Studies on the Mode of Action of Polyoxins

Part III. Relation of Polyoxin Structure to Chitin Synthetase Inhibition

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(1) Chitin-UDP acetylglucosaminyltransferase (E.C. 2.4.1.16., chitin synthetase) in the cell-free system from phytopathogenic fungus *Piricularia oryzae*, and effects of various polyoxins and related compounds on the enzyme activity were studied. Polyoxins A~M, polyoxin A derivatives, polyoxin C derivatives, 5'-amino-5'-deoxyuridine, uridine and thymidine inhibited equally the incorporation of N-acetylglucosamine (GlcNAc) from UDP-N-acetylglucosamine (UDP-GlcNAc) into chitin.

(2) Competition between the above inhibitors and UDP-GlcNAc was observed by kinetic studies. The K_m for UDP-GlcNAc was determined to be $3.3 \times 10^{-3} M$ and the K_i values for polyoxins A~M, except polyoxin C, were found to be in the range of $3.3 \times 10^{-5} M$ to $3.4 \times 10^{-6} M$. For polyoxin C, 5'-amino-5'-deoxyuridine and uridine, the K_i values of $2.7 \times 10^{-3} M$, $8.0 \times 10^{-3} M$ and $3.0 \times 10^{-3} M$ were given, respectively. The inhibitor constants for other related compounds were also calculated.

(3) The values of binding affinity, $-\Delta G$, for formation of substrate- or inhibitor-enzyme complexes were calculated from the K_m or K_i values. In addition, partial binding affinities, $-\Delta g$, for certain moieties or groups of polyoxins were estimated from the $-\Delta G$. For instance, the $-\Delta G$ values for UDP-GlcNAc and polyoxin L were 5.7 kcal/mole and 9.2 kcal/mole, respectively. And the $-\Delta g$ values for the nucleoside moiety (part I*), the carbamylpolyoxamic acid moiety (part II*) and the carboxyl group* at C5' position of polyoxin L were 5.2, 3.5 and 0.7 kcal/mole, respectively.

(4) From the results obtained, the mechanism of action and relation between chemical structure and competitive inhibition of chitin synthetase were discussed.

Polyoxins A~M,¹⁾ which belong to nucleoside antibiotics produced by *Streptomyces cacaoi* var. *asoensis*, are now in practical use as an agricultural fungicides in Japan.

In previous papers,^{2,3)} Ohta *et al.* reported that polyoxin D, a component of polyoxins,

inhibited cell wall chitin synthesis of some phytopathogenic fungi and induced the accumulation of UDP-GlcNAc, an intermediate of chitin biosynthesis.

Recent studies using cell-free systems of *P. oryzae*³⁾ and *Neurospora crassa*⁴⁾ showed that polyoxin D strongly inhibited chitin synthetase in competition with UDP-GlcNAc.⁴⁾

Because polyoxins are the first antibiotics which inhibit fungal cell wall synthesis, it

* See Scheme 2.

1) K. Isono, K. Asahi and S. Suzuki, *J. Am. Chem. Soc.*, **91**, 7490 (1969).

2) S. Sasaki, N. Ohta, I. Yamaguchi, S. Kuroda and T. Misato, *Nippon Nogeikagaku Kaishi*, **42**, 633 (1968).

3) N. Ohta, K. Kakiki and T. Misato, *Agr. Biol. Chem.*, **34**, 1224 (1970).

4) A. Endo and T. Misato, *Biochem. Biophys. Res. Commun.*, **37**, 718 (1969).

should be very interesting to study their effect on chitin synthesis in cell free system. It is expected that an analogy between polyoxins and UDP-GlcNAc provides a structural basis for the inhibition of chitin synthesis.¹⁾

In the present paper, we attempt to elucid-

ate the relationships among chitin synthetase, UDP-GlcNAc and polyoxins. Polyoxins A~M, polyoxin A derivatives, polyoxin C derivatives and other related compounds, which structures are shown in Scheme 1, are used in the present studies.

Polyoxin	R ₁	R ₂	R ₃
A	CH ₂ OH	*	OH
B	CH ₂ OH	HO	OH
D	COOH	HO	OH
E	COOH	HO	H
F	COOH	*	OH
G	CH ₂ OH	HO	H
H	CH ₃	*	OH
J	CH ₃	HO	OH
K	H	*	OH
L	H	HO	OH
M	H	HO	H

	R
Polyoxin C	CH ₂ OH
Polyoxin C acid	COOH
Uracil polyoxin C	H
Thymine polyoxin C	CH ₃

SCHEME 1

MATERIALS AND METHODS

Chemicals. Polyoxins A, B, D, E, F, G, K, L, M, uracil polyoxin C¹¹ and polyoxin C acid were obtained from Kaken Chemical Co., Ltd. Polyoxins C, H, J, N-acetylpolyoxin A,¹¹ deaminopolyoxin A,¹¹ thymine polyoxin C¹¹ and other related substances were generous gifts from Dr. K. Isono, Institute of Physical and Chemical Research. D-Glucosamine-1-¹⁴C was purchased from Radiochemical Centre, England, and UDP-GlcNAc-¹⁴C was prepared according to the method described previously.⁵⁾ Chitinase and unlabeled UDP-GlcNAc were purchased from Nutritional Biochemicals and SIGMA, respectively. Soluble chitodextrin was prepared by acetolysis of commercial chitin (solvent mixture of acetic anhydride/conc sulphuric acid, (20:1), was used).⁶⁾

Organisms and growth conditions. *Piricularia oryzae* P₂, which was obtained from National Institute of Agricultural Sciences, Tokyo, was grown in Y.G. liquid medium (glucose 2%, yeast extract 0.4%) for 44 hr at 28°C with reciprocal shaking. *Neurospora crassa* wild strain IFO 6068 was grown in Vogel's medium⁷⁾ under the same conditions as *P. oryzae*.

Preparation of enzyme. Mycelia (10 g) of *P. oryzae* were harvested by filtration and washed three times with ice cold 1/20 M Tris-HCl buffer (pH 7.8) containing 5 mM MgCl₂ and 0.5 mM EDTA. The washed mycelia were suspended in 20 ml of the same buffer and then disrupted by grinding with 10 g of sands in a glass homogenizer at 0°C for 10 min. This homogenate was centrifuged at 15,000 × g for 30 min and the supernatant fraction was again centrifuged at 150,000 × g for 60 min. The precipitate obtained by the second centrifugation was suspended in 2 ml of the above buffer and this suspension was used as enzyme solution.

Assay of enzyme activity. Incubation mixtures contained 25 mM Tris-HCl, 2.5 mM MgCl₂, 0.25 mM EDTA, 25 mM GlcNAc, 1.5 mM UDP-GlcNAc-¹⁴C,

0.25 mg of soluble chitodextrin and 50 μl of the enzyme solution in a total volume of 100 μl. Incubations were made for 15 min at 24°C or for 120 min at 20°C. The reaction was stopped by heating the mixtures in a boiling water bath for 1 min. The reaction mixtures were spotted on the cellulose powder (Avicel SF) thin layer plate. Chromatography was performed overnight in isobutyric acid/1N ammonium hydroxide (5:3). Radioactive products remained at origins of the TLC plate (see Fig. 1) were cut out and counted on Beckman liquid scintillation spectrometer.

FIG. 1. A Radioautogram on Synthesis of Chitin and Effect of Polyoxin D.

Tubes of 1, 2 and 3, 4 were incubated for 5 min and 15 min, respectively. Polyoxin D (19 μM) was contained in the tubes 2 and 4. Other conditions of the experiment were the same as described in Fig. 5.

RESULTS

Formation of water insoluble material

When the particulate enzyme from *P. oryzae*

5) A. Endo, K. Kakiki, M. Hori, H. Abe and T. Misato, *Biochem. Biophys. Res. Commun.*, **39**, 718 (1970).

6) P. Perchemliden, T. Osawa, E. A. Davidson, and R. W. Jeanloz, *Carbohydrate Res.*, **3**, 463 (1963); J. Conchie, G. A. Levvy and C. A. Marsh, *Advances in Carbohydr. Chem.*, **12**, 157 (1957).

7) H. J. Vogel, *Microbial Genet. Bull.*, **13**, 42 (1956).

was incubated under the conditions described above, formation of a chromatographically immobile product was observed (Fig. 1). This material was water insoluble and digesting with chitinase, gave a degradation product which was identified as GlcNAc by Avicel SF TLC (isobutyric acid/1 N ammonium hydroxide, 5:3).

Effects of addition of GlcNAc and soluble chitodextrin

The addition of GlcNAc resulted in about 5-to 10-fold stimulation of chitin synthetase activity (Fig. 2). Half maximal activation of the enzyme was obtained at 9.5×10^{-3} M GlcNAc. The soluble chitodextrin also stimulated the formation of chitin in the presence of 50 mM GlcNAc (Fig. 3). In the presence of both GlcNAc and the soluble chitodextrin, stable chitin synthetase activity was usually obtained. On the other hand the enzyme

activity was very unstable without the addition of the soluble chitodextrin.

FIG. 3. Effect of Soluble Chitodextrin on Chitin Synthesis.

The reaction mixture contained 1 mM UDP-GlcNAc- 14 C (10,000 cpm), 25 mM Tris-HCl, 2.5 mM MgCl_2 , 0.25 mM EDTA, 50 mM GlcNAc and 20 μ l of the enzyme solution from *P. oryzae* in a total volume of 40 μ l, pH 7.8. Soluble chitodextrin was added as indicated. Incubation was for 15 min at 24°C.

FIG. 2. Effect of N-Acetylglucosamine (GlcNAc) on Chitin Synthesis.

The reaction mixture contained 2 mM UDP-GlcNAc- 14 C (8000 cpm), 25 mM Tris-HCl, 2.5 mM MgCl_2 , 0.25 mM EDTA and 50 μ l of the enzyme solution from *P. oryzae* in a total volume of 100 μ l, pH 7.8. GlcNAc was added as indicated. Incubation was for 120 min at 20°C.

FIG. 4. Time Course of Chitin Synthesis.

The experiment was carried out as described in Fig. 2 and GlcNAc was added at the concentration of 25 mM.

Time course of chitin synthesis

The synthesis of chitin was almost linear during the first 120 min under the conditions described in Fig. 4, while a linearity was observed during the first 15 min under the another conditions in Fig. 5.

FIG. 5. Time Course of Chitin Synthesis.

The reaction mixture contained 1.5 mM UDP-GlcNAc- ^{14}C (19,000 cpm), 25 mM Tris-HCl, 2.5 mM MgCl_2 , 0.25 mM EDTA, 50 mM GlcNAc, 0.1 mg of soluble chitodextrin and 10 μl of the enzyme solution from *P. oryzae* in a total volume of 25 μl , pH 7.8. Incubation was at 24°C for the periods indicated.

Michaelis constant

From the data in Fig. 6, it was calculated that the K_m for UDP-GlcNAc was $3.3 \times 10^{-3} \text{ M}$ in the presence of GlcNAc and soluble chitodextrin.

Effects of polyoxins and related compounds**

Polyoxins A~M, except polyoxin C, caused 50% inhibition of chitin synthetase at the concentrations in the range of 5 to 25 $\mu\text{g}/\text{ml}$ (Table I). By removal or acetylation of the amino group at C2'' position, however, the inhibitory activity of polyoxin A was greatly

TABLE I. EFFECTS OF VARIOUS POLYOXINS ON CHITIN SYNTHESIS IN *P. oryzae*.

The reaction mixture contained 2 mM UDP-GlcNAc- ^{14}C (4,000 cpm), 25 mM Tris-HCl, 2.5 mM MgCl_2 , 0.25 mM EDTA, 25 mM GlcNAc, 0.5 mg of soluble chitodextrin and 50 μl of enzyme solution from *P. oryzae* in a total volume 100 μl , pH 7.8. Incubation was for 120 min at 20°C.

Amount of polyoxins and related substances added, ($\mu\text{g}/\text{ml}$)	UDP-GlcNAc incorporated ($\mu\text{mole}/2 \text{ hr}$)	Percent inhibition
None (control)	11.6	
Polyoxin A 10	7.2	39
100	1.5	87
B 10	4.2	64
100	0.6	95
C 10	12.3	0
100	10.7	8
D 10	6.1	47
100	1.3	89
E 10	9.9	15
100	6.8	41
F 10	6.0	48
100	1.6	86
G 10	7.7	34
100	2.0	82
K 10	7.4	37
100	1.8	85
L 10	5.4	53
100	1.5	87
M 10	9.6	17
100	6.5	44
N-Acetylpolyoxine A 1000	9.7	17
Deaminopolyoxin A 1000	8.3	29

lowered. Although it has so far been regarded that polyoxin C⁸⁾ and its derivatives have no biological activity, the inhibition of chitin synthetase by them were observed at the high concentration as shown in Table II. Uridine and 5'-amino-5'-deoxyuridine⁹⁾ also inhibited the enzyme, but by uracil, carbamylpolyoxamic acid¹⁾ and polyoxamic acid,¹⁾ the synthetic

8) K. Isono, J. Nagatsu, K. Kobinata, K. Sasaki and S. Suzuki, *Agr. Biol. Chem.*, **31**, 190 (1967).

9) K. Isono, T. Azuma and S. Suzuki, *Chem. Pharm. Bull.*, **19**, 505 (1971).

* See Scheme 1.

TABLE II. EFFECTS OF POLYOXIN C, POLYOXIN C DERIVATIVES AND OTHER RELATED SUBSTANCES ON CHITIN SYNTHESIS IN *P. oryzae*

The experiment was carried out as described in Table I.

Amount of polyoxins and related substance added ($\mu\text{g/ml}$)	UDP-GlcNAc incorporated ($\text{m}\mu\text{mole/2 hr}$)	Percent inhibition
Exp. I.		
None	42.6	
Polyoxin D 25	13.8	68
C 1000	29.8	30
Uracil polyoxin C 1000	37.8	11
Polyoxin C acid 1000	33.4	21
Exp. II		
None	25.0	
Polyoxin D 10	15.3	39
Uridine 1000	13.0	48
5'-Amino-5'-deoxyuridine 1000	21.4	14
Uracil 1000	25.0	0
Polyoxamic acid 1000	23.8	5
Carbamylpolyoxamic acid 1000	25.0	0

reaction was not inhibited at the concentration of 1,000 $\mu\text{g/ml}$ (Table II).

The effects of the various inhibitors of chitin synthetase on the reaction velocity at various UDP-GlcNAc concentrations were investigated and the data obtained were plotted according to Lineweaver and Burk¹⁰⁾ (Fig. 6, Fig. 7, Fig. 8, Fig. 9).

It was found that the relationships between UDP-GlcNAc and the all inhibitors were evidently competitive. In addition, the finding that uridine was a competitor for UDP-GlcNAc was also confirmed by Dixon plot¹¹⁾ (Fig. 10).

In addition to uridine, some other nucleosides were used to examine their effects on the chitin synthetase. As shown in Table III, uridine and thymidine inhibited the enzyme at the concentrations of 250 and 1,000 $\mu\text{g/ml}$.

FIG. 6. Competitive Inhibition of Chitin Synthetase by Polyoxins.

Assays were carried out as described in the text except that the reaction mixture contained UDP-GlcNAc-¹⁴C (15,000 cpm) at the various concentrations. Polyoxins A, B, D, E and M were added at the concentrations indicated. Incubation was for 15 min at 24°C. Data were plotted according to Lineweaver and Burk.

However other nucleosides including cytidine, which have a pyrimidine base like the above two nucleosides showed no inhibition.

Other antibiotics such as penicillin, bacitracin, cycloserine, cycloheximide and griseofulvin did not inhibit chitin synthetase in this system at the concentration of 800 $\mu\text{g/ml}$.

Inhibitor constant

From the data in Fig. 6~9, the K_i values for various polyoxins and other related substances were calculated (Table IV).

In the case of polyoxins A~M, except polyoxin C, the K_i values were in the range of $3.3 \times 10^{-5} \text{ M}$ to $3.4 \times 10^{-6} \text{ M}$ were about 100 to 1,000 times higher than the K_m value for UDP-GlcNAc ($3.3 \times 10^{-3} \text{ M}$). When the amino group at C2'' position of polyoxin A was removed (deaminopolyoxin A) or acetylated (N-acetylpolyoxin A), the K_i for the derivatives were increased about 100 times. In polyoxins

10) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

11) M. Dixon, *Biochem. J.*, **55**, 170 (1953).

FIG. 7. Competitive Inhibition of Chitin Synthetase by Polyoxins.

The experiment was carried out as described in Fig. 6. Polyoxins F, G, H, J, K and L were used as indicated.

with a methyl group as a substituent at the R_1 position, such as polyoxins H and J, the inhibitory activities of them were approximately doubled compared with those of polyoxins A, B, D, F and L. The K_i values for polyoxins E, G and M were about 3 times larger than those for polyoxins with a hydroxyl group as R_3 such as polyoxins B, D and L.

On the K_i values for a series of compounds related to polyoxin C, several interesting findings were obtained. The K_i for polyoxin C was $2.7 \times 10^{-3} M$ and was the same order as the K_m value. The K_i for uracil polyoxin C and its decarboxylated compound, 5'-amino-5'-deoxyuridine (see Scheme 1), were $2.5 \times 10^{-3} M$ and $8.0 \times 10^{-3} M$, respectively. From the data in either Figs. 9 and 10, the K_i for uridine was calculated as $3.0 \times 10^{-3} M$.

FIG. 8. Competitive Inhibition of Chitin Synthetase by Polyoxins.

Assays were carried out as described in the text except that reaction mixture contained UDP-GlcNAc- ^{14}C (15,000 cpm) at the various concentrations indicated. Polyoxin C, polyoxin C acid, thymine polyoxin C and uracil polyoxin C were added at the concentrations indicated. Incubation was for 15 min at $24^\circ C$.

TABLE III. EFFECTS OF VARIOUS NUCLEOSIDES ON CHITIN SYNTHESIS IN *P. oryzae*

The experiment was carried out as described in the text except that the incubation was for 30 min at $24^\circ C$ and the concentration of UDP-GlcNAc- ^{14}C (10,500 cpm) was 1.25 mM.

Amount of nucleosides added (ug/ml)	UDP-GlcNAc incorporated (mμmole/30 min)	Percent inhibition
None (control)	42.5	
Adenosine 1000	40.5	
250	41.3	
Cytidine 1000	43.0	
250	40.4	
Guanosine 1000	43.8	
250	42.6	
Inosine 1000	42.4	
250	43.2	
Thymidine 1000	31.7	24
250	36.7	12
Uridine 1000	19.4	55
250	23.6	43
Xanthosine 1000	44.1	
250	43.5

FIG. 9. Competitive Inhibition of Chitin Synthetase by Uridine and 5'-Amino-5'-deoxyuridine.

The experiment was carried out as described in Fig. 8 except that uridine and 5'-amino-5'-deoxyuridine were added instead of polyoxins at the concentrations indicated.

FIG. 10. Competitive Inhibition of Chitin Synthetase by Uridine.

The experiment was carried out as described in the text except that the reaction mixture were contained UDP-GlcNAc-¹⁴C (10,500 cpm) and uridine at the various concentrations indicated. Incubation was for 15 min at 24°C. Data were plotted according to the method of Dixon.

Binding affinity

As a measure of the strength of the binding between the enzyme and UDP-GlcNAc

TABLE IV. VALUES OF INHIBITOR CONSTANT, K_i , AND BINDING AFFINITY, $-\Delta G$, FOR POLYOXINS AND RELATED COMPOUNDS

The K_m and the $-\Delta G$ for substrate UDP-GlcNAc were 3.3×10^{-3} M and 5.7 kcal/mole, respectively.

Competitive inhibitors of chitin synthetase	K_i (M)	$-\Delta G$ (kcal/mole, 24°C)
Polyoxin A	1.1×10^{-5}	9.0
B	5.6×10^{-6}	9.4
D	1.2×10^{-5}	9.0
E	3.3×10^{-5}	8.4
F	7.5×10^{-6}	9.2
G	1.5×10^{-5}	8.7
H	3.9×10^{-6}	9.6
J	3.4×10^{-6}	9.7
K	1.1×10^{-5}	9.0
L	8.1×10^{-6}	9.2
M	2.1×10^{-5}	8.6
N-Acetylpolyoxin A	1.1×10^{-3}	6.4
Deaminopolyoxin A	8.6×10^{-4}	6.5
Polyoxin C	2.7×10^{-3}	5.8
Uracil polyoxin C	2.5×10^{-3}	5.9
Thymine polyoxin C	1.4×10^{-3}	6.2
Polyoxin C acid	1.5×10^{-3}	6.2
Uridine	3.0×10^{-3}	5.8
5'-Amino-5'-deoxyuridine	8.0×10^{-3}	5.2

TABLE V. VALUES OF PARTIAL BINDING AFFINITY, $-\Delta g$, FOR VARIOUS MOIETIES^{a)} OR GROUPS^{a)} OF POLYOXINS

	Calculation methods ($-\Delta G$) polyoxin X- ($-\Delta G$) polyoxin Y	$-\Delta g$ (kcal/mole, 24°C)
Part I ($R_1=H$)		5.2
Part II ($R_3=OH$)	(B)-(C) (L)-(uracil C) (J)-(thymine C)	3.6 3.3 3.5 } 3.5 ^{b)}
Part II ($R_3=H$)	(G)-(C) (M)-(uracil C)	2.9 2.9 } 2.9 ^{b)}
-COOH at C5'	(uracil C)-(5'-amino-5'-deoxyuridine)	0.7
-NH ₂ at C2''	(A)-(deamino A) (A)-(N-acetyl A)	2.2 2.0 } 2.1 ^{b)}
-OH at C3''	(B)-(G) (D)-(E) (L)-(M)	0.6 0.6 0.6 } 0.6 ^{b)}
-CH ₃ at C5	(H)-(K) (J)-(L)	0.6 0.5 } 0.6 ^{b)}

^{a)} See Scheme 2.

^{b)} Mean of $-\Delta g$ values.

or polyoxins, we used a so-called binding affinity,¹²⁾ $-\Delta G^*$, which could be calculated from the K_m ($\equiv K_s$) value or the K_i value. The larger the value of $-\Delta G$, the greater the affinity of UDP-GlcNAc or polyoxins for the enzyme. From the K_i values described above the $-\Delta G$ values were calculated (Table IV). In addition, since it was assumed that polyoxins were bound to the enzyme through several moieties and groups of their molecules the values of partial binding affinity,¹²⁾ $-\Delta g^{**}$, for certain moieties and groups of polyoxins were calculated from the above $-\Delta G$ values (Table V).

As shown in Scheme 2, polyoxin structures can be divided into three parts. In the case of polyoxin L, the $-\Delta G$ value was 9.2 kcal/mole. This value and the sum of the $-\Delta g$ values for part I (5.2 kcal/mole), part II (3.5 kcal/mole) and carboxyl group at C5' position (0.7 kcal/mole) were approximately equal. The

$-\Delta G$ values for polyoxin C and a series of related compounds of it were in the range of 5.2~6.2 kcal/mole. The $-\Delta g$ value for the amino group at C2'' position was 2.1 kcal/mole and was a fairly great value compared with the $-\Delta G$ value for whole molecule of polyoxin.

When it was assumed that $K_s \equiv K_m$, the $-\Delta G$ value for UDP-GlcNAc was 5.7 kcal/mole.

Particulate enzyme from *N. crassa*

Enzyme was prepared according to method as used in *P. oryzae*. The particulate fraction obtained from the second centrifugation was suspended in 8 ml of 1/20 M Tris-HCl buffer, pH 7.8, containing 5 mM $MgCl_2$ and 0.5 mM EDTA, and was used as an enzyme solution.

When the particulate enzyme was incubated under the conditions shown in Fig. 11, the

FIG. 11. Time Course of Chitin Synthesis Catalyzed by the Particulate Enzyme from *N. crassa*.

The reaction mixture contained 1.25 mM UDP-GlcNAc- ^{14}C (10,000 cpm), 25 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.25 mM EDTA, 10 mM GlcNAc and 50 μ l of the enzyme solution from *N. crassa* in a total volume of 100 μ l, pH 7.8. Incubation was at 24°C for the periods indicated.

12) W. Kauzmann, *Advance in Protein Chemistry*, **14**, 1 (1959); D.M. Chipman and N. Sharon, *Science*, **165**, 454 (1969); Y. Nitta, *Protein Nucleic Acid Enzyme*, **15**, 1262 (1970).

* $-\Delta G = -RT \ln K + 2.4$ (kcal/mole, 24°C)

** $-\Delta G = -(\Delta g_1 + \Delta g_2 + \Delta g_3 + \dots)$

formation of insoluble chitin was observed and the rate of the formation was linear over a period of 30 min (Fig. 11).

The effects of polyoxins A~M, deaminopolyoxin A and uridine on the synthesis of chitin were investigated. The enzyme from *N. crassa* seemed to be somewhat sensitive for polyoxins compared with that from *P. oryzae* (Table VI). The data in Fig. 12 show-

TABLE VI. EFFECTS OF VARIOUS POLYOXINS ON CHITIN SYNTHESIS IN *N. crassa*

The experiment was carried out as described in Fig. 11. Incubation was for 10 min at 24°C.

Amount of polyoxins added, $\mu\text{g/ml}$	UDP-GlcNAc incorporated (m $\mu\text{mole/10 min}$)	Percent inhibition
None (control)	12.8	
Polyoxin A 5	8.1	37
B 5	6.3	51
C 240	10.9	15
D 5	8.0	37
E 5	10.4	19
F 5	10.2	20
G 5	7.6	41
H 5	7.7	40
J 5	5.5	57
K 5	8.5	34
L 5	7.2	44
M 5	9.9	23
Deaminopolyoxin A 250	9.7	24

ed that uridine was a competitor for UDP-GlcNAc and the K_i value was $1.0 \times 10^{-3} \text{M}$. This value was about one third of that obtained in the case of *P. oryzae* and differed scarcely from the K_m value for UDP-GlcNAc ($1.43 \times 10^{-3} \text{M}$), which has been reported by Endo *et al.*⁴¹

DISCUSSION

It has been considered that polyoxin C has no antifungal activity.⁸⁾ Ohta *et al.*³¹⁾ reported that polyoxin C did not inhibit the incorporation of glucosamine-¹⁴C into cell wall

FIG. 12. Competitive Inhibition of Chitin Synthetase from *N. crassa* by Uridine.

The experiment was carried out as described in Fig. 11 except that the reaction mixture contained UDP-GlcNAc-¹⁴C (10,000 cpm) and uridine at the various concentrations indicated. Incubation was for 10 min at 24°C. Data were plotted according to the Dixon plot.

chitin at the concentration of 50 $\mu\text{g/ml}$. The findings described in this paper showed that polyoxin C and its derivatives inhibited the synthesis of chitin in cell free system in competition with UDP-GlcNAc. However, the K_i for polyoxin C is the same order as the K_m for UDP-GlcNAc and is about 10^3 times higher than those for other polyoxins. The reason why polyoxin C is inactive for growing or resting cells is that UDP-GlcNAc is presumably present at rather high concentration in intact fungal cells. At any rate, findings that 5'-amino-5'-deoxyuridine which is a basal structure of polyoxins is a competitive inhibitor of the enzyme but carbamylpolyoxamic acid which corresponds to the part II of polyoxins has no activity, suggest the ab-

solute need of the part I moiety for the inhibitory action of polyoxins.

It is suggested that the difference between activities of polyoxin C and other polyoxins is due to the affinity of the part II moiety for the enzyme. In polyoxin L, for instance, the $-\Delta g$ value for the part II is 3.5 kcal/mole and is about 40% of the $-\Delta G$ value for the whole molecule. And polyoxin C does not contain this moiety in its molecule. Amino group ($-\Delta g=2.1$ kcal/mole) at C2'' position plays main role for the binding of Part II with the enzyme and hydroxyl group ($-\Delta g=0.6$ kcal/mole) as a substituent for R_3 is also important. Depending upon the varieties of substituents for R_1 and R_2 , minor differences among the activities of polyoxins, except polyoxin C, were observed. The component, which showed the highest activity, was polyoxin J containing a methyl group as R_1 and hydroxyl groups as R_2 and R_3 . The sum of the $-\Delta g$ values for these three groups is 1.9 kcal/mole and is about 20% of the $-\Delta G$ value for polyoxin J.

As mentioned in "Results," $-\Delta G$ value for certain polyoxins and the sum of $-\Delta g$ values for part I, part II and some groups constituting a polyoxin molecule were almost equal. In other words, the relation $-\Delta G \doteq -(\Delta g_1 + \Delta g_2 + \Delta g_3 + \dots)$ could be obtained. These results indicate that the so-called binding affinity concept is applicable to our investigation purposes.

In the inhibition process of chitin synthetase, inhibitor uridine and substrate UDP-GlcNAc are competitive and the $-\Delta G$ values for them are almost equal. From these facts, it is presumed that UDP-GlcNAc is bound tightly to the enzyme with its nucleoside moiety,* while the residual part* of UDP-GlcNAc is bound loosely. It seems likely, therefore, that there are binding site and catalytic site on the chitin synthetase molecule and the uridine moiety of UDP-GlcNAc can attach to the binding

site. And presumably the inhibition of chitin synthetase by polyoxins is due to the competition between the part I of polyoxins and the nucleoside moiety of UDP-GlcNAc at the binding site on the enzyme molecule. In addition, the part II and the substituents for R_1 and R_2 , may enhance markedly the binding of the part I to the enzyme. However, it is uncertain whether the part II and the R_2 substituent attach to the catalytic site. A structure of the binding site is presumably complementary to uridine and its derivatives with a substituent such as $-\text{CH}_3$, $-\text{CH}_2\text{OH}$ and $-\text{COOH}$ at C5' position. The finding that the inhibitory activity of thymidine, a nucleoside containing methyl group at C5' position, was lower than that of uridine (Table III) was possibly due to lacking of the hydroxyl group at C2' position of ribose moiety. In other word, it is assumed that the hydroxyl groups of ribose moiety of polyoxins contribute to the binding. Further studies will be necessary to elucidate the more defined role of the part II or the R_2 substituents on the competitive inhibition of chitin synthetase.

The particulate enzyme from *P. oryzae* catalyzed the synthesis of insoluble chitin from UDP-GlcNAc in the presence of GlcNAc and soluble chitodextrin. Glaser *et al.*¹³⁾ reported that soluble chitodextrin was a primer of the reaction and soluble chitin synthetase had an absolute primer requirement. From our experiments, it is presumed that primer content in the particulate enzyme fraction depends upon preparation methods and varieties of fungi. When the primer content is not sufficient for chitin synthesis, the addition of soluble chitodextrin may be required.

Two particulate enzymes obtained from *P. oryzae* and *N. crassa* are different each other in some characteristics such as sensitivity for inhibitors, the K_m for UDP-GlcNAc and

* See Scheme 2.

13) L. Glaser and D. H. Brown, *J. Biol. Chem.*, **228**, 729 (1957).

the requirement of activators. But it is not clear where these differences have derived from. In any case, the inhibition of chitin synthetase in *N. crassa* by polyoxins can be explained in the same manner as described in the case of *P. oryzae*.

The results of the experiments described in a series of our papers indicate that the primary action of polyoxins is the competitive inhibition of chitin synthetase. And in views of the chemical structure and the mechanism

of action, polyoxin is a new type of the inhibitor of cell wall synthesis.

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