STEROLS IN RELATION TO THE INHIBITION OF FUNGI BY NYSTATIN.

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STEROLS IN RELATION TO THE INHIBITION OF FUNGI BY NYSTATIN

DISSERTATION

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By

Edison Rudolph Fowlks, B.S., M.S.

The Ohio State University

1965

Approved by

[Signatures]

Adviser
Department of Botany and Plant Pathology

[Signatures]

Adviser
Department of Agricultural Biochemistry
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VITA

August 29, 1938  Born - Terrell, Texas

1960 .......... B.S., Prairie View A & M College, Prairie View, Texas

1962 .......... M.S., Michigan State University, East Lansing, Michigan

1962-1965 . . Teaching Assistant, Department of Botany and Plant Pathology, The Ohio State University, Columbus, Ohio
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INTRODUCTION

The polyene antibiotics, notably inhibitory to fungi, also inhibit the growth of a broad spectrum of organisms (3,9,12,13,18). Bacteria and blue green algae appear to be insensitive to these materials, however (4,9,18). Representative polyene antibiotics are amphotericin A and B, ascosin, candididin, candidin, endomycin, filipin, flavacid, fungichromin, fumagillin, nystatin, rimocidin, and sistomycosin.

It is noteworthy that of all the fungi tested, the few which are not affected by these antibiotics are pythiaceous types. Because of this selectivity, polyene antibiotics have become useful in the isolation of Pythium and Phytophthora from soil or plant parts (5,22).

The mode of action of polyene antibiotics is not fully delineated. Sterols have been implicated as playing a role in the sensitivity of various organisms to these materials. It has been suggested that organisms containing sterols in their cellular membranes are inhibited, whereas those with no sterols are not affected (4,16,18). Evidence indicates that polyene antibiotics form a complex with the sterol present in the membranes of cells of sensitive organisms, thus altering the permeability (9,15,19). As a result, vital constituents are lost from the cell and lysis occurs. A review of sterols and steroids in relation to microorganisms has been published recently (3).

The purpose of the present work is to study the mechanism of action of nystatin on two fungi, using a pythiaceous species that is not
inhibited and a non-pythiaceous type which is inhibited by this antibiotic. Particular attention is given to experiments with sterols and to protein synthesis in cell free systems.
Culturing of fungi

*Pythium aphanidermatum* (Edson) Fitz (isolate No. 547) and *Colletotrichum lagenarium* (Pass.) Ell. & Halst. (isolate No. 525) were selected as tools for this study because they were pathogenic to cucumber (*Cucumis sativus* L.), the *Pythium* producing a root rot and the *Colletotrichum* a leaf spot on seedlings of the variety "Green Prolific" under specific greenhouse conditions (Curt Leben, Department of Botany and Plant Pathology, Ohio State University, unpublished results). Stock cultures of the *Pythium* were maintained at 24°C under mineral oil on PA agar and the *Colletotrichum* on CL agar. Transfers for routine use were made each month. PA agar was composed per liter of 2 g of sucrose, 0.5 g of asparagine, 0.15 g of KH₂PO₄, 0.15 g of K₂HPO₄, 0.1 g of MgSO₄, 0.058 g of ethylenediamine tetraacetic acid (Na form), 0.001 g of thiamine, 0.010 g of ascorbic acid, 0.020 g of cholesterol, 10 ml of mineral trace stock (0.278 g of CaCl₂, 1.438 g of MnCl₂, 0.838 g of ZnCl₂, 0.048 g of FeCl₃ made to 1 liter with water) and 20 g of Difco agar (Difco Laboratories, Detroit, Michigan). CL agar was composed per liter of 20 g of dextrose, 1.1 g of NaNO₃, 0.06 g of KH₂PO₄, 0.078 g of K₂PO₄, 0.049 g of MgSO₄, 2.0 ml of mineral trace stock (same formulation as used in PA agar) and 20 g of agar.

Most of the experiments on the growth of the test fungi were conducted in 250 ml Erlenmeyer flasks containing 100 ml of a basal
liquid medium supplemented as indicated in the section on results. The medium was autoclaved for 15 min at 121°C. Seeded flasks were shaken for 3 days at 24°C on a reciprocal shaker operating at 110 oscillations per min. The basal medium (pH, 6.8) consisted per liter of 8 g of nutrient broth (Difco) and 10 g of dextrose. There were three replicate flasks for each treatment in each test. At the end of the incubation period, the mycelium was separated from the medium by filtration through Whatman No. 1 filter paper. The mat was removed from the paper and washed several times with deionized water. For sterol extractions, the mat was dried ca. 12 hr in a forced air circulation incubator at 50°C. The moist mat was used for studies on protein synthesis.

For the preparation of inoculum, Pythium was cultured for 5 days on 25 ml of PA agar in flasks. Twenty-five ml of sterile, deionized water was added to each flask and allowed to stand 5 hours at 16°C to permit the release of zoospores. This suspension, which contained ca. $6 \times 10^4$ zoospores per ml, was used as seed material in a proportion of 0.5 ml per 100 ml of medium, unless specified differently. The medium and methods employed for zoospore production were recommended by A. F. Schmitthenner. Colletotrichum was cultured 9 to 21 days at 24°C on 25 ml of CL agar in 250 ml Erlenmeyer flasks. Conidia were scraped from the bottom of a flask with an inoculation needle and suspended in 25 ml of sterile, deionized water.

All culturing procedures were done at 24°C.

A single lot of nystatin (Lot No. 42262-042, 3870 units/mg) was used. The cholesterol and ergosterol were obtained from Calbiochem Company (Los Angeles) and Nutritional Biochemicals Corporation (Cleveland),
respectively. Sterols and nystatin were dissolved in dimethyl formamide before addition to the medium. Tests, not herein reported, demonstrated that this solvent was not inhibitory to either test fungus at the concentration that was used (not over 2% of total volume).

**Extraction of sterols from test fungi.**—Kieber's (14) method of sterol extraction was used. Fifteen g of 3-day-old dried mycelium from ca. 4 liters of basal liquid medium was saponified by adding 200 ml of 5% NaOH and autoclaving for 1 hr at 121°C. The suspension was cooled to room temperature, 100 ml of petroleum ether (b.p. 30-60°C) added, and the mixture placed on a reciprocal shaker for 1 hr. The ether layer was allowed to separate by placing the mixture in a 0°C room for 12 hours. The ether layer was removed and evaporated under nitrogen. The crude sterol-containing residue was dissolved in ca. 0.25 ml of a 3:1 (v/v) chloroform-methanol solution. The following methods were used for sterol detection in this material: (a) thin layer chromatography, (b) gas chromatography, and (c) Lieberman-Burchard test (2).

The method of Waldi (25) for preparing thin layer chromatoplates was modified as follows: 100 g of alumina was shaken in 200 ml of a 7:1 (v/v) chloroform-methanol solution. Microscope slides were dipped in the resulting suspension and dried. An aliquot (ca. 0.1 microliter) of the sterol-containing residue was placed at the bottom of a coated slide. Aliquots of pure ergosterol were used for comparisons. Slides were developed in a Stendor microscope slide staining jar containing 10 ml of benzene. The bottom of the slide rested in benzene; filter paper was placed in the jar around the sides to maintain a saturated atmosphere. Slides remained in the equilibrated jar for ca. 2 minutes; they were
dried and then exposed to an iodine vapor atmosphere, which reacted with the sterols to form dark yellow spots.

The gas chromatograph instrument (Barber-Colman Co., Rockford, Illinois) had the following set features: column, 1% neopentyl glycol succinate on gas-chrom P (80-100 mesh); temperature, 225°C; injector and detector temperature, 300°C; nitrogen pressure, 25 psi; hydrogen pressure, 20 psi; and oxygen pressure, 42 psi. Volume of samples was 0.8 microliters. An ergosterol standard was run under the same conditions.

**Cell-free protein synthesis**

The influence of nystatin on the incorporation of \(3-C^{14}\) phenylalanine into mycelium protein was determined with *Pythium* and *Colletotrichum*, using the method of Nirenberg and Matthaei (21). Ten g (wet weight) of 24-hour-old mycelium was washed with a standard buffer, at pH 7.8 (per liter: 10 ml of 1 M tris [hydroxymethyl] aminomethane, 4.473 g of KCl, 2.14 g of magnesium acetate, and 0.4687 g of mercaptoethanol). Ground mycelium was centrifuged twice in a Servall ultracentrifuge for 20 minutes at 20,000 x g and once at 30,000 x g for 30 minutes. After each centrifugation, the pellet was discarded. The final supernatant was dialyzed against the buffer (10 ml of supernatant to 600 ml of buffer). This dialyzed supernatant was used throughout the experiments; it was assumed to contain ribosomes, nucleic acids, and enzymes which catalyzed protein synthesis.

The following ingredients, made to final volume of 1 ml with double distilled water, were added to 12 ml centrifuge tubes: 50 \(\mu\)M of tris (hydroxymethyl) aminomethane, 50 \(\mu\)M of potassium chloride, 10 \(\mu\)M of
magnesium acetate, 1 μM of adenosine triphosphate-Na salt, 5 μM of phosphoenolpyruvate-K salt, 20 μg of phosphoenolpyruvate kinase, 0.03 μM of guanosine triphosphate, 6 μM of mercaptoethanol, and 0.50 μC of 3-C^{14}DL phenylalanine (Calbiochem Co., specific activity, 10 μC/μM). Dialyzed supernatant was added in an amount of 0.39 ml (2-3 mg of protein on dry weight basis). Nystatin, puromycin, or tetracycline was added to the reaction mixture. The latter two are known to be inhibitory to protein synthesis by cell-free fractions of Escherichia coli (Migula) Castellani and Chalmers in this system (20,23). Ingredients were preincubated at 37°C for 5 minutes, then the dialyzed supernatant was added and incubated for 1 hour. One ml of 10% trichloroacetic acid (TCA) was added to stop the reaction and precipitate the proteins. The protein precipitate was centrifuged and the pellet was washed once with 4 ml of 4% TCA and resuspended in 4 ml of the same material. The suspension was then incubated in an oil bath at 90°C for 15 minutes to dissolve the nucleic acids. The mixture was centrifuged and supernatant discarded. The pellet was mixed with 4 ml of 2:2:1 (v/v/v) ethanol-ether-chloroform solution and the mixture centrifuged. The resulting pellet was then washed with ether, centrifuged, and dried at room temperature. Dried samples were weighed and placed in vials to which 1 ml of hydroxide of hyamine was added. These samples were incubated for 1 hour at 50-55°C in a water bath to dissolve the protein. Ten ml of scintillator fluid, 1:1 (v/v) mixture of 4% 2,5-diphenyloxazole and 0.01% 1,4 bis-2-(4-methyl-5-phenyloxazoyl)-benzene dissolved in a 1:1 (v/v) mixture of toluene-cellosolve solution, was added to each vial and placed overnight in a

**Determination of protein-ribonucleic acid ratio.**—For determining the amount of ribonucleic acid in a given sample, the method of Gottlieb et al. (9) was used; i.e., 0.2 ml of dialyzed supernatant was diluted with 3 ml of double distilled water and read at 260 and 280 μν on a Zeiss spectrophotometer. RNA in *E. coli* cells was determined by the same method with 0.03 ml of dialyzed supernatant from this organism. The amount of protein was determined by precipitation with hot TCA for 15 minutes at 90°C. The precipitate was washed with cold TCA, dried, and weighed.
RESULTS

Influence of nystatin on Pythium and Colletotrichum

Prior to studies with supplements, growth of each fungus in the basal medium was examined. Flasks containing Pythium were harvested at 12-hr intervals after seeding. Colletotrichum was harvested at 24-hr intervals. The steepest portion of the growth curve for both fungi was between 12 and 96 hr (Figure 1). Pythium grew densely as intertwined hyphae; Colletotrichum formed uniform pellets ca. 4 mm in diameter. Results indicated in Figure 1 are from one test; those from a second were substantially the same.

Nystatin dose-response curves were determined for Pythium and Colletotrichum by supplementing the basal medium with the antibiotic. Pythium was cultured in the basal medium containing 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 μg/ml of nystatin. The concentration used with Colletotrichum ranged from 0.1 to 1.0 μg/ml. As expected from a previous report (5), the growth of Pythium was not affected at any concentration. On the other hand, Colletotrichum was inhibited by nystatin at a concentration as low as 0.1 μg/ml (Figure 2). Data in Figure 2 are from one test; in the other the results were similar.

The influence of nystatin on zoospore production of Pythium was studied by incorporating nystatin at 20 μg/ml in PA agar. In two tests zoospore production was not inhibited.
Fig. 1.—Growth of *Pythium* and *Colletotrichum* in the basal medium.
Fig. 2.--The inhibition of *Colletotrichum* by nystatin.
Reversal of nystatin inhibition by sterols

Since only Colletotrichum was inhibited by nystatin, studies on the possible reversal of the inhibition by cholesterol and ergosterol were conducted. It was demonstrated that the sterols, at concentrations ranging from 10 to 50 \( \mu \text{g/ml} \), partly reversed the inhibition of Colletotrichum by 10 \( \mu \text{g/ml} \) of nystatin (Table 1). At all levels of sterols, there was ca. 40\% reversal. A similar plateau effect was noted when nystatin was used at 20 \( \mu \text{g/ml} \). Similar tests were done in petri plates containing 10 ml of CL agar. No reversal of nystatin (20 \( \mu \text{g/ml} \)) was observed. This may be due to a lack of diffusion or the different media used for the tests. For each type of tests, results are based on two experiments, each with parallel results; data in Table 1 are from one test.

**TABLE 1.** The reversal by sterol of nystatin inhibition

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Mean dry weight (mg) of mycelium per flask of basal medium supplemented with 10 ( \mu \text{g/ml} ) of nystatin* and the following amount of sterol, ( \mu \text{g/ml} ):</th>
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<td></td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>148</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>154</td>
</tr>
</tbody>
</table>

*No growth was observed in the medium supplemented only with nystatin. Mean dry weights for cholesterol and ergosterol controls with no nystatin were 357 and 359 mg per flask, respectively.
Experiments were conducted to determine if *Pythium*, cultured in a cholesterol-containing medium, was inhibited by nystatin. *Pythium* was cultured in the basal medium containing 40 μg/ml of cholesterol for 12 hrs. Forty μg/ml of nystatin then was added to each flask. After an additional 60 hrs the amount of *Pythium* was the same as in control flasks not supplemented with nystatin.

Not only did sterols partly reverse the inhibition of nystatin to *Colletotrichum*, but they appeared to stimulate the growth of this fungus when supplemented into the basal medium. This was demonstrated in three tests in which cholesterol or ergosterol was added at concentrations of 10, 20, 30, 40, or 50 μg/ml. At all concentrations, the growth of *Colletotrichum* was increased about 40% over the controls. On the other hand, these compounds did not affect the growth of *Pythium* in parallel tests. Other workers have also noted increased growth of fungi by sterols (3,10).

Andreasen and Stier (1) noted that a yeast that did not grow anaerobically would grow under this condition when ergosterol was present. Tests were run to ascertain if *Pythium* and *Colletotrichum* responded similarly. Experiments were done in 500 ml stationary flasks containing 200 ml of basal medium. Forty μg/ml of cholesterol or ergosterol was added per flask. Nitrogen was bubbled through a train of flasks during the experiment. In two tests with each fungus, *Pythium* and *Colletotrichum* did not grow under anaerobic conditions even though sterols were present.
Influence of digitonin on Pythium and Colletotrichum

Since sterols partly reversed the inhibition of Colletotrichum by nystatin, an experiment was devised to determine if Pythium or Colletotrichum was influenced by a sterol-complexing agent, digitonin. Digitonin reacts with 3α-hydroxysterols, whether the configuration is equatorial or axial, or the compound is saturated or unsaturated (7). Each fungus was cultured in the basal medium supplemented with digitonin. Data in Table 2 indicate that digitonin and nystatin act similarly; i.e., Pythium was not inhibited at concentration as high as 50 μg/ml, whereas Colletotrichum was partly inhibited at 10 μg/ml and completely at 20 μg/ml or more. Results of the two tests were similar; data in Table 2 are from one.

TABLE 2. The influence of digitonin on the growth of Pythium and Colletotrichum

<table>
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<tr>
<th>Fungus</th>
<th>Mean dry weight (mg) with digitonin at the following concentrations in μg/ml:</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Colletotrichum</td>
<td>228</td>
</tr>
<tr>
<td>Pythium</td>
<td>319</td>
</tr>
</tbody>
</table>

Detection of sterols in the mycelium of Pythium and Colletotrichum

Sterols were detected in both fungi after 3 days of growth. In ten tests with thin layer chromatography, one spot with an Rf value of 0.70 developed from Pythium extract. Four spots developed from Colletotrichum extract. The Rf values were 0.10, 0.38, 0.66, and 0.89. Pure
ergosterol had an $R_f$ value of 0.66, which was identical to that of one of the spots developed from the extract of *Colletotrichum*. With gas chromatography, in one test, one peak was observed with *Pythium* extract and four were apparent when *Colletotrichum* extract was used. One of the peaks of the *Colletotrichum* extract had a retention time identical to that of the ergosterol standard. Such a peak was absent in *Pythium* extract. The dry weight of the *Pythium* extract was ca. 0.1 mg/g of mycelium; that of *Colletotrichum* was ca. 1 mg/g of mycelium (two determinations). The Lieberman-Burchard test was positive for sterols for both fungal extracts in 10 tests; intensity of color was much greater with the *Colletotrichum* extract.

It appeared possible that *Pythium* was not inhibited by nystatin because this fungus produced substances in the medium that inactivated the antibiotic. Tests were run to explore this possibility. *Colletotrichum* was cultured in a nystatin-containing medium in which *Pythium* had grown. Nystatin had been added to the basal medium at 10 or 20 $\mu$g/ml. After *Pythium* had grown 3 days, the mycelium was removed and the filtrate adjusted to the original pH, 6.8. The filtrate then was sterilized by filtering through a Seitz filter, placed in heat-sterilized flasks, and seeded with *Colletotrichum*. Since there was no growth of *Colletotrichum* in the flasks containing nystatin and *Pythium* filtrate after three days, it was concluded that *Pythium* medium did not contain nystatin inhibitors.

**Influence of nystatin on cell-free protein synthesis**

The incorporation of $3-O^{14}$-phenylalanine in *Pythium* or *Colletotrichum* supernatant was ca. 100 CPM/mg protein. In 10 tests, nystatin at concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 $\gamma$/ml did
not inhibit the incorporation of labelled phenylalanine into protein of either fungus. Furthermore, puromycin or tetracycline did not inhibit incorporation. It was felt that knowledge of protein-RNA ratios of the fungus supernatants might suggest a reason for lack of inhibition by puromycin and tetracycline. This ratio was 3.21 (9.30 mg protein/2.20 mg RNA) for *Pythium* and 5.36 (7.20 mg protein/1.34 mg RNA) for *Colletotrichum*. In comparison, the ratio for *E. coli* was 2.38 (14.8 mg protein/6.2 mg RNA). *Pythium* and *Colletotrichum* contained more protein than *E. coli* relative to the amount of RNA; however, the difference does not appear to suggest why protein synthesis by *E. coli* was inhibited by puromycin and tetracycline (20,23) and that of the fungi was not. However, it may explain why the *E. coli* supernatant was more active (ca. 300 CPM/mg protein) compared to fungi (ca. 100 CPM/mg protein).
DISCUSSION

The results of this study support the hypothesis that microorganisms inhibited by polyene antibiotics contain sterols and that organisms not possessing sterols are not affected, a view discussed recently (4, 25). Thus nystatin-insensitive *Pythium* contained only a small amount of an unknown sterol, and the nystatin-inhibited *Colletotrichum* contained quantities of ergosterol and other materials suspected of being sterols. Moreover, digitonin, like nystatin, inhibited *Colletotrichum* and not *Pythium*, perhaps because the small amount of sterol in *Pythium* was not in the membrane or the configuration of the unknown sterol was such that it was not bound by digitonin or nystatin. It also is possible that digitonin and nystatin did combine with the *Pythium* sterol and that the complex did not alter membrane permeability.

Other workers have reported that the growth of other polyene-sensitive organisms was inhibited by digitonin (8, 25).

Feingold (6) and Weber and Kinsky (25) found that *Mycoplasma laidlawii*, ordinarily not affected by filipin, was inhibited by this antibiotic if cultured in the presence of cholesterol, presumably because the cholesterol had become incorporated in the membrane. This type of evidence in favor of the "sterol hypothesis" was not supported by the present work, however, since a sterol-containing medium in which *Pythium* had grown supported growth of this fungus in the presence of nystatin. Experiments with *Pythium* culture filtrates indicated that this fungus
did not produce sterols or other substances that reversed the inhibitory effects of nystatin on *Colletotrichum*. The inhibition of *Colletotrichum* by nystatin was not completely reversed by sterols; it is suggested, therefore, that the "sterol hypothesis" does not explain fully the mechanism by which this fungus was inhibited.

The growth of *Colletotrichum* was stimulated by cholesterol or ergosterol. It thus appears that this fungus not only synthesized mycelial sterols but was stimulated by an exogenous supply. The growth of *Pythium*, on the other hand, was not influenced by added sterols in the same medium. This observation suggests that the sterol content of the medium was optimal or that *Pythium* was not stimulated by these materials. It has been reported that sterols increased the growth of various pythiaceous (10,11) and other fungi (3). Sterols have a profound effect upon the reproduction of pythiaceous types (10,11,17).

The observation that puromycin and tetracycline did not inhibit amino acid incorporation into protein by cell-free fractions of *Pythium* and *Colletotrichum* is evidence for an unusual mode of protein synthesis in these organisms. Puromycin and tetracycline are known inhibitors in this type of system (20,23). Kinsky (15) commented that since puromycin and chloroamphenicol (another inhibitor of protein synthesis) did not inhibit the growth of *Neurospora* in culture, this may have been owing to a lack of penetration of the antibiotics, to their inactivation, or to an alternate mode of protein synthesis. My work supports the last view. Further studies on cell-free fungal systems may thus lead to the discovery of an undescribed mechanism of protein synthesis or a shunt system through which synthesis may occur.
SUMMARY

Pythium was not inhibited in a liquid medium at concentrations of nystatin up to 100 ug/ml; Colletotrichum was partly inhibited at 0.1 ug/ml. Digitonin, a sterol-complexing agent, inhibited Colletotrichum and not Pythium. Cholesterol and ergosterol partly reversed the inhibition of Colletotrichum by nystatin. Sterols were detected in petroleum ether extracts from Pythium and Colletotrichum mycelium by means of three techniques: thin layer chromatography, gas chromatography, and the Lieberman-Burchard test. Ergosterol and other materials suspected of being sterols were found in Colletotrichum mycelium. A lesser amount of an unknown sterol was in Pythium. Pythium did not produce nystatin-inactivating substances in the medium, as assayed with Colletotrichum.

Nystatin, puromycin, and tetracycline did not inhibit the incorporation of 3-C\textsuperscript{14} phenylalanine into protein in the cell-free extracts of the test fungi. Since puromycin and tetracycline are known inhibitors of protein synthesis, it is suggested that an undescribed mechanism of synthesis may exist in these fungi. This work adds support for the hypothesis that polyene antibiotics inhibit by complexing with sterols in cell membranes of sensitive organisms.
LITERATURE CITED


