

ON THE MODE OF ACTION OF A NEW ANTIFUNGAL ANTIBIOTIC, PYRROLNITRIN

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Pyrrolnitrin inhibits the syntheses of protein, RNA and DNA to almost the same degree. It also inhibits respiration but this site does not seem to be the primary action. It does not inhibit the biosynthesis of porphyrin. It is not an uncoupler of oxidative phosphorylation. It inhibits the transport of many substances, and causes the leakage of $A_{260m\mu}$ absorbing materials inside the cells. Pyrrolnitrin bursts the protoplasts of *Bacillus megaterium* KM strain at the growth inhibitory concentration. This action is completely neutralized by an approximately equimolar concentration of some phospholipids in the cell membrane components. It is concluded that the primary damage by pyrrolnitrin occurs in the cell membrane through a combination of pyrrolnitrin and some phospholipids.

Pyrrolnitrin is an antifungal antibiotic produced by *Pseudomonas pyrocinia*. It was discovered in our laboratory in 1964¹⁾. Its chemical structure is 3-(2'-nitro-3'-chlorophenol)-4-chloro-pyrrol. Pyrrolnitrin strongly inhibits the growth of fungi, less actively yeast and Gram-positive bacteria, but does not inhibit the growth of Gram-negative bacteria. Today, pyrrolnitrin is available as a remedy for trichophytoses.

We have been investigating the mode of action of pyrrolnitrin using a few sensitive microorganisms. The results are presented in this publication.

Methods and Materials

1. Strains: *Candida utilis* IAM 4215, *Rhodopseudomonas spheroides* and *Bacillus megaterium* KM strain.

2. Media:

BURKHOLDER medium for *Candida utilis*: asparagine 2 g, sodium lactate (or glucose) 20 g, $(NH_4)_2SO_4$ 2 g, KH_2PO_4 1.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2 \cdot 2H_2O$ 0.33 g, KI 0.1 g, trace element solution 1 ml, vitamin mixture 0.1 ml per liter.

Medium for *Rhodopseudomonas spheroides*: sodium glutamate 3.8 g, DL-malic acid 2.7 g, KH_2PO_4 0.5 g, K_2HPO_4 0.5 g, $(NH_4)_2HPO_4$ 0.8 g, $MgSO_4 \cdot 7H_2O$ 0.2 g, $CaCl_2$ 0.04 g, Fe-citrate 0.003 g, nicotinic acid 0.001 g, thiamine hydrochloride 0.001 g, biotin 10 mcg, yeast extract 2 g per liter.

Bouillon medium for *Bacillus megaterium*: dry bouillon powder 20 g per liter.

3. Growth of bacteria: The turbidity or absorbancy at 555 $m\mu$ was measured. The number of living cells was counted by the dilute plating method.

4. Respiration: Oxygen uptake was measured by the Warburg manometric method or oxygen consumption electrode (Yanagimoto Co., Ltd., Japan).

5. Protein, RNA and DNA syntheses: To determine cellular nucleic acid and protein, 5 ml of the culture was centrifuged at 7,000 g for 10 minutes and the cells harvested were washed three times with 5 ml of cold 5% trichloroacetic acid. The precipitate was then extracted with 2 ml of 5% trichloroacetic acid at 90°C for 15 minutes. The supernatant extract was utilized for nucleic acid determination. RNA was determined by the method of BROWN²⁾, and DNA by that of BURTON³⁾. The precipitate for protein analysis was dissolved in 0.5 N KOH and determined by the method of LOWRY⁴⁾.

6. Active transport: The experiment was done using ¹⁴C-amino acids and ¹⁴C-uracil. Log phased cells were incubated in phosphate buffer containing a carbon source and the incorporation of ¹⁴C-substance into the cell protein or RNA was very low compared with that into the whole cell.

7. Chlorophyll synthesis and the accumulation of coproporphyrin from δ -aminolevulinic acid by *Rhodospseudomonas spheroides*: The synthesis of chlorophyll was measured by the absorbancy at 800 and 850 m μ of the intact cell suspensions (semi-anaerobically grown cells under the tungsten light) by Cary spectrophotometer⁵⁾. The light-grown cells convert δ -aminolevulinic acid into coproporphyrin when they are incubated under the light in the following reaction mixture: δ -aminolevulinic acid $2 \cdot 10^{-3}$ M, MgSO₄ 10^{-3} M, Na₂HPO₄ 10^{-4} M, pH 8.0. Periodic sampling of 4 ml from the mixture, adding 0.44 ml of 1.5 N HCl and holding the sample in the dark for 4 hours allowed us to measure, after centrifugation, coproporphyrin by the amount of the absorbancy at 400 m μ of the supernatant⁵⁾.

8. Preparation of mitochondria: Early stationary phased cells of *Candida utilis* were disrupted by French press (450~500 atms) in 0.55 M sucrose containing 0.01 M tris buffer and $5 \cdot 10^{-4}$ M EDTA, centrifuged at 4,000 g for 10 minutes and the supernatant was then centrifuged at 60,000 g for 30 minutes by Spinco model L ultracentrifuge. The precipitate was washed once and resuspended in the same sucrose solution to make the mitochondria fraction. To determine oxidative phosphorylation in the mitochondria, we used the Warburg manometric method with the following reaction mixture: main chamber, 1.8 ml containing 3.6 mg bovine serum albumin, 32 mg glucose, 1.33 mg MgSO₄, 2.45 mg KH₂PO₄, 1.8 mg Na₂-ATP, 5.4 mg sodium succinate and 308 mg sucrose; 0.048 ml ³²P-phosphate (50 μ c) and 0.004 ml pyrrolnitrin (or pentachlorophenol, ethanol as control); side arm, 0.2 ml (hexokinase final concentration 1.4 units 0.1 ml and mitochondria fraction 0.1 ml—about 5 mg dry weight); center well, 20% KOH 0.2 ml. The determination was done by the modified method of LEHNINGER¹⁷⁾.

9. Preparation of protoplasts of *Bacillus megaterium* KM strain: Overnight-cultured cells were harvested, washed with M/15 phosphate buffer at pH 6.8 containing $5 \cdot 10^{-3}$ M MgSO₄ and suspended in the same buffer containing 0.7 M sucrose and 100 mcg/ml lysozyme. After incubation at 30°C for 30~60 minutes, more than 95% of cells became protoplasts. We always ascertained this phenomenon microscopically. The protoplast suspension was diluted by the same sucrose phosphate buffer without lysozyme when it was used. These protoplasts were very stable but we used them within 8 hours after the preparation. The decrease of the absorbancy at 555 m μ shows the burst of protoplasts, and the protoplast bursting action by pyrrolnitrin is expressed as the percentage of the absorbancy against the initial (the time of adding pyrrolnitrin) absorbancy at 555 m μ .

10. Preparation of the membrane fraction: The protoplast suspension was centrifuged at 10,000 g for 30 minutes and disrupted by a homogenizer. After washing and recentrifuging at 21,000 g for 30 minutes, the precipitate was obtained as the membrane fraction. Membrane lipids were extracted by the addition of a CHCl₃-CH₃OH (2:1) solution to the membrane fraction, agitating at room temperature for 5 hours, and drying at low pressure. Phosphatidylethanolamine, analytical grade, was purchased from Tokyo Kasei Co., Ltd., phosphatidylcholine was obtained from Meiji Seika Co., Ltd., and cardiolipin from Sumitomo Chemical Co., Ltd.

Results

1. Effect on Growth, Protein Synthesis and Nucleic Acid Synthesis

Fig. 1 and Table 1 show that the growth of *Candida utilis* is inhibited by pyrrolnitrin (10 mcg/ml). The lethal effect is not rapid. The shape of the treated cells with pyrrolnitrin during 2 hours was slender and its protoplasm seemed not to be homogeneous, *i. e.*, spotted.

Fig. 2 shows that pyrrolnitrin inhibits the synthesis of protein and nucleic acids to almost the same degree. The same results were obtained in other micro-organisms (*Penicillium chrysogenum*, *Rhodospseudomonas spheroides*, *Bacillus megaterium*).

Fig. 3 shows the primary action of pyrrolnitrin not to be the site of respiration. Because the inhibitory concentration of pyrrolnitrin of the respiration was a little higher than that of pyrrolnitrin on the growth, and at 50 mcg/ml of pyrrolnitrin the respiration was completely inhibited at first but it was restored soon after 10 minutes.

Fig. 1. Effect of pyrrolnitrin on the growth of *Candida utilis*

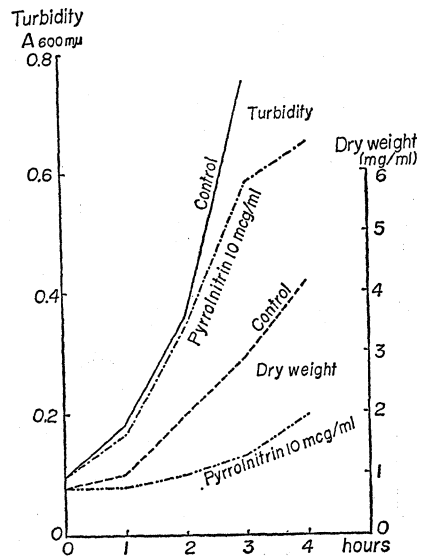


Fig. 2. Effect of pyrrolnitrin on the synthesis of protein, RNA and DNA of *Candida utilis*

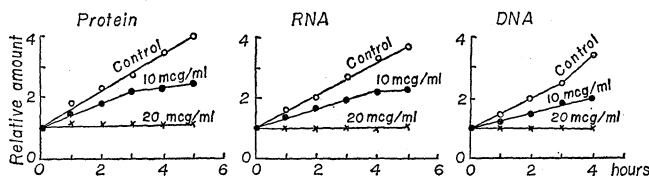


Fig. 3. Effect of pyrrolnitrin on the respiration of *Candida utilis*

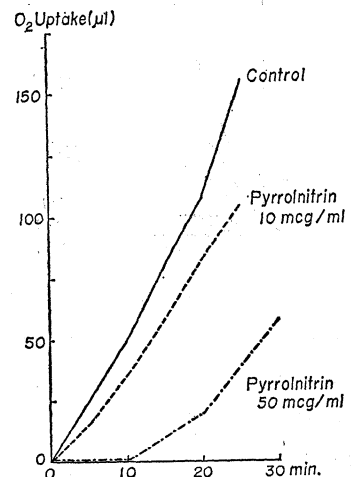


Table 1. Effect of pyrrolnitrin on the viable counts of *Candida utilis*

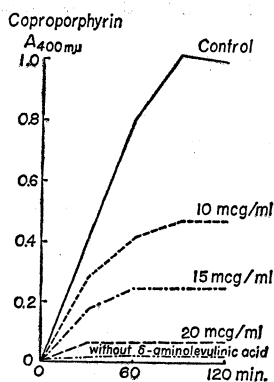
	Cells per ml			
	0	30 min.	60 min.	5 hrs.
Control	$1.4 \cdot 10^7$	$1.7 \cdot 10^7$	$2.3 \cdot 10^7$	$5 \cdot 10^7$
Pyrrolnitrin 10 mcg/ml	$1.3 \cdot 10^7$	$1.2 \cdot 10^7$	$1.0 \cdot 10^7$	$6.5 \cdot 10^5$

2. Effect on the Biosynthetic Pathway of Porphyrin

Because pyrrolnitrin has a pyrrol ring, we investigated the possibility of that it affects the formation of the pyrrol ring or of tetrapyrrol. End products of this pathway are hemes and chlorophylls. Here we examined the chlorophyll formation of *Rhodospseudomonas spheroides*. This bacterium is Gram-negative and can grow

aerobically and anaerobically (photosynthetically). The growth of this strain is inhibited by 10 mcg/ml pyrrolnitrin, both aerobically and anaerobically. Plotting the ratio of the amount of chlorophyll against the cell turbidity, chlorophyll synthesis was seen to be slightly inhibited by pyrrolnitrin (Fig. 4). We investigated the effect of pyrrolnitrin on the conversion of δ -aminolevulinic acid into coproporphyrinogen, because we considered this conversion was the most possible step influenced in the presence of pyrrolnitrin from the viewpoint of the structure of pyrrolnitrin. In intact cells, the conversion was inhibited by pyrrolnitrin (Fig. 5), but in cell-free systems the inhibition was not clear (Table 2). This result indicates that pyrrolnitrin cannot inhibit this step but can inhibit the transport of δ -aminolevulinic acid inside the cell.

Fig. 5. Effect of pyrrolnitrin on the conversion of δ -aminolevulinic acid into coproporphyrin by the intact cells of *R. spheroides*.



3. Effect on the Transport of Substances inside the Cell

Fig. 6 shows the inhibitory effect of pyrrolnitrin on the transport of δ -aminolevulinic acid into the cells. Besides δ -aminolevulinic acid, pyrrolnitrin inhibited the transport of all the tested amino acids (^{14}C -glutamic acid, ^{14}C -proline, ^{14}C -methionine) and ^{14}C -uracil. The same results were obtained in other microbes, for example, *Candida utilis* and *Bacillus megaterium* KM.

This phenomenon may be due to either inhibition of the energy producing systems concerned with active transport, or to damage of the cell membrane's permeability.

4. Effect on the Energy-producing Systems

To determine the inhibition of oxidative phosphorylation by pyrrolnitrin, another

Fig. 4. Amount of bacteriochlorophyll against the increase of cell mass of *Rhodospseudomonas spheroides*.

Aerobically grown cells (log phase) were harvested and re-suspended in fresh medium. They were incubated semianaerobically under the light at 30°C. After about 18 hrs the synthesis of chlorophyll ($A_{850m\mu}$) and the growth were observed. In the presence of pyrrolnitrin 10 mcg/ml the growth rate was half as much as that of control.

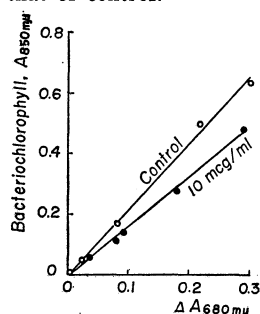


Table 2. Effect of pyrrolnitrin on the conversion of δ -aminolevulinic acid into coproporphyrin in cell-free system of *R. spheroides*.

	Coproporphyrin $A_{400m\mu}$
Control	1.14
Pyrrolnitrin 10 mcg/ml	1.11
Pyrrolnitrin 100 mcg/ml	1.12
No δ -aminolevulinic acid	0.005

Light grown cells (log phase) were harvested and washed twice with 0.9% NaCl. Alumina powder (1.5 g) was added to grind the cells (dry weight 100 mg). Enzyme was extracted in 3 ml of $m/10$ phosphate buffer (pH 7.6) containing 0.9% NaCl. By centrifugation at 10,000 g for 30 minutes, the residue and alumina were taken away and the supernatant was obtained as the enzyme solution.

control, pentachlorophenol, an uncoupler, was used. With the intact cells of *Candida utilis*, ^{32}P -phosphate incorporation into organic phosphate was inhibited by pyrrolnitrin, but in the cell-free system, that is, with mitochondria, we could not see the inhibitory action of pyrrolnitrin, although pentachlorophenol showed its inhibitory action (Table 3). We concluded that pyrrolnitrin's action is not on oxidative phosphorylation.

Table 3. Effect of pyrrolnitrin of the oxidative phosphorylation of the mitochondria fraction of *Candida utilis*.

	O_2 uptake (μmoles)	Po formed (μmoles)	P/O ratio	%
Control	7.5	15.4	1.03	100
Pyrrolnitrin 10 mcg/ml	7.2	13.7	0.95	89
Pyrrolnitrin 100 mcg/ml	7.0	13.3	0.95	89
Pentachlorophenol 10^{-4}M	6.7	1.23	0.09	8

5. Effect on the Cell Membrane

Cell membrane damage by pyrrolnitrin is measured by examining the effect of the antibiotic on cell permeability. At first, the active transport of amino acids is inhibited, as shown in Fig. 6. Moreover the leakage of cellular substances (260 $m\mu$ absorbing materials) was observed in the presence of pyrrolnitrin (Fig. 7). The value of $A_{260m\mu}$ absorbing materials at 120 minutes was about 30% of the total free pool (hot water extractable fraction). Based on these results, we feel that pyrrolnitrin acts by injury of the cell membrane.

6. Effect on the Protoplasts of *Bacillus megaterium* KM Strain

To examine directly the effect of the antibiotic on the cell membrane, protoplasts which are free of cell walls seemed to be one of the best experimental materials. The protoplasts prepared by us were very stable but we used them as soon as possible.

Fig. 8A shows that at high concentration of pyrrolnitrin protoplast bursting was immediate and rapid but at low concentration it was delayed and slow. Fig. 8B shows the relationship of the bursting velocity of protoplast to the concentration of pyrrolnitrin. In Fig. 8C, the time required for the initiation of the bursting was plotted against the concentration of pyrrolnitrin.

7. Effect of the Temperature on Protoplast Lysis

Fig. 9 shows that the protoplast bursting by pyrrolnitrin is dependent on the

Fig. 6. Effect of pyrrolnitrin on the incorporation of ^{14}C - δ -aminolevulinic acid into the cell of *R. spheroides*.

The reaction mixture was composed of $\text{M}/20$ tris buffer (pH 8.0) 5 ml, $\text{M}/5$ K_2HPO_4 0.04 ml, MgCl_2 ($\text{M}/10$) 0.08 ml, ^{14}C - δ -aminolevulinic acid 30 μmoles (0.3 μC) and light grown cells (1 mg dry weight). They were incubated at 33°C under the tungsten light (200 W, 25 cm distant from this light).

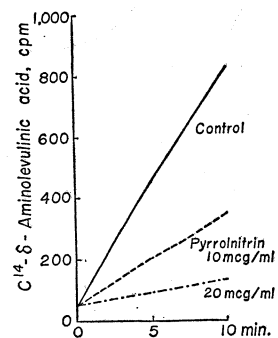


Fig. 7. Leakage of $A_{260m\mu}$ absorbing materials in the presence of pyrrolnitrin 10 mcg/ml by *C. utilis*.

The amount of $A_{260m\mu}$ at the zero time is that of pyrrolnitrin itself. Log phased cells were washed twice with 0.8% NaCl and suspended in $\text{M}/100$ KH_2PO_4 50 ml containing MgSO_4 40 μM . They were incubated with or without pyrrolnitrin at 30°C .

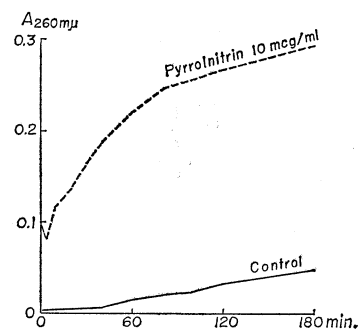


Fig. 8A. Effect of pyrrolnitrin on the protoplasts of *Bacillus megaterium*. Protoplasts were incubated at 30°C.

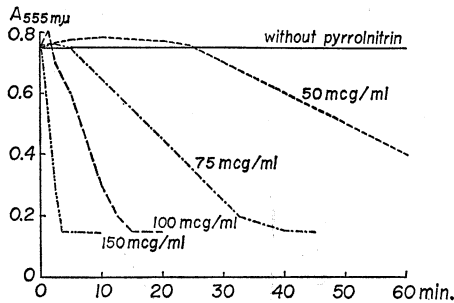


Fig. 8B. Relationship between the bursting velocity of protoplasts and the concentration of pyrrolnitrin.

This figure was replotted from Fig. 8A.

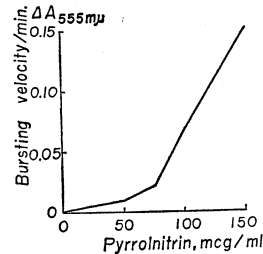
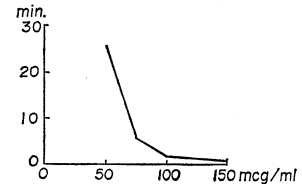


Fig. 8C. Relationship between the time required for the initiation of the bursting after the addition of pyrrolnitrin and the concentration of pyrrolnitrin.

This figure was replotted from Fig. 8A.



temperature though the solubility of the antibiotic in the reaction mixture does not vary at 5~30°C. The solubility was measured by the absorbancy of the solution at 260 m μ , the absorption maximum of ultraviolet spectra of pyrrolnitrin. In contrast, a surfactant, Tween 20, is almost independent of temperature in its lytic action on protoplast bursting. This indicates that the protoplast bursting action of pyrrolnitrin is not identical with that of a surfactant. Moreover, the surface tension of water examined by the drop method⁶⁾ was not decreased by pyrrolnitrin.

Fig. 9. Effect of the temperature on the protoplast bursting by pyrrolnitrin 100 mcg/ml at pH 7.2.

Vertical line is expressed as percent of $\Delta A_{555m\mu}$ during one minute after the initiation of the protoplast bursting against $A_{555m\mu}$ at zero time.

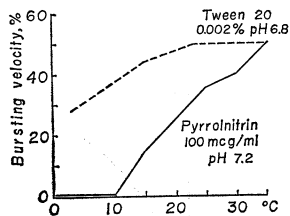
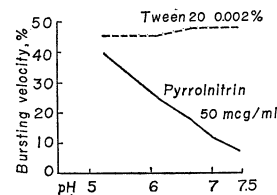


Fig. 10. Effect of pH on the protoplast bursting by pyrrolnitrin 50 mcg/ml at 30°C.

Vertical line is expressed as percent of $\Delta A_{555m\mu}$ during one minute after the initiation of the protoplast bursting against $A_{555m\mu}$ at zero time.



8. Effect of pH on Protoplast Bursting by Pyrrolnitrin

Fig. 10 shows that the bursting action by pyrrolnitrin is greater at an acidic pH than at an alkaline one. Pyrrolnitrin is neutral and not easy to solubilize much in water, and we cannot increase the solubility in acid.

9. Effect of Some Inhibitors of Energy-producing Systems on the Protoplast Bursting Action

The action of pyrrolnitrin was not influenced by KCN, NaN₃ and pentachlorophenol, which did not burst the protoplasts in itself (Table 4).

Table 4. Effect of some inhibitors on the protoplast bursting action of pyrrolnitrin

Additions	Bursting velocity $\Delta A_{555m\mu}$	%
Pyrrolnitrin 100 mcg/ml	0.155	100
Pyrrolnitrin 100 mcg/ml+KCN 10 ⁻³ M	0.140	90
Pyrrolnitrin 100 mcg/ml+NaN ₃ 10 ⁻³ M	0.125	81
Pyrrolnitrin 100 mcg/ml+PCP 10 ⁻⁴ M	0.150	97

10. Adsorption of Pyrrolnitrin to the Cell Membrane

Protoplasts treated with pyrrolnitrin were fractionated and the pyrrolnitrin was detected as outlined in Table 5. Over 50 % of the pyrrolnitrin was adsorbed by the cell membrane and was extracted with ethanol. It is thought that pyrrolnitrin is adsorbed by the cell membrane and that its combination with cell membrane is such as to be extractable with organic solvents.

Table 5. Adsorption of pyrrolnitrin to the cell membrane. Bioassay of pyrrolnitrin was according to the paper disk method using *Penicillium chrysogenum*

Fraction	Pyrrolnitrin		
	15 mcg/ml	50 mcg/ml	100 mcg/ml
Supernatant 1	5 mcg/ml	20 mcg/ml	40 mcg/ml
Supernatant 2	3.5	15	37
EtOH	0	5	5
EtOH extract	9	20	55
CHCl ₃ -MeOH extract	0	0	0

11. Action Site of Pyrrolnitrin

The membrane fraction of protoplasts decreased the bursting action of pyrrolnitrin. Moreover, the membrane lipid fraction decreased or neutralized this action, while the protein did not. The membrane protein was denatured by the process of the lipid extraction, so instead of the membrane protein we used cytochrome C and ovalbumin as a pure protein (Fig. 11). Fatty acids of the lipids did not decrease the

Fig. 11. Adverse effect of the membrane fraction on the protoplast bursting action by pyrrolnitrin.

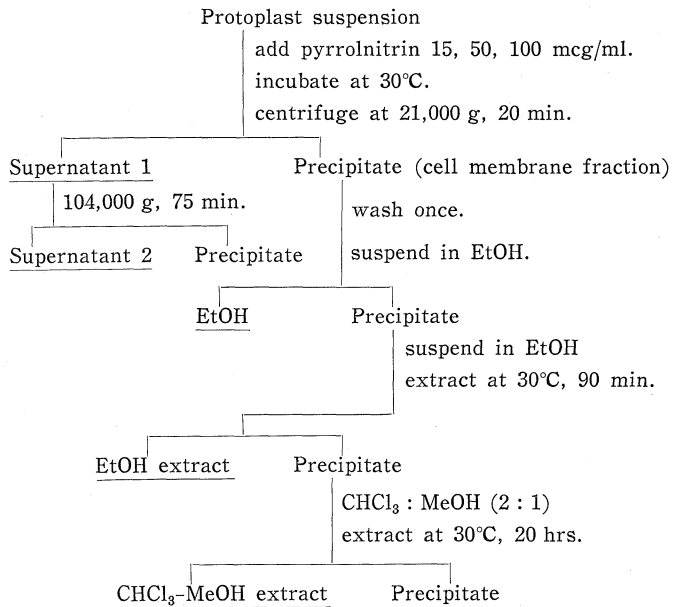
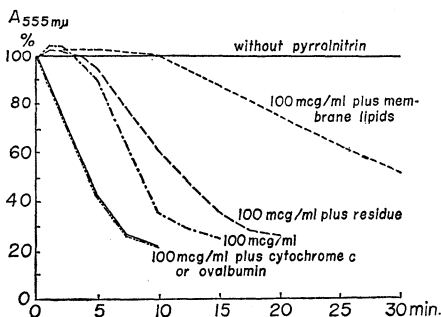
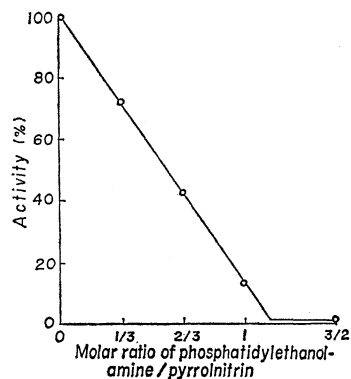


Fig. 12. Neutralization of pyrrolnitrin's action by phosphatidylethanolamine.

Protoplasts were incubated with pyrrolnitrin 100 mcg/ml ($4 \cdot 10^{-4}M$), pyrrolnitrin $4 \cdot 10^{-4}M$ plus phosphatidylethanolamine $1.3 \cdot 10^{-4}M$, pyrrolnitrin $4 \cdot 10^{-4}M$ plus phosphatidylethanolamine $2.7 \cdot 10^{-4}M$, pyrrolnitrin $4 \cdot 10^{-4}M$ plus phosphatidylethanolamine $4 \cdot 10^{-4}M$ and pyrrolnitrin $4 \cdot 10^{-4}M$ plus phosphatidylethanolamine $6 \cdot 10^{-4}M$.



action of pyrrolnitrin, but the phospholipids, the main components of the cell membrane lipids, could neutralize the protoplast bursting action of pyrrolnitrin. The relationship between phospholipids and the concentration of pyrrolnitrin is shown in Fig. 12. That is, equivalent concentrations of phosphatidylethanolamine can decrease the bursting action of pyrrolnitrin. The purity of phosphatidylethanolamine used in this experiment was about 80 %, measured by thin-layer chromatography.

Besides phosphatidylethanolamine, phosphatidylcholine and cardiolipin can neutralize the bursting action of the antibiotic, but phosphatidylinositol can not do so. Phosphatidylinositol is a surfactant, and thus bursts the protoplasts by itself, and in the presence of both pyrrolnitrin and phosphatidylinositol the protoplasts were lysed more rapidly.

12. Effect of Protoplast Stabilizer on the Bursting Action

We studied the effect of various protoplast stabilizers on the action of pyrrolnitrin. Table 6 shows the results. In each case the protoplasts were stable and the decrease at $A_{555m\mu}$ was not observed during 60 minutes in the absence of pyrrolnitrin. In 5 % sucrose used as the stabilizer, the concentration of pyrrolnitrin to burst the protoplasts decreased to be 25 mcg/ml, and in 1 % NaCl the protoplasts were bursted slowly at the concentration of the growth inhibition (15 mcg/ml). Therefore the concentration for growth inhibition is the same as that for protoplast bursting.

Table 6. Effect of various protoplast stabilizer on the action of pyrrolnitrin 50 mcg/ml. Protoplasts were incubated at 30°C in $M/15$ phosphate buffer (pH 6.8) containing $MgSO_4 \cdot 5 \cdot 10^{-3}M$ and protoplast stabilizer.

Protoplast stabilizer	Bursting velocity Max. decrease at $A_{555m\mu}/min.$
1 % NaCl	0.440
0.5 M Phosphate buffer (pH 6.8)	0.390
10 % Glucose	0.130
5 % Sucrose	0.110
10 % Raffinose	0.030
7.5 % Polyethyleneglycol	0.000
25 % Sucrose	0.010

Discussion

We consider that the primary action of pyrrolnitrin is an attack on the cell membrane, by combination with the phospholipids. Then cell permeability is changed and synthesis of protein, nucleic acids, *etc.*, is inhibited, and the microbes are killed.

A number of antibiotics are known to have a destructive influence on the cell membrane. Several antibiotics act as detergents: (for example, the polypeptides including tyrocidin⁷), gramicidin⁸), polymyxin^{9,10}) and the polyene antifungal antibiotics including nystatin¹¹⁻¹³), filipin¹¹⁻¹³), *etc.* Polymyxin adsorbs to the membrane of protoplasts and the specific binding loci have been suggested to be the polyphosphates of the membrane. Nystatin is reported to combine with cholesterol in the cell membrane of fungi. Moreover, streptomycin¹⁴), novobiocin¹⁵) and azalomycin¹⁶) have been found to change cell permeability.

It is interesting that, though pyrrolnitrin is neither a polypeptide nor a polyene antibiotic, there is a resemblance among them in the mode of action.

Our results were obtained on Gram-positive bacteria and the action on fungi remains to be determined. The fact that the content of phospholipids in the cell membrane of Gram-positive bacteria (pyrrolnitrin sensitive) is less than that of Gram-negative bacteria (pyrrolnitrin resistant) and the data that pyrrolnitrin was neutralized with phospholipids, may explain why pyrrolnitrin exerts its action most strongly against fungi, less against Gram-positive and least against Gram-negative bacteria.

We are now investigating the nature of the complex between pyrrolnitrin and phospholipids.

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