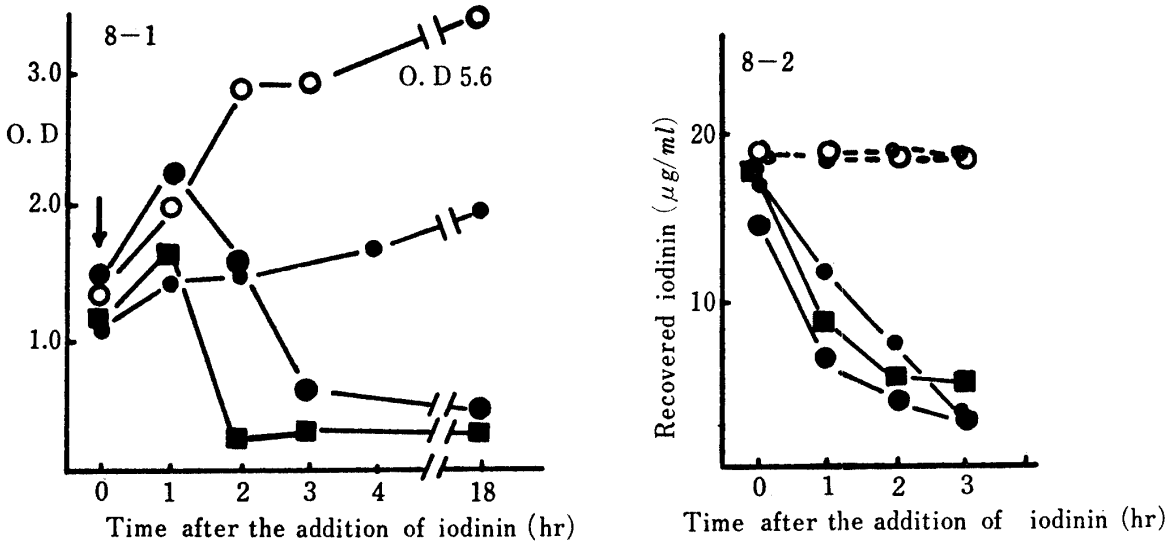


Fig. 8. Lytic action by iodinin in the nutrient broth supplemented with yeast extract and the amounts of iodinin recovered from the culture broth.

○ : yeast extract (1%), control (no iodinin)
● : yeast extract (1%), iod. (20 *μg/ml*) added
■ : yeast extract (2%), iod. (20 *μg/ml*) added
● : nutrient broth , iod. (20 *μg/ml*) added
○----○ : yeast ext. (1%), (no inoculation of microorganisms)
○----○ : nutrient broth, (no inoculatron of microorganisms)

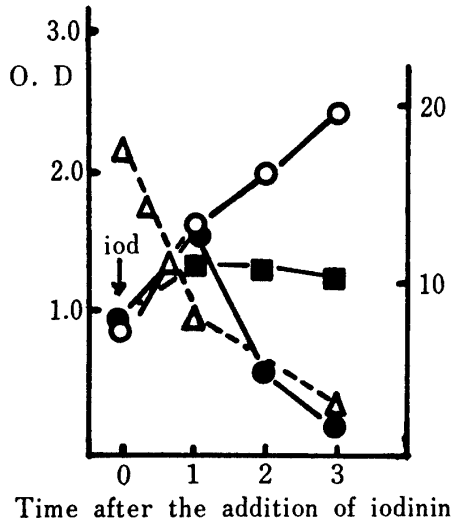


Fig. 9. Effect of iodinin on the growth of *B. megaterium* IAM 1030 in nutrient broth supplemented with polypepton (2 %).

○—○ : control ●—● : iod. 20*μg/ml*
■—■ : iod. 25 *μg/ml* (nutrient broth)
△---△ : recovered iodinin.

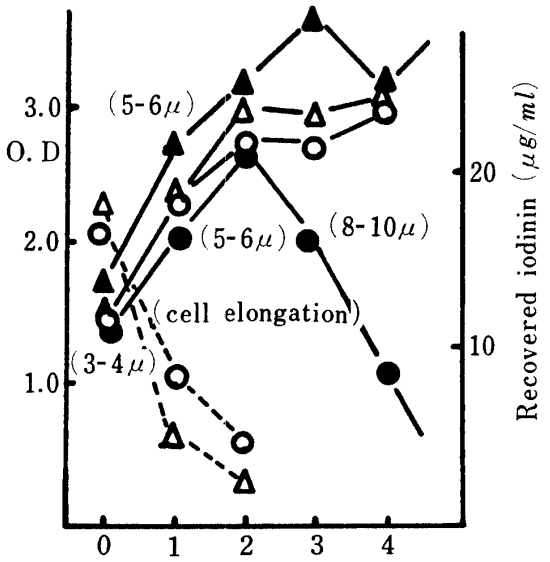


Fig. 10. Effect of iodinin on the growth of *B. megaterium* IFO 3003 and *B. cereus* IFO 3001 in nutrient broth, containing yeast extract (1%).

(*B. megaterium*) (*B. cereus*)
○—○ : control △—△ : control
●—● : iod.(25*μg/ml*) ▲—▲ : iod.(25*μg/ml*
○---○ : iod. recovered, △---△ : iod. recovered.

nutrient broth containing riboflavin, however, an increase in the optical density was also observed after the addition of iodinin and the growth inhibition by iodinin seemed to be removed by the addition of riboflavin.

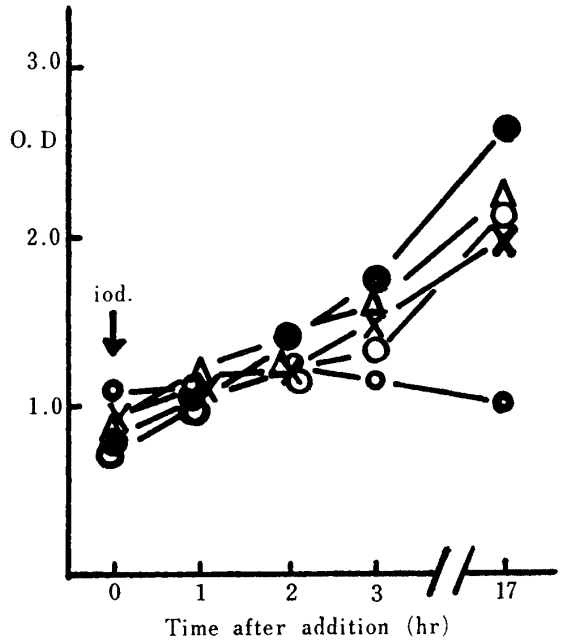


Fig. 11. Effect of iodinin on the growth of *B. magaterium* IAM 1030 in nutrient broth supplemented with various concentrations of riboflavin.

● : ribofiavin 50*μg/ml* (control). × : riboflavin 10*μg/ml*, iod. 20*μg/ml*.
△ : riboflavin 20*μg/ml*, iod. 20*μg/ml*. ○ : riboflavin 50*μg/ml*, iod. 20*μg/ml*.
○ : nutrient broth, iod. 20*μg/ml*.

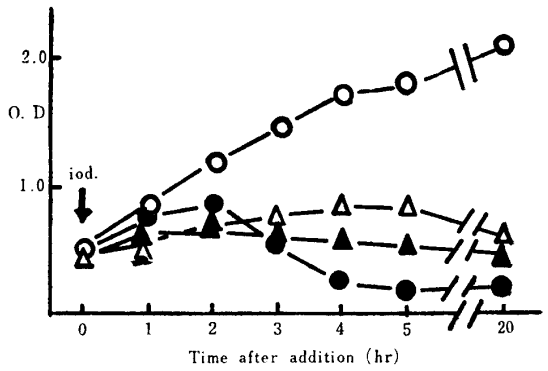


Fig. 12. Effect of chloramphenicol on lytic action by iodinin.
Used strain : *B. megaterium* IAM 1030.
○ : control. ● : iod. 25 *μg/ml*.
△ : chloramphenicol 50 *μg/ml*.
▲ : chloramphenicol 50 *μg/ml*, iod. 25 *μg/ml*.

Effect of chloramphenicol on the lysis of the cells by iodinin

Iodinin acts only on the actively growing cells at the early stage of logarithmic growth phase. It was researched whether chloramphenicol is to have influence on the lysis of bacterial cells by iodinin, or not. When both iodinin and chloramphenicol were added at the same time to the growing culture of *Bac. megaterium* at an optical density of 0.5, an antilytic action by chloramphenicol was observed, as shown in Fig. 12. The fact indicates that lysis of bacterial cells by iodinin is not to be brought forth, when growth of the bacterial cells at the early stage of logarithmic growth phase was inhibited previously by chloramphenicol.

Effect of *iodinin upon the bacterial respiration*

The differential absorption spectrum of cytochromes of *Bac. cereus* IFO 3001 was shown in Fig. 13. Non-enzymatic reduction of cytochromes was performed by sodium hydrosulfite. The absorption-bands of the reduced form of cytochrome *b* are γ band at 430 *nm*, β band at 524 *nm* and α band at 560 *nm*, that of cytochrome *a* being a band at 605 *nm*. The results obtained with the other strains were shown in Table 4. There was no significant difference between the spectra of cytochromes of iodinin-sensitive strains and those of iodinin-insensitive strains. As high absorption-peaks of the reduced cytochrome *b* were observed with iodinin-insensitive strains, *Bac. cereus* IAM 1029, *Bac. cereus* IFO 3001, and *Microbacterium flavum* IAM 1642, these strains were assumed to have a large amount of cytochrome *b* and consequently the ability to reduce iodinin strongly.

Effiects of iodinin on enzymatic reduction of cytochromes with the intact cells and the sonicate of *Bac. firmus* IFO 3330, employing succinate or NADH as a substrate, were shown in Figs. 14 and 15, respectively. The absorption spectrum of the reduced form of cytochrome *b* and that of cytochrome *c* showed a peak at 523 *nm* and a peak at 552 *nm*,

Table 4. Absorption bands in the differential spectrum of cytochromes of bactaria

Inhibition	Microorganism	(cytochrome b)						(cytochrome a_1)	
			γ-band		β-band		α-band		
±	Aerom. hydrophila IAM 1018	(± 405)	++ 430		+ 528	(+ 540)	++ 556	(+ 575)	
+	Bac. alvei IFO 3343	(± 420)	+ 428		± 530	—	+ 563	—	
±	Bac. c. var.mycoides IFO 3015	(+$_w$430)	+$_w$430		+ 530	—	+ 560	—	± 606
+	Bac. circulans IFO 3329	(± 415)	+ 434		+$_w$530	(+ 540)	+ 560	(+ 575)	± 605
+	Bac. firmus IFO 3330	(+ 420)	+ 433		+$_w$532	(+ 540)	+ 562	(+ 575)	—
+	Bac. megaterium IAM 1030	(+ 420)	—		+$_w$530	—	+ 560	—	± 605
+	Bac. megaterium IFO 3003	(± 422)	+ 424		+ 528	—	++ 558	—	—
+	Bac. natto IFO 3339	(++ 426)	++ 430		—	—	+ 562	—	—
+	Bac sphaericus IFO 3341	(+ 413)	± 432		—	(± 536)	—	(+$_w$573)	—
+	Bac. subtilis IFO 3007	(++ 422)	+ 430		+ 530	(± 540)	++ 560	(± 590)	+ 606
+	Bac. s. var. niger IFO 3108	(+ 417)	++ 431		+ 530	—	++ 562	(+ 585)	± 610
±	Cor. equi IAM 1038	(+ 425)	+ 434	(+ 458)	± 530	(± 544)	+$_w$565	(± 590)	
+	Micrococ. luteus IAM 1097		—		—	+	+ 560	(± 588)	—
+	Sar. lutea IAM 1099				+ 545	(+ 545)	+$_w$590	(+ 590)	—
+$_{24}$	Staph. aureus IAM 1011		—		+$_w$534	(+ 548)	+ 560	(+ 570)	+ 600
+$_{24}$	Br. ammoniagenes IAM 1641	(+ 415)	—		+$_w$534	(+$_w$540)	++ 560	(+$_w$590)	± 600

—	Bac. cereus IAM 1029	(卄 430)	卅 430		卄 526	—	卅 560	—	+ 605
—	Bac. cereus IFO 3001	(卅 430)	卅 430		卄 524	—	卄 560	(± 590)	卄 604
—	Microbac. flavum IAM 1642	(+ 408)	卅 435		卄 523	(+ 540)	卅 562	(+ 575)	+ 604 ($+_w$650)
—	E. coli IAM 1016	(+ 415)	卄 428		± 535	(+ 542)	卄 563	($+_w$578)	—
—	E. coli IFO 3806	(+ 418)	卄 434		$+_w$535		卄 565		± 600
—	Ps. aeruginosa Pk-199	(+ 410)	卅 430		+ 532	(+ 540)	卄 560	(+ 575)	—
—	Ps. iodinum IFO 3558		± 435		+ 530		卄 565		—

卅 : Relative absorption, above 20%. $+_w$: weak absorption.
卄 : Relative absorption, 10-20%. ± : very weak absorption.
\+ : Relative absorption, below 10%.
* A number in a parenthesis shows differential spectrum of cytochrome CO compounds.

respectively, at the time when succinate or NADH was added to the intact cells or the sonicate. Inhibition of cytochrome reduction by iodinin was not observed either with the intact cells or with the sonicate. Iodinin did not affect oxygen consumption of the intact cells of *Bac. firmus*, as shown in Fig. 16. These results suggest that growth inhibition by iodinin is not caused by inhibition of the respiration of the cells.

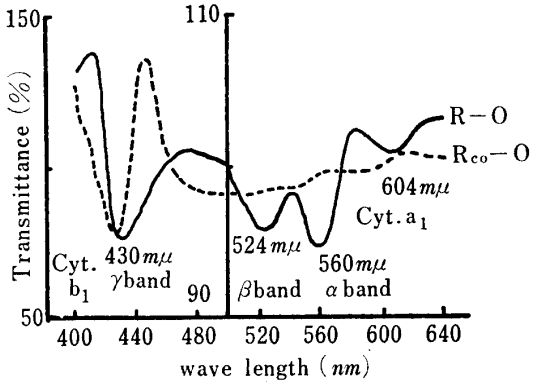


Fig. 13. Differential spectrum of cytochromes of *B. cereus* IFO 3001 (non-enzymatic reduction).
Reducing reagent : $Na_2S_2O_4$.

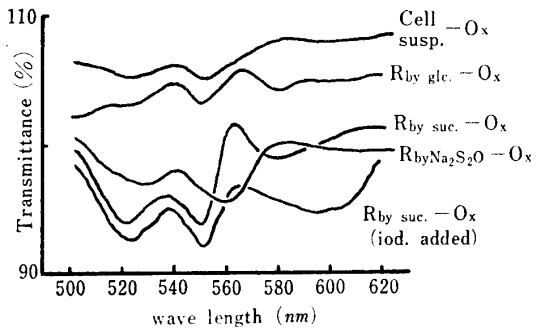


Fig. 14. Differential spectrum of cytochromes of *B. firmus* IFO 3330.
Substrate : succinate, and glucose.

SUMMARY

Various kinds of gram-positive bacteria, especially members of the genus *Bacillus*, are susceptible to growth inhibition by iodinin, 1,6-phenazinediol-5,10-di-N-oxide, therefore the reactions of bacterial cells with iodinin were investigated. No rapid lysis of the resting cells of the bacteria was caused by iodinin, but a lysis was caused by it in case of the cells of *Bac. megaterium* IAM 1030 and *Bac. megaterium* IFO 3003, at the early stage of the logarithmic growth phase. Iodinin was reduced by the intact cells of bacteria into the reduced form of iodinin, 1,6-phenazinediol, but not by the sonicate.

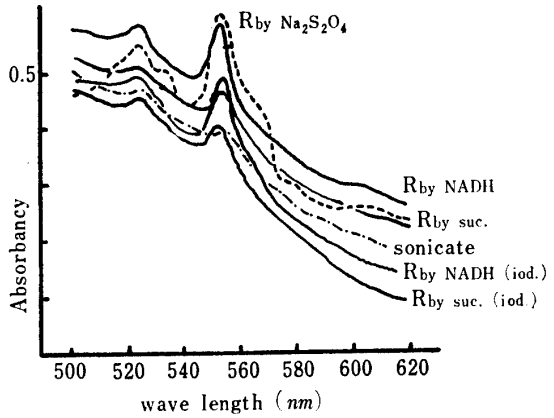


Fig. 15. Reduction of the sonicate of *B. firmus* IFO 3330
Substrate : 0.2*M* succinate, 0.2 *ml*, and 1 *mM* NADH, 0.01 *ml*
iod. conc.: 20 *μg/ml*.

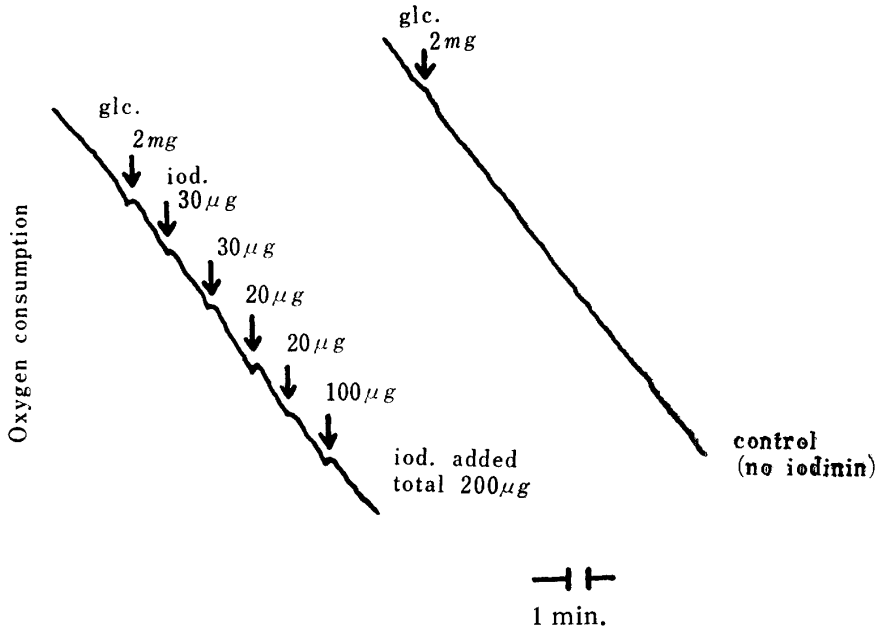


Fig. 16. Polarograph of oxygen consumption by the resting cells of *Bac. megaterium* IAM 1030 on glucose as substrate.

* Mesurement of oxygen consumption was taken with the oxygen consumption recorder at 30°*C*. Cells of *Bac. megaterium* IAM 1030 were suspended in *M*/10 phosphate buffer (adjusted to O. D. 1.0). One tenth percent of glucose solution (0.1*ml*) and iodinin were added into 2.4 *ml* of cell suspension.

Lactic acid fermentation of the intact cells of the lactic acid bacteria was not inhibited by iodinin. Therefore, it was considered that iodinin was not inhibitory to the permeation of glucose and to the glycolysis in the bacterial cells.

There was no significant difference in the differential absorption spectrum of the cytochromes between the iodinin-sensitive strains of bacteria employed and the iodinin-insensitive strains of bacteria employed. Iodinin was not inhibitory to the respiration of the resting cells of *Bac. megaterium* IAM 1030.

REFERENCES

1. TANABE, I., IIO, N., IMAMURA, R. and OBAYASHI, A.: *presented at the Annual Meeting of the Agricultural Society of Japan in 1970.* Abstracts, p. 320.
2. SEKIZAWA, Y., WATANABE, T. and ODA, M.: *Ann. Phytopathol. Soc. Japan*, **30**, 145-152 (1965).
3. ODA, M., SEKIZAWA, Y. and WATANABE, T.: *Appl. Microbiol.*, **14**, 365-367 (1966).
4. LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L. and RANDAI,I R. J.: *J. Biol.Chem.*, **163**, 265-275 (1951).
5. BARKER, S. B. and SUMMERSON, W. H.: *J. Biol. Chem.*, **138**, 535-554 (1941).
6. TANABE, I. and OBAYASHI, A.: *Mem. Fac. Agr. Kagoshima Univ.*, **8**, 373-389 (1971).
7. CARD, G. L., GEORGI, C. E. and MILITZER, W. E.: *J. Bacteriol.*, **97**, 186-192 (1969).
8. LANG, D. R. and LUNDGREN, D. G.: *J. Bacteriol.*, **101**, 483-489 (1970).
9. WONG, D. T., HORNG, J.-S. and GORDEE, R. S.: *J. Bacteriol.*, **106**, 168-173 (1971).
10. MCILWAIN, H.: *Nature*, **37**, 265-271 (1943).
11. WOOLLEY, D. W.: *J. Biol. Chem.*, **154**, 32 (1944).