Studies on the Mode of Action of Polyoxin D

Part II. Effect of Polyoxin D on the Synthesis of Fungal Cell Wall Chitin

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In the previous papers we reported that the antibiotic Polyoxin D induced the characteristic swelling of the mycelia of fungi,^{1,2)} and strongly inhibited the incorporation of ¹⁴C-glucosamine into the fungal cell wall chitin.³⁾ The present work has been conducted to further investigate the influence of this antibiotic on the fungal cell wall biosynthesis.

Polyoxin D did not inhibit the incorporation of ¹⁴C-glucose, ¹⁴C-amino acids and ¹⁴C-sodium acetate into the cell wall. In addition, UDP-N-acetylglucosamine, a precursor of chitin biosynthesis of cell wall, was accumulated in the Polyoxin D-treated mycelia of *Cochliobolus miyabeanus* more than 150 to 160% of that accumulated in the untreated one.

Chitin synthetase prepared from *Piricularia oryzae* which is not treated with Polyoxin D was completely inhibited by the addition of 0.1 ppm of Polyoxin D. The fungitoxicity of Polyoxins A to G was positively related to their inhibition of ¹⁴C-glucosamine incorporation into the cell wall chitin of *C. miyabeanus*. From above results, it became evident that the antibiotic Polyoxin complex inhibited the biosynthesis of fungal cell wall chitin.

Nucleoside antibiotic Polyoxin groups, consisted of 12 closely related compounds,⁴⁾ have potential fungitoxicity against Phytopathogenic filamentous fungi (*i. e.*; *Cochliobolus miyabeanus*, *Pellicularia sasakii*, *Alternaria kikuchiana* and *Piricularia oryzae* etc.), but they were not toxic against bacteria and yeast.¹⁾ By the microscopical observation, it found that the tips of growing mycelia and germ tubes were

swollen as Penicillin-treated bacteria.^{5,6)} In the previous papers, the authors reported Polyoxin D strongly inhibited the incorporation of ¹⁴C-glucosamine into the fungal cell wall without affecting the respiration and protein and nucleic acid synthesis. From these results, it was suggested that Polyoxin D might be an inhibitor of the biosynthesis of fungal cell wall chitin.³⁾

Penicillin,⁷⁾ Vancomycin⁸⁾ and other antibi-

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otics were known as inhibitors of bacterial cell wall synthesis, and amino sugar intermediates of cell wall synthesis were accumulated by the treatment of bacterial cells with these antibiotics.⁹⁾ We thought that Polyoxin D might cause the similar abnormal accumulation of the intermediates and tried to extract amino sugar derivatives from the Polyoxin D-treated mycelia according to the method of Park.¹⁰⁾

Many reports have been published on the cell-free biosynthesis of cell wall chitin of *Neurospora crassa*,¹¹⁾ *Venturia inaequalis*,¹²⁾ *Alomyces macrogynus*¹³⁾ and *Blastocladiella emersonii*.¹⁴⁾ In these cell-free systems, chitin synthetase catalyzed the transfer of *N*-acetylglucosamine from UDP-*N*-acetylglucosamine to chitin. But very little work has been published on its inhibitor. We attempted to prepare chitin synthetase from *P. oryzae*, a sensitive strain against Polyoxin D, and studied the effect of Polyoxin D on the enzyme activity.

METHODS AND MATERIALS

Chemicals. Polyoxins A, B, C, D, E, F and G (more than 99% purity) were obtained from Kaken Chemical Co., Ltd., ¹⁴C-amino acids, U-¹⁴C-glucose, sodium acetate-¹⁴C and ¹⁴C-glucosamine were obtained from Japan Radio Isotope Association. UDP-*N*-acetylglucosamine-¹⁴C was prepared from Polyoxin D- or Kitazin (*S*-benzyl *O*, *O* diethyl phosphorothioate)-treated mycelia of *C. miyabeanus* and *P. oryzae*, and purified on the filter paper and by thin-layer chromatography.¹⁵⁾

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Pronase (7000 puk/mg) was obtained from Kaken Chemical Co., Ltd.

Organisms. *C. miyabeanus* and *P. oryzae* P₂ were obtained from National Institute of Agricultural Sciences, (Tokyo). *Streptomyces aerocolonigenes* which produces the chitinase was obtained from Fermentation Research Institute, (Chiba) and *Bacillus circulans* which produces β -1, 3-glucanase was obtained from The Institute of Physical and Chemical Research.

Growth conditions and preparation of mycelial suspension. *C. miyabeanus* was cultured for ten days at 27°C on the potato agar medium. Spores harvested were suspended in a glucose-yeast liquid medium (2% glucose and 0.3% yeast, pH 5.0) and incubated for 40 hr at 27°C. The mycelia were thoroughly washed with 0.05 M phosphate buffer, Tris-HCl buffer or Czapeck solution, pH 6.0, and homogenized for 30 seconds with the same buffer of solution. This mycelial suspension was used for further experiments.

Fractionation of cell wall of *C. miyabeanus* and incorporation of ¹⁴C-glucosamine, ¹⁴C-glucose, ¹⁴C-amino acids and sodium acetate-¹⁴C into the cell wall fraction. Mycelial suspension of *C. miyabeanus* in the Czapeck solution was preincubated at 28°C and ¹⁴C-glucosamine and Polyoxin D were added. The mixed suspension was incubated again and after the appropriate time the reaction was stopped, and cell wall fraction was prepared and washed by centrifugation according to the method described in Fig. 1 until about 90% purity. The cell wall fraction was treated with 1 N KOH and ether-ethanol (1:3) to remove proteins and lipids respectively, and was hydrolyzed in 2 N HCl for 5 hr at 100°C, and then in 6 N HCl for 6 hr at 100°C. Radioactivity of the hydrolyzate was counted by gas-flow counter. Amino sugars were determined according to the method of Elson and Morgan.¹⁶⁾

Incorporation of ¹⁴C-amino acids into the protein of cell wall fraction was carried out with the same method as that of the incorporation of ¹⁴C-glucosamine. After removing lipids and nucleic acids the labelled cell wall fraction was dissolved in 1 N KOH. Radioactivity in this fraction was counted, and protein was determined according to the Folin's Method.¹⁷⁾

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FIG. 1. Fractionation of Cell Wall.

Incorporation experiment of sodium acetate-¹⁴C into lipids of cell wall fraction was also carried out with the same method except that mycelia was suspended in Tris-HCl buffer, pH 6.0. Lipids of cell wall fraction were extracted twice with ether-ethanol (1:3) for 10 min at 60°C, and radioactivity in the extract was counted by gas-flow counter. Dry weight of the residue was measured. Specific radioactivity was expressed as counts per minute per mg of the dried residue.

Incorporation of ¹⁴C-glucose into the glucan of cell wall fraction was carried out with the mycelia in 0.05 M phosphate buffer (pH 6.0). The cell wall fraction was washed with 6 N acetic acid at room temperature and hydrolyzed with 1 N HCl for 5 hr at 100°C. Radioactivity in the lysate was counted by gas-flow counter, and the sugar was determined according to the method of Somogyi.¹⁸⁾

Analysis of cell wall with chitinase. The cell wall which incorporated ¹⁴C-glucosamine in the presence of various concentrations of Polyoxin D was treated with 1 N KOH and ether-chloroform (1:3) successively. Then the sample was treated with β-1, 3-glucanase for 15 hr at 30°C, which was prepared

from the culture broth of *B. circulans* according to the method of Horikoshi, *et al.*¹⁹⁾ The residue which was not hydrolyzed by glucanase was hydrolyzed with crude chitinase preparation, which was prepared from the culture broth of *St. aerocolonigenes* according to the method of Nakagami *et al.*²⁰⁾ The reaction mixture was consisted of 2 ml of the suspension containing 5 mg of cell wall in 0.1 M phosphate buffer (pH 6.0), and 2 ml of the crude chitinase solution. The reaction mixture was incubated for 15 hr at 45°C. The lysates obtained by this procedure were spotted on the filter paper, and were cochromatographed with authentic *N*-acetylglucosamine and glucosamine using *n*-butanol-acetic acid-water (4:1:5) as the solvent. The chromatogram was cut into 0.5 cm piece. Radioactivity in each piece was measured with liquid scintillation system. Then the position of the radioactive material on the chromatogram was determined. On the other hand, radioactivity in the lysate was directly counted with gas-flow counter, and *N*-acetylglucosamine was determined according to the method of Reissig *et al.*²¹⁾

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FIG. 2. Extraction of the Amino Sugar Derivatives.

Specific radioactivity incorporated into the lysate was then calculated.

Extraction of ¹⁴C-amino sugar derivatives and effect of Polyoxin D on the accumulation of the derivatives. To an aliquot of the mycelia in the Czapeck solution, Polyoxin D (final concentrations 0 to 50 ppm) and ¹⁴C-glucosamine were added. The mixture was then incubated at 27°C and the sample was withdrawn every 60 min and filtered. Mycelia separated by filtration were washed thoroughly with ice cold water. The amino sugar derivative was extracted and fractionated by the method described in Fig. 2. Radioactivity of the final ethanol precipitate was counted with gas-flow counter. Specific radioactivity of the accumulated substance per dry weight of mycelia was obtained.

Chromatography of the final ethanol precipitates and identification of the accumulated substance. The final ethanol precipitate obtained by the method shown in Fig. 2, was dissolved in a known volume of water and the aliquot was spotted on to a filter paper strip with a microsyringe and chromatographed using 1 N ammonium hydroxide-isobutyric acid (1:3) as the solvent. Ultraviolet absorbing spots were detected under UV light. The position of radioactive materi-

als on the chromatograms was determined by counting the radioactivity of each 0.5 cm piece of the filter paper by a liquid scintillation system. The main radioactive spot was again extracted from the chromatogram and cochromatographed with authentic UDP-*N*-acetylglucosamine as a marker. The following four solvent systems were used: *n*-propanol-pyridine-acetic acid-water (8:8:1:4), ethylacetate-pyridine-water (2:2:1), methanolpyridine-acetic acid-water (6:6:1:4) and 1 N ammonium hydroxide-isobutylic acid (3:5).

Preparation of chitin synthetase from the mycelia of P. oryzae. Spores of *P. oryzae* cultured for seven days at 27°C on a starch-yeast slant medium (2% soluble starch, 0.2% yeast extract and 1.3% agar) were suspended in glucose-yeast liquid medium (2% glucose and 0.3% yeast). After incubation for 72 hr, the mycelia were transferred to the same fresh medium and incubated for 24 hr at 27°C. The mycelia were harvested and washed with ice cold water. Mycelial pellets (10 to 50 g) were homogenated for 30 seconds with 2 to 3 fold volume (w/v) of ice cold buffer (pH 7.5) consisted of 0.05 M Tris, 0.01 M MgCl₂, 0.001 M EDTA and 0.003 M mercaptoethanol. The homogenate was further treated by French Press (400

FIG. 3. Preparation of Chitin Synthetase.

kg/cm²) and separated into three fractions by centrifugation at 2500×*g* for 10 min, 10,000×*g* for 30 min and 140,000×*g* for 1 hr successively. The final pellets obtained were resuspended in 3 to 7 ml of buffer solution containing 0.05 M Tris, 0.01 M MgCl₂, 0.001 M EDTA and 0.005 M ascorbic acid (pH 7.5). All these procedures for the enzyme preparation were carried out at 4°C (Fig. 3).

Assay of chitin synthetase activity and effect of Polyoxin D. The standard reaction mixture was shown in Fig. 4. The reaction was carried out at 25°C and stopped by immediate heating in a boiling water bath for 1 min. The reaction mixture was spotted directly on the filter paper and chromatographed using methanol-pyridine-water (2:2:1) as the solvent. Then the position of radioactive materials on the chromatograms was determined by gas-flow thin-layer chromatography. Radioactive material remained at the origin on chromatogram was treated with chitinase after deproteinized with pronase (100 p.u./ml). The lysate was chromatographed again with authentic *N*-acetylglucosamine and glucosamine using methanol-pyridine-water (2:2:1) as the solvent. On the other hand, the reaction mixture was centrifuged and the precipitate was thoroughly washed four to five times with

FIG. 4. Reaction Mixture for the Chitin Synthetase Assay.

0.05 M phosphate buffer by centrifugation. The precipitate was resuspended into 2 ml of 0.05 M phosphate buffer and was incubated with 2 ml of chitinase solution for 10 hr at 45°C. Radioactivity in the lysate was counted with liquid scintillation system.

RESULTS

Table I shows the effect of Polyoxin D on the incorporation of ^{14}C -labeled amino acids, sodium acetate, glucose and glucosamine into protein, lipids, glucan and chitin fractions of fungal cell wall respectively. At 5 ppm of Polyoxin D, incorporation of glucosamine was inhibited more than 85%. Incorporation of amino acids was also inhibited about 50%, whereas the incorporations of glucose and sodium acetate were not affected. At 50 ppm of Polyoxin D incorporation of glucosamine was completely inhibited but inhibition of amino acids incorporation was 60%. On the other hand, transport of all labeled substances from culture medium into cytoplasm was not inhibited in the presence of 50 ppm of Poly-

oxin D. Therefore, our investigation was focussed on the inhibition of chitin synthetase by the antibiotic.

Recently, it has been confirmed that polyoxin group consists of A, B, C, D, E, F, G, H, I, J, K and L.⁴¹ Table II shows the minimum inhibitory concentrations against *C. miyabeanus* of Polyoxins A to G determined by agar dilution method.¹¹ Polyoxins A, B and G having 5-hydroxymethyluracil as chromophore and Polyoxins D, E and F having uracil-5-carboxylic acid showed strong growth inhibitory activity against mycelia of *C. miyabeanus* at low concentrations of 3 and 6 ppm, but Polyoxin C having 5-hydroxymethyluracil does not show inhibition. Effect of the Polyoxin group on the incorporation of ^{14}C -glucosamine into the cell wall of

TABLE I. EFFECT OF POLYOXIN D ON INCORPORATION OF ^{14}C -LABELED SUBSTRATES INTO THE PROTEIN, LIPIDS, GLUCAN AND CHITIN OF CELL WALL OF *C. miyabeanus*

Polyoxin D	Radioactivity			
	^{14}C -Amino acids	^{14}C -Sodium acetate	^{14}C -Glucose	^{14}C -Glucosamine
ppm	cpm/O.D. _{640mμ}	cpm/1 mg dry weight	cpm/O.D. _{520mμ}	cpm/O.D. _{540mμ}
0	18050	262	2500	11750
5	9300	276	1885	2020
50	7250	230	1719	210

The reaction mixture was incubated at 27°C for 60 min and radioactivity was counted with gas-flow counter.

TABLE II. MOLECULAR CONSTITUENTS OF POLYOXINS AND MINIMUM INHIBITORY CONCENTRATIONS AGAINST *C. miyabeanus*

Polyoxin	Chromophore	Polyoximic acid	Polyoxamic acid	Desoxy-polyoxamic acid	M.I.C. ^{a1} (ppm)
A	5-Hydroxymethyluracil	+	+	—	3.125
B	5-Hydroxymethyluracil	—	+	—	3.125
C	5-Hydroxymethyluracil	—	—	—	> 100
D	Uracil-5-carboxylic acid	—	+	—	6.25
E	Uracil-5-carboxylic acid	—	—	+	6.25
F	Uracil-5-carboxylic acid	+	+	—	6.25
G	5-Hydroxymethyluracil	—	—	+	3.125

^{a1} Minimum inhibitory concentrations against *C. miyabeanus* of Polyoxins was determined by agar dilution method.¹¹

FIG. 5. Effect of Polyoxins on Incorporation of ^{14}C -Glucosamine into the 6N HCl Hydrolysate of *C. miyabeanus*.

FIG. 6. Effect of Polyoxin D on the Incorporation of ^{14}C -Glucosamine into the Cell Wall of *C. miyabeanus*.

Control
+ Polyoxin D, 5 ppm
+ Polyoxin D, 50 ppm

mycelia of *C. miyabeanus* was also investigated. Polyoxins A, B and G, D, E and F which showed highly antifungal activities, also caused 80 to 90% inhibition of the incorporation of ^{14}C -glucosamine at the concentration of 50 ppm, but Polyoxin C having no antifungal activity did not cause the inhibition at the same concentration (Fig. 5). These results showed the close relation between the inhibition of incorporation of ^{14}C -glucosamine into the cell wall and the antifungal activity of Polyoxin group.

FIG. 7. Paper Chromatogram of Chitinase Hydrolysate of Cell Wall.

Solvent; *n*-butanol-acetic acid-water (4:1:5)

GA: glucosamine, N-Ac-GA: N-acetylglucosamine. Radioactivity was counted with liquid scintillation system.

The cell wall labeled with ^{14}C -glucosamine in the presence of various concentrations of Polyoxin D was hydrolyzed with chitinase (Fig. 6). The lysate was chromatographed with authentic *N*-acetylglucosamine and glucosamine (Fig. 7). The spot of radioactive material was accorded with authentic *N*-acetylglucosamine, but radioactivity was not detected at the position of authentic glucosamine. Polyoxin D (5 to 50 ppm) inhibited

FIG. 8. Time Course of the Accumulation of ¹⁴C-Glucosamine Derivatives.

●—● Control
 ●—● + Polyoxin D, 5 ppm
 ●---● + " 50 ppm

FIG. 9. Effect of Polyoxin D on the Incorporation of ¹⁴C-Glucosamine into the Residue after TCA Extraction.

●—● Control
 ●—● Polyoxin D, 5 ppm
 ●---● Polyoxin D, 50 ppm

the incorporation of ¹⁴C-glucosamine into the cell wall fraction at 85 to 95% and the most radioactivity of cell wall fraction was released by chitinase treatment. From these results, it was suggested that ¹⁴C-glucosamine was incorporated into the chitin layer of cell wall and the incorporation was inhibited by Polyoxin.

The mycelia of *C. miyabeanus* which were labeled with ¹⁴C-glucosamine in the presence of 0 to 50 ppm of Polyoxin D were extracted with 5% TCA according to the method described in Fig. 2. The ethanol precipitate was hydrolyzed with 1N HCl for 1 hr at 100°C. The radioactive substance in the lysate was identified as ¹⁴C-glucosamine by chromatography. The effect of Polyoxin D on the formation of ¹⁴C-amino sugar derivative is shown in Figs. 8 and 9. Maximum accumulation of ¹⁴C-amino sugar derivative was obtained after 120 min. At this time, mycelia treated with 5 ppm of Polyoxin D contained about 150 to 160% of the derivative as compared with the untreated one, and at

50 ppm, the accumulation was almost the same degree. After that, the amount of accumulated amino sugar derivatives decreased gradually in untreated and 5 ppm Polyoxin D-treated mycelia. The decrease was probably due to the utilization of the accumulated material for the resynthesis of cell wall chitin, but this decrease was not observed in 50 ppm Polyoxin D-treated mycelia. This might be due to the inhibition of the resynthesis of cell wall chitin by high concentration of Polyoxin D. After extraction with TCA radioactivity of the cell wall fraction was counted. The incorporation of ¹⁴C-glucosamine into the cell wall was inhibited by Polyoxin D at 5 to 50 ppm. These findings have proved that the accumulation of amino sugar derivatives results from the inhibition of chitin synthesis by Polyoxin D.

A fraction, which was obtained by TCA extraction and ethanol precipitation of mycelia incubated with ¹⁴C-glucosamine and Polyoxin D for 120 min, gave four ultraviolet absorbing spots on paper chromatogram developed with a mixture of isobutyric acid and

Fig. 10. Paper Chromatogram of TCA-Extracted Substances.

Solvent system; isobutyric acid-1N ammonium hydroxide (5:3), UDP-N-AcGA; UDP-N-acetylglucosamine, N-Ac-GA; N-acetylglucosamine, GA; glucosamine.

○—○ 1.0 ppm of Polyoxin D treated
●—● Control

1N ammonium hydroxide (5:3); the chromatogram is shown in Fig. 10. One radioactive spot was also found. The corresponding spot from untreated mycelia had lower radioactivity, and this difference would indicate the difference in the amount of accumulated derivatives.

This spot had the same R_f value as that of authentic UDP-*N*-acetylglucosamine.

The spot of UDP-*N*-acetylglucosamine-like substance was extracted and cochromatographed with authentic UDP-*N*-acetylglucosamine. As shown in Table III the accumulated substance gave identical R_f value with that of UDP-*N*-acetylglucosamine in four solvent systems.

From the above results, it is suggested that Polyoxin D induces the accumulation of UDP-*N*-acetylglucosamine in cytoplasm of mycelia and this antibiotic may inhibit the synthetic

TABLE III. IDENTIFICATION OF THE ACCUMULATED SUBSTANCE BY PAPER CHROMATOGRAPHY

	R_f value			
	Solv. A	Solv. B	Solv. C	Solv. D
Accumulated substance	0.10	0.13	0.69	0.152
Authentic UDP- <i>N</i> -acetylglucosamine	0.09	0.13	0.69	0.150
Solv. A:	<i>n</i> -Propylalcohol-pyridine-acetic acid-water (8:8:1:4)			
Solv. B:	Ethyl acetate-pyridine-water (2:2:1)			
Solv. C:	Methanol-pyridine-acetic acid-water (6:6:1:4)			
Solv. D:	Isobutyric acid-1N ammonium hydroxide (5:3)			

Spots on paper chromatograms were detected with UV absorption and radioactivity.

process from UDP-*N*-acetylglucosamine to chitin.

Effect of Polyoxin D on the activity of chitin synthetase

Chitin synthetase was prepared from the mycelia of *P. oryzae* according to the method shown in Fig. 3. The particulate enzyme used in the present study did not require chitobiose as a primer. The chitin synthesis from UDP-*N*-acetylglucosamine- ^{14}C was also carried out. After stopping reaction, the reaction mixture was chromatographed using methanol-pyridine-water (2:2:1) as the solvent, and positions of radioactive materials on the chromatogram were determined with gas-flow thin-layer scanner. The results are shown in Fig. 11. The amount of radioactive material remained at the origin increased with the lapse of time. Radioactivity at the origin after 120 min incubation was 1,666 cpm and this value corresponded to about 10% of UDP-*N*-acetylglucosamine added. The radioactive material remained at the origin did not move when chromatographed using two solvent systems: methanol-chloroform-water (30:15:2) and methanol-chloroform-acetic

FIG. 11. Activity of Chitin Synthetase Prepared from Mycelia *P. oryzae*.

	Radioactivity at origin
A. original reaction mixture	24 cpm
B. incubated for 30 min	216 "
C. incubated for 60 min	576 "
D. incubated for 120 min	1666 "

acid-water (8:8:1:1). Radioactive spot at the origin was cut and treated with pronase to remove protein and, with chitinase for 10 hr at 45°C. The hydrolyzate by chitinase was chromatographed again in methanol-pyridine-water (2:2:10) the chromatogram was cut at 0.5 cm of pieces and the radioactivity was counted by liquid scintillation system. In addition to the original spot, two radioactive spots were found. High radioactive spot was accorded with the position of *N*-acetylglucosamine, and the weak radioactivity was detected at the position of glucosamine (Fig. 12).

The susceptibility to chitinase of the radioactive substance remained at origin confirmed that this substance was chitin and the cell-free system of *P. oryzae* had the activity of chitin synthetase. Effect of Polyoxin D on the enzymatic chitin synthesis was investigated and the result was shown in Fig. 13.

FIG. 12. Paper Chromatogram of the Chitinase Hydrolyzate Enzymatically Synthesized Chitin.

GA; glucosamine. N-Ac-GA; N-acetylglucosamine. Solvent system; *n*-butanol-acetic acid-water (4:1:5).

●—● Chitinase treated
—○— non treated

FIG. 13. Effect of Polyoxin D on the Enzymatic Synthesis of Chitin.

●—● Control
●—● + Polyoxin D, 2×10^{-7} M
●---● + " 2×10^{-6} M
●----● + " 1×10^{-5} M

Synthesis of chitin from UDP-*N*-acetylglucosamine-¹⁴C in the cell-free system of *P. oryzae* was inhibited by Polyoxin D. Eighty and 98% inhibition was observed in the presence of 2×10^{-7} M (0.1 ppm) and 1×10^{-6} M (5 ppm) of Polyoxin D, respectively. 2×10^{-7} M Polyoxin D is sufficient to induce the swelling of the mycelia of *P. oryzae* *in vivo*.

DISCUSSION

It is known that penicillin and many other antibiotics inhibit the synthesis of bacterial cell wall.²²⁾ Penicillin inhibits the peptidoglycantranspeptidase and D-alaninecarboxypeptidase activities of gram negative bacteria^{9,23)} and induces the swelling and bursting of the bacterial cells. On the other hand, chitin synthetase (UDP-*N*-acetylglucosamine:chitin acetylglucosaminyltransferase) has been prepared from *N. crassa*, *etc.*,¹¹⁻¹⁴⁾ but its inhibitor has not been reported. 2×10^{-7} M Polyoxin D inhibited the activity of chitin synthetase prepared from *P. oryzae* more than 80%. This concentration of Polyoxin D was enough to induce *in vivo* swelling of growing cell of *P. oryzae*. Therefore, it is suggested that the inhibition of chitin synthetase activity by Poly-

oxin D induced the swelling and bursting of the cell as penicillin did in bacteria.

So far, the lipid intermediates of chitin synthesis has not been reported and *N*-acetylglucosamine seemed to be directly incorporated from UDP-*N*-acetylglucosamine into the chitin by chitin synthetase in cell-free system. In the present study, we also could not find the lipid intermediate of chitin synthesis. However, in intact cells, the transport of UDP-*N*-acetylglucosamine synthesized in cytoplasm into cell wall chitin layer must involve an intermediate for permeation through cytoplasmic membrane. Further study on the lipids intermediate may be conducted in next paper of this series.

Isono *et al.* reported the stereo structure of Polyoxin is very similar to that of UDP-*N*-acetylglucosamine;⁴⁾ therefore, it is very interesting to know whether Polyoxin is an competitive inhibitor for UDP-*N*-acetylglucosamin and the relation between the Polyoxin derivatives and their inhibitory on chitin synthetase activity.

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