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*ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES I. Pleurotus griseus**

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In a survey[1] conducted in this laboratory it was noted that 213 of the 332 species of Basidiomycetes studied produced substances that inhibited the growth of *Staphylococcus aureus*. Among the species that seemed worthy of further investigation was *Pleurotus griseus*.[2]

P. griseus grew with medium rapidity on a thiamine-peptone agar[1] and a modified Czapek-Dox agar to which corn steep solids had been added. It formed a pink pigment which diffused into the agar. After the fungus had grown for one to several weeks, discs 5.5 mm. in diameter were cut on a radius extending from the center of the colony into the agar adjacent to the colony. These discs were placed on a yeast-peptone agar seeded with *Staph. aureus* and the plates incubated overnight at 37 ± 2°C. Clear zones of inhibition were found around the discs indicating that some substance or substances had diffused from the agar discs into the surrounding seeded medium in sufficient concentration to inhibit the growth of the staphylococci in the agar. A disc cut from the agar at as great a distance as 20 mm. from the edge of the colony frequently produced a zone of inhibition. The zones, while small, were clear, indicating that bacteria resistant to the antibiotic substance or substances were absent. Disc tests, with strains of *Staph. aureus* resistant to other antibiotic substances, indicated that *P. griseus* produced an antibiotic substance which was different from penicillin and different from the active substances produced by several other Basidiomycetes. The antibiotic substance or substances from *P. griseus* did not inhibit the growth of *Escherichia coli*.

P. griseus was grown on several types of nutrient media, differing from one another in the source of nitrogen and carbon, to determine the effect of the medium on the production of the antibacterial substances by the fungus. Cane sugar, dark brown sugar, galactose, lactose, mannitol and corn steep solids were ineffective carbon sources; maltose and soluble starch were poor; dextrose and levulose were the best and nearly equal so far as the

production of antibacterial substances was concerned. Various amounts of dextrose ranging from 10 to 100 g. per l. were tested; 40 g. per l. seemed somewhat superior to 20 or 60 g. Nitrates and asparagine in the presence of dextrose, were unsatisfactory sources of nitrogen; corn steep solids, an extract of corn steep solids made with 90 per cent methanol, N-Z-CASE, amigen and neopeptone were all effective. On the basis of this survey it was decided that a practical medium to use for liquid culture was a modified Czapek-Dox mineral solution[3] to which 40 g. of dextrose and 5 g. of corn steep solids were added per liter of solution.

Antibiotic Material in Liquid Culture.—The fungus was grown at 25°C. in Fernbach flasks on coils of beech shavings, using one liter of the above medium per flask. After a growth period of about one month, the fungous mat had nearly covered the entire liquid surface; the liquid had an activity of about 1000 dilution units per ml. when assayed with *Staph. aureus* (Heatley strain). The culture fluid was then decanted and replaced by one liter of fresh corn steep medium. After a further incubation period of from 5 to 7 days, the antibacterial activity of the culture fluid in the reflooded flask was usually at least 256 dilution units. This culture fluid was then decanted and replaced by fresh nutrient solution. This reflood technique saved from seven to fifteen days in the production of each lot of active culture fluid and was especially useful for those Basidiomycetes which grow slowly.[4] Flasks which had been reflooded nine times have shown as rapid and as great a production of antibacterial substance during the last period of reflooding as during the first.

Isolation of an Antibiotic Substance.—Pooled culture fluids with an activity of from 256 to 1024 dilution units per ml. were strained through cheesecloth to remove bits of mycelium and wood. They were then extracted by shaking with a one-tenth volume of chloroform. The chloroform-in-water emulsion was removed and centrifuged to break the emulsion. The chloroform portion was extracted three times with one-tenth volume of one per cent sodium bicarbonate solution to remove acids. The chloroform solution was then reduced to a few milliliters by distillation under reduced pressure, transferred to a small beaker and left at room temperature until all the chloroform had evaporated. A reddish gum in the beaker was dissolved in the minimum volume of hot ethanol, ether was added, the beaker covered and let stand at room temperature to permit slow evaporation of the solvents. Orange-colored crystals formed soon after the ether was added. The crystals were removed, washed with ether and air-dried. The product was recrystallized from a chloroform-ether solution. Large amber colored crystals were formed when evaporation of the solvents took place very slowly. Fine needle-like yellow crystals were formed as a result of rapid evaporation of the solvents. From 100 to 180 mg. of the crystalline material were obtained per l. of culture fluid.

A solution prepared from the crystals showed activity after 24 hrs. against *Staph. aureus* at a concentration of 1 μg. per ml. of solution and no activity against *E. coli*. The crystalline antibiotic substance was named pleurotin.

Chemical Properties of Pleurotin.—Pleurotin began to melt with decomposition at temperatures between 200° and 215°C. depending upon the rate of heating. Qualitative tests indicated the absence of ash and of the following elements: halogens, nitrogen and sulfur.

Microanalysis[5] gave the following: C, 70.83, H, 6.43; Mol. wt. (Rast) 343; MeO, 0.0. The computed values for an empirical formula of $C_{20}H_{22}O_5$ were C, 70.16; H, 6.47; Mol. wt. 342.4. Pleurotin was optically active with $\alpha]_D^{23°} = -20°$; C = 0.59 m. chloroform. We are indebted to Dr. J. D. Dutcher of The Squibb Institute for Medical Research for the molecular weight, analysis and optical rotation.

The absorption spectrum had a single absorption peak in the ultra violet at 2500 A for which the molecular extinction co-efficient was 13,680 (molecular weight assumed to be 342) for a solution in 4 per cent ethanol. The absorption in the visible was not measured.

The solubility of pleurotin at 25° was in water 0.125 mg. per ml., in 95% ethanol 6.8 mg. per ml., in 5% ethanol, 0.37 mg. per ml., in ether 3.5 mg. per ml. and in chloroform more than 200 mg. per ml. It was relatively insoluble in dilute acids, dilute solutions of sodium bicarbonate and in petroleum ether. It was more soluble in acetone.

Pleurotin did not give a color reaction with ferric chloride. It liberated iodine from acidified potassium iodide solution. Its reaction with a solution of potassium cyanide to give a blue color was used as the basis of a colorimetric method suitable for the quantitative determination of pleurotin in culture fluids and other solutions.

Pleurotin was adsorbed from culture fluids by Norit A and eluted from the air-dry carbon by chloroform. Pleurotin is a neutral substance which reacts with alkali to give an acidic product devoid of antibacterial activity.

Pleurotin was not thermostable. Solutions of pleurotin in 0.1 M. phosphate buffer when boiled for ten minutes lost 50 per cent of their biological activity at pH 3, 75 per cent at pH 6.5 and all their activity at pH 8.5 and higher. Pleurotin was 75 per cent destroyed in one hour at pH 8.5 and 25°C. An aqueous solution containing 100 μg. of pleurotin per ml. lost 30 per cent of its pleurotin as determined chemically after autoclaving at 120°C. for 15 min. Pleurotin in solution was rendered inactive by exposure to light for a few hours. It was filterable through a Seitz filter pad.

The chemical and physical properties of pleurotin are sufficient to establish it as different from all other antibiotic substances that have been prepared in pure form. The low solubility of pleurotin in aqueous solution and its instability suggest that its possible therapeutic value is unlikely.

Antibacterial Action of Pleurotin.—The antibacterial activity of pleurotin was determined by the methods in use in this laboratory.[6] The bacteria used were: *Staph. aureus, Klebsiella pneumoniae, Photobacterium fischeri,* and the following standard tester strains of S. A. Waksman: *E. coli, B. mycoides* and *B. subtilis.* The following table gives the minimum antibacterial concentrations of pleurotin after an incubation period of 24 hours:

BACTERIA	μG. PER ML.
Staphylococcus aureus	0.8
Bacillus mycoides	1.6
Bacillus subtilis	0.2
Escherichia coli	500.0
Klebsiella pneumoniae	500.0
Photobacterium fisheri	6.0

The activity for incubation periods of 16 to 18 hrs. was somewhat higher and for 48 hrs. was one-half or one-quarter the values given.

Pleurotin was active only on the gram-positive bacteria. The culture fluid from *Pleurotus griseus* was active only on the gram-positive bacteria indicating the absence of an appreciable amount of a second antibiotic substance with antibacterial properties markedly different from those of pleurotin. The antibacterial activity of a culture fluid was equal to that of a solution of pure pleurotin of the same concentration, the pleurotin content of the culture fluid being determined by the potassium cyanide method.

Toxicity for Mice.—Tests made by Dr. G. Rake at the Squibb Institute for Medical Research indicated that a single dose of pleurotin was not toxic when given intravenously to white mice at the rate of 24 mg. per kilogram of body weight. Pleurotin is so insoluble in aqueous solutions that larger amounts could not be given. For the twenty studies 11.4 mg. of pleurotin were dissolved in 1.0 ml. of warm ethanol and diluted with 19.0 ml. of warm saline solution. By maintaining the solution at 37° crystallization was delayed long enough to permit intravenous injection.

Activity on M. tuberculosis.—Through the courtesy of Dr. Ralph R. Mellon, Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh, Pa., tests were carried out by Miss Jean Onslow on *Mycobacterium tuberculosis.* Discs cut from a 29-day old colony of *P. griseus* grown on the modified Czapek-Dox medium containing corn steep solids showed inhibition of an avirulent strain of *M. tuberculosis* H 234 on a yeast peptone agar. The activity was confined to the discs from the colony and was less than for *Staph. aereus.* A comparison for the two organisms is given in the following table where the inhibition in mm. is given for 6 discs taken on a radius extending from the center of the colony. Discs 1 and 2 came from the fungous colony.

ORGANISM \ DISC	1	2	3	4	5	6
Staph. aureus	17	17	11	9	8	Halo
M. tuberculosis H 234	11	7	0	0	0	0

Agar discs saturated with pleurotin produced no zones of inhibition. Pleurotin at a concentration of 100 μg. per ml. of Youmans' medium[7] or Kirchner's medium[8] was ineffective.

Discs from a colony *P. griseus* 9 days old were tested on virulent *M. tuberculosis* H 37 grown on Herrold egg medium. A disc taken from within the limits of the fungous colony produced an inhibition zone of 7 mm. Discs from the edge of the colony or in the agar adjacent to the colony were ineffective.

Relative Activity of Pleurotus griseus and pleurotin.—Some observations made during the course of our investigation indicated that all of the antibacterial activity of *P. griseus* was not accounted for by pleurotin. Disc cut from within the limits of a colony of *P. griseus* gave zones of inhibition on *Staph. aureus* and *M. tuberculosis* considerably larger than those obtained with similar discs saturated with pleurotin. The presence in the mycelium of an enzyme capable of forming an antibacterial substance during the incubation of the discs at 37° or of a factor which enhanced the activity of pleurotin was not demonstrated by our experiments.

On the other hand, an aqueous extract of the mycelium tested by the cup method against *Staph. aureus* was considerably more effective than a saturated solution of pleurotin and an acid fraction.[9] prepared from culture liquid gave large zones of inhibition.

The acid fraction showed some activity (1333 dilution units per g.) on *M. tuberculosis* H 234, although a saturated solution of pleurotin was inactive. The activity of this fraction on *Staph. aureus* was 8000 dilution units per g.

This evidence was taken to indicate the production by *P. griseus* of an antibacterial substance or substances other than pleurotin. Since the unidentified substances were not isolated, we do not know whether they fall within the antibiotic range (effective at a dilution of 1 to 40,000 or less). In any event, the quantity in the culture liquid must be small since within the limits of error the antibacterial activity of the culture liquid can be accounted for on the basis of its pleurotin content as determined chemically.

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[1] Robbins, W. J., Hervey, A., Davidson, R. W., Ma, R., and Robbins, W. C., *Bull. Torrey Bot. Club*, **72**, 165–190 (1945).

[2] The fungus was obtained from Dr. Ross W. Davidson and is numbered 14616-R in his collection. Another isolation of *Pleurotus griseus* obtained from Dr. Mildred K. Nobles was also active. No difference in the pleurotin formed by the two strains was detected.

[3] The mineral solution contained per liter, 3 g. $NaNO_3$, 1 g. KH_2PO_4, 0.5 g. KCl, 0.5 g. $MgSO_4.7H_2O$ and 0.01 g. $Fe_2(SO_4)_3$.

[4] Robbins, W. J., Kavanagh, F. A., Hervey, A., *Ann. N. Y. Acad. Sci.*, **48**, 67–72 (1946).

[5] The microanalyses and molecular weight determinations were carried out by Mr. J. J. Alicino of The Squibb Institute for Medical Research.

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*ANTIBIOTICS FROM BASIDIOMYCETES II. Polyporus biformis**

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In a survey of fungi reported earlier,[1] *Polyporus biformis*[2] was found to produce antibacterial substances. Its activity on *Staphylococcus aureus* (H) as evidenced by the disc test, encouraged us to investigate it further.

This fungus grew well at 25°C. on malt agar, thiamine-peptone agar and a modified Czapek-Dox agar to which corn steep solids had been added,[1] and produced antibacterial substances on all three media. Tested by the streak method, inhibition zones extending 12 to 25 mm. from the edge of the fungus colony were observed. The inhibition of *E. coli* was less than that of *Staph. aureus*. When the antibacterial activity was tested by the agar disc[1] method, inhibition areas as large as 25 mm. in diameter with a 5.5 mm. disc were obtained with *Staph. aureus*.

Culture liquids of *P. biformis* evidenced antibacterial activity. The fungus was grown at 25°C. in 2800 ml. Fernbach flasks containing 1 l. of modified Czapek-Dox medium with dextrose and corn steep solids[3] on coils of beech wood shavings which furnished mechanical support for the mycelium. After a growth period of 2 weeks or more, the activity of the culture liquid on *Staph. aureus* ranged from 64 to 256 dilution units. When the activity of the culture fluid approached 256 dilution units, the liquid was decanted with suitable precautions to prevent contamination of the cultures and each mycelial mat was reflooded with 1 l. of fresh sterile culture solution. Within 6 to 10 days the activity justified further decantation and reflooding. Several mats were reflooded as many as 20 times in the