POLYOXIN D, A COMPETITIVE INHIBITOR OF UDP-N-ACETYLGLUCOSAMINE: CHITIN N-ACETYLGLUCOSAMINYLTRANSFERASE IN NEUROSPORA CRASSA

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Summary -- The antibiotic polyoxin D inhibited the incorporation of glucosamine-^{14}C into cell wall chitin in Neurospora crassa. In addition, it caused the accumulation of labeled UDP-N-acetylglucosamine (UDP-GlcNAc) under the same conditions, indicating an inhibition in the UDP-GlcNAc: chitin N-acetylglucosaminyltransferase (chitin synthetase) reaction. Kinetic studies of the cell free system showed that polyoxin D strongly inhibited the incorporation of N-acetylglucosamine from UDP-GlcNAc into chitin. The relation between polyoxin D and UDP-GlcNAc was a competitive one. The Km for UDP-GlcNAc and the Ki for polyoxin D was 1.43×10^{-3}M and 1.40×10^{-6}M, respectively.

Polyoxin D, a component of the uracil antibiotic polyoxin complex, is produced by Streptomyces cacaoi var. asoensis (Isono et al., 1967; Isono and Suzuki, 1968). The antibiotic inhibits the growth of some filamentous fungi, but has no effect on bacteria and yeasts. Ohta et al. (1969) have reported that polyoxin D inhibited the uptake of glucosamine-^{14}C into the cell wall chitin in the plant pathogen Cochliobolus miyabeanus. During the course of an investigation of the biosynthesis of fungal cell wall, it was shown that polyoxin D at a concentration of 100 μg/ml strongly inhibited N. crassa. At this concentration it reduced the incorporation of glucosamine-^{14}C into the cell wall fraction while it showed no effect on the incorporation of labeled inorganic phosphate into nucleic acid and of labeled glutamic acid into protein. The present communication provides evidence

that the primary effect of polyoxin D is the competitive inhibition of chitin synthetase (E C 2.4.1.16).

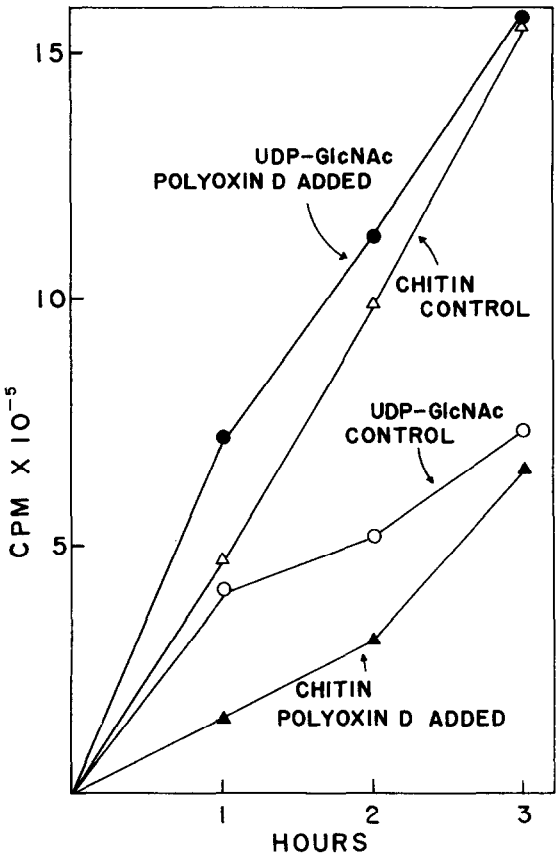

Fig. 1. Effect of polyoxin D on the incorporation of glucosamine-^{14}C into chitin and UDP-GlcNAc in N. crassa. Young mycelia of N. crassa were obtained by inoculating Vogel's medium (1956) with a heavy spore suspension and incubating on a reciprocal shaker at 27°. After 16 hours mycelia were harvested by centrifugation and suspended in 0.05 M Tris-maleate buffer, pH 6.0 (3 mg dry weight/ml). Five ml of the mycelial suspension was transferred to a flask and shaken at 27° for 5 minutes. Incubation was started by addition of 5 μC (1.2 μmoles) of glucosamine-^{14}C and 0.19 mM polyoxin D (where indicated) to the suspension (final volume 5 ml). Incubation was terminated by addition of 5 ml of 10% trichloroacetic acid to the incubation mixture after different times. The mixture was then centrifuged. The resultant pellet was treated with hot trichloroacetic acid to extract nucleic acid, followed by digestion with chitinase (Nutritional Biochemicals Corp.). The chitinase-solubilized fraction was collected (referred to as chitin) and counted. Nucleotides in the acid-soluble fraction obtained above were adsorbed on charcoal and eluted with ammoniacal ethanol, and the UDP-GlcNAc was isolated by paper electrophoresis in pyridine-acetic acid-water (1:10:69), pH 3.5.

Glucosamine-^{14}C was obtained from Radiochemical Centre, England. Polyoxin D was a generous gift from Dr. S. Suzuki of the Institute of Physical and Chemical Research, Saitama, Japan.

Fig. 1 shows that polyoxin D at a concentration of 100 μg/ml (0.19 mM) nearly halved the incorporation of glucosamine-^{14}C into chitin. All of the radioactivity in the chitin fraction was contained in N-acetylglucosamine after the digestion with chitinase and subsequent paper chromatography in n-butanol-pyridine-water (2:1.5:0.75) or in isobutyric acid-1N NH_4OH (50:30). On the other hand, polyoxin D almost doubled the UDP-GlcNAc-^{14}C level in the culture under the same conditions (Fig. 1). UDP-GlcNAc-^{14}C was the only labeled nucleotide in the culture, as ascertained by paper chromatography in ethanol-1M ammonium acetate (75:35), pH 7.5, paper electrophoresis in pyridine-acetic acid-water (1:10:69), pH 3.5, and the determination of its activity as a substrate in the chitin synthetase system of N. crassa (see below). From the data in Fig. 1, the sum of radioactivity of the UDP-GlcNAc and the chitin fraction at different times was found to be essentially the same in the control and the polyoxin D-added experiment. These results indicate that there was an inhibition in chitin synthetase reaction, since glucosamine must be converted to UDP-GlcNAc before it can be incorporated into chitin.

TABLE I

Effect of polyoxin D on the chitin

Synthetase of N. crassa

Variation of complete system	UDP-GlcNAc incorporated (mμmoles/5 min.)	Per cent inhibition
None	16.0	
+ 4.7 μM polyoxin D	4.3	73
+ 23.5 μM polyoxin D	1.5	91

Ten grams of 48-hour mycelia of N. crassa were ground with sands and 30 ml of 0.05 M Tris-HCl, pH 7.5 in a mortar at 0°. The mixture was centrifuged at 20,000 x g for 30 minutes. The supernatant solution was then centrifuged at 100,000 x g for 2 hours. The particles obtained were dispersed in 8 ml of 0.05 M Tris-HCl, pH 7.5. The complete reaction mixture contained per 0.2 ml final volume: 37.5 mM Tris-HCl, pH 7.5, 1.8 mM UDP-GlcNAc (46,800 cpm), 5 mM $MgCl_2$, 5 mM GlcNAc, 0.05 ml of the particle suspension. After 5 minutes of incubation at 24° the reaction was stopped by heating for 1 minute at 100°. The precipitate was collected by centrifugation, washed twice with cold water and counted. UDP-GlcNAc-^{14}C labeled in the glucosamine moiety was prepared according to Glaser and Brown (1955).

Polyoxin D markedly inhibited chitin synthetase in the cell free system (Table I). The kinetics of the inhibition were of the competitive type (Fig. 2), i.e., polyoxin D increased the Km for UDP-GlcNAc without changing the maximum velocity. From the data in Fig. 2, the Km for UDP-GlcNAc was calculated to be 1.43 x 10^{-3}M, and the Ki for polyoxin D, 1.40 x 10^{-6}M.

Polyoxin D at a concentration of 100 µg/ml (0.19 mM) showed no effect on UDP-GlcNAc pyrophosphorylase (E C 2.7.7.23) of N. crassa, which was assayed by using UDP-GlcNAc as a substrate, indicating that the antibiotic was not a competitor for UDP-GlcNAc in the pyrophosphorylase enzyme.

Recently lipid intermediates have been shown to be involved in the biosynthesis of cell wall polysaccharides of bacteria and yeast (Anderson et al., 1965; Weiner et al., 1965; Scher et al., 1968; Tanner, 1969). Various attempts to detect a lipid intermediate in the chitin synthetase system of N. crassa, however, were unfruitful so far.

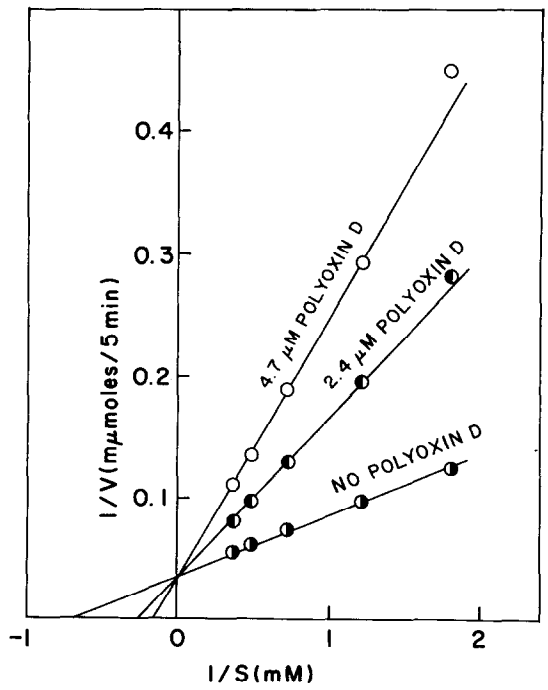

Fig. 2. Competitive inhibition of chitin synthetase by polyoxin D. Conditions were the same as in Table I except that the reaction mixture contained varying amounts of UDP-GlcNAc-^{14}C. Data were plotted according to Lineweaver and Burk (1934).

The chitin synthetase system was far more sensitive to polyoxin D than was the intact mycelia (Fig. 1 and Table I). The existence of a barrier to the penetration of the antibiotic to the site of the sensitive enzyme is probably at least a partial explanation of the relative insensitivity of intact mycelia. The results reported above indicate that polyoxin D selectively inhibits the synthesis of cell wall chitin in N. crassa at levels which are comparable with those required for antifungal activity and that the primary effect is the competitive inhibition of chitin synthetase. That the ratio of Km to Ki was about 1000 emphasizes the efficiency of the antibiotic as a competitor for the substrate UDP-GlcNAc.

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