Fungicidin, an Antibiotic Produced by a Soil Actinomycete. (18397)

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In a search among freshly isolated actinomycetes for antagonists to pathogenic fungi (1-3), an antibiotic substance, designated *fungicidin,* has been obtained from a strain (No. 48240) isolated from a farm soil in Fauquier County, Va.(4). Fungicidin is both fungistatic and fungicidal; and apparently lacks antibacterial activity.

Actinomycete No. 48240, belongs to the genus *Streptomyces*(5); the species has not been determined. Oval spores are produced in chains from curved and spiral-forming hyphae. (Fig. 1). Grown on Sabouraud's glucose agar at ±28°C for 7 days, the colony is heaped and folded and the aerial mycelium is white, but later at sporulation it is gray; on the reverse, the color at first is light tan, but after 2 weeks it is dark brown and the medium is darkened throughout. There is no hemolysis on blood agar medium. Fig. 2 shows zones of inhibition produced by this streptomyces against the 2 assay species *Cryptococcus neoformans* and *Candida albicans.* When grown in a glucose-tryptone broth(3) in shallow layers,* the stationary culture yields two antibiotics both active against fungi. From the liquors one substance resembling actidione(2,6) has been obtained. From the surface growth a second substance with much broader antifungal activity has been extracted; this is designated fungicidin.

Isolation. The surface growth from 5- to 7-day-old broth cultures is harvested, heated at 70°-72°C for 10 minutes, pressed between filter papers to remove adhering medium, and extracted several times with methanol. The antibiotic in the pooled extracts is purified by a process that involves repeated fractional precipitations with ethyl acetate, washing of the precipitates with 0.85% NaCl solution, dissolution in methanol, and fractional precipitation with ether. All procedures are carried out in the cold so far as practicable.

Chemical and physical properties. Crude fungicidin is obtained in a yield of 30 to 50 mg per liter of culture medium. It is a fine yellow powder that can be stored in the cold for several months without loss of activity. It is only sparingly soluble in methanol and ethanol, and relatively insoluble in most organic solvents and water. Very

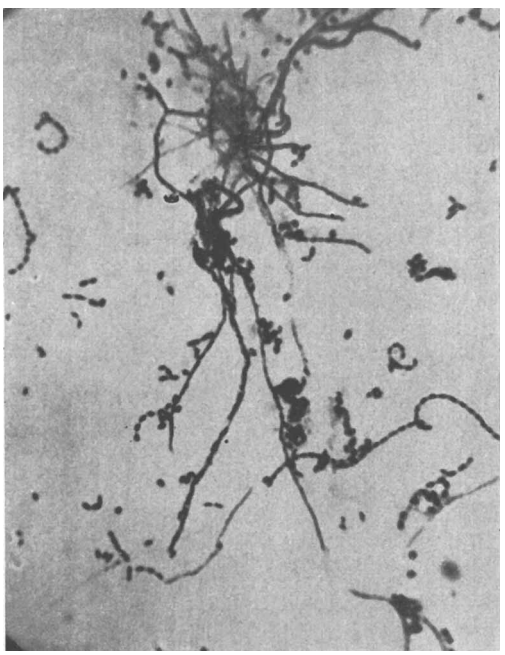

FIG. 1.
Streptomyces sp. No. 48240 grown on Sabouraud's glucose agar.

1. Schatz, Albert, and Hazen, E. L., *Mycologia,* 1948, v40, 461.

2. Brown, Rachel, and Hazen, E. L., *Annual Report, Division of Laboratories and Research, New York State Department of Health,* 1949, p19.

3. Brown, Rachel, and Hazen, E. L., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 454.

4. Hazen, E. L., and Brown, Rachel, *Science,* 1950, v112, 423.

5. Waksman, S. A., *The Actinomycetes: their nature, occurrence, activities, and importance,* Waltham, Mass., Chronica Botanica Co., 1950, 230p.

* For this work 0.1% agar was added to the broth.

6. Whiffen, A. J., *J. Bact.,* 1948, v56, 283.

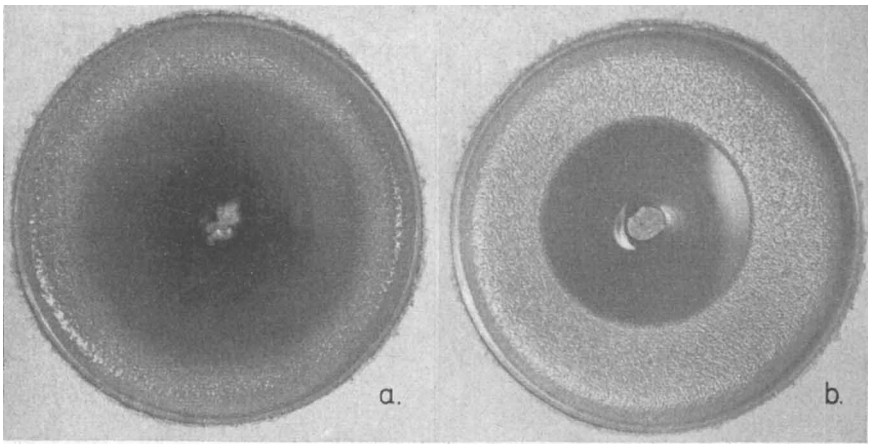

FIG. 2. Zones of inhibition of *Streptomyces* sp. No. 48240 against: a. *C. neoformans*. b. *C. albicans*.

unstable aqueous solutions, however, can be prepared at pH 2 or pH 9. In the present crude state, preparations of fungicidin contain about 1.5% of Kjeldahl nitrogen. Sulfur and the halogens have not been detected. Fungicidin shows strong reducing properties. It fails to react in tests for carbohydrate or protein. The molecule is relatively small since it may be ultrafiltered through fine gradocol membranes.

In vitro activity. Materials and methods. A stock suspension of fungicidin containing 5 mg ml is prepared by dissolving the powder in N 50 HCl and adjusting to pH 7.2 with 0.1% Na_2CO_3. This is kept in a refrigerator and used for only 72 hours. Dilutions are prepared in sterile distilled water. The sensitivity of *C. neoformans* and *C. albicans* to fungicidin was determined in broth cultures. Serial 2-fold dilutions were made in 4.5 ml glucose-tryptone broth and heated for 10 minutes at 70° C for sterilization. To 2 series of tubes was added, respectively, 0.5 ml of a saline suspension of *C. neoformans* and *C. albicans* in concentrations of 1,000,000 and 300,000 cells per ml. Readings of visible growth were made after 5, 24, 72, and 96 hours' incubation at ±28° C. The sensitivity (7) was recorded as the least amount of fungicidin inhibiting growth, as evidenced by absence of gross turbidity after 96 hours.

The antimicrobial activity of fungicidin was determined by the agar dilution method of Reilly, Schatz, and Waksman(8) except that infusion agar was employed for the bacterial cultures and the temperature of incubation was 35°-36° C. The effect of fungicidin upon the growth of *C. neoformans* was determined in broth cultures. Two sets of serial 2-fold dilutions in glucose-tryptone broth were inoculated, respectively, with 10,000,000 and 1,000,000 microorganisms per ml. After incubation for 3, 4, 5, 24, and 48 hours, aliquot portions were removed and plated on glucose-tryptone agar. Colony counts were made after 48 hours of incubation. The effect of defibrinated horse blood and serum was determined as follows: 0.1 ml of fungicidin diluted in sterile saline was added to a series of tubes containing 0.25 ml of whole blood or serum and to each dilution was added 0.05 ml of a 48-hour glucose-tryptone broth culture of *C. neoformans* containing 26,000,000 microorganisms/ml. The tests were incubated for 48 hours with frequent shaking, after which the contents were streaked on plates of Sabouraud's glucose agar which were incubated for 48 hours.

Results. The data presented in Tables I and II indicate that fungicidin is strongly fungistatic against a wide variety of saprophytic and pathogenic forms. Amounts such

7. Lenert, T. F., and Hobby, G. L., PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 235.

8. Reilly, H. C., Schatz, Albert, and Waksman, S. A., *J. Bact.*, 1945, v49, 585.

TABLE I. Fungistatic Action of Fungicidin.

Test species	Sensitivity: μg of fungicidin per ml at +28°C 72 hr	96 hr
C. neoformans	1.56	1.56
C. albicans	3.12	3.12

TABLE II. Antifungal Spectrum of Fungicidin.

Fungi	Strain No.	Least amt inhibiting growth, μg/ml
Cryptococcus castellanii	45232	3.13
Cryptococcus glutinis	4676	1.56
Candida guilliermondi	45211	3.13
Candida krusei	45214	6.25
Candida stellatoidea	45213	3.13
Saccharomyces cerevisiae	45217	3.13
Sporobolomyces salmonicolor	4550	3.13
Schizosaccharomyces octosporus	45231	1.56
Endomycopsis fibuliger	45230	3.13
Geotrichum lactis	47462	6.25
Aspergillus fumigatus	50248	6.25
Penicillium notatum	43281	3.13
Penicillium sp.	50526	13
Penicillium claviforme	49470	3.13
Rhizopus nigricans	50524	3.13
Fusarium sp.	50527	3.13
Alternaria sp.	50523	1.56
Cephalosporium sp.	45226	25
Phoma sp.	50522	6.25
Ceratostomella ulmi (plant pathogen)	50525	6.25
Hormodendrum sp.	4893	3.13
Histoplasma capsulatum (yeastlike)	4894	1.56
Blastomyces dermatitidis (yeastlike)	45223	1.56
Paracoccidioides brasiliensis (yeastlike)	45224	1.56
Coccidioides immitis (spherules)	50521	6.25
Cryptococcus neoformans	45215	1.56
Candida albicans	4657	3.13
Trichophyton mentagrophytes	45141	6.25
Trichophyton rubrum	4516	6.25
Trichophyton rosaceum	4974	3.13
Epidermophyton floccosum	44253	1.56
Microsporum audouini	4896	3.13
Microsporum canis	4817	13
Sporotrichum schenckii (mycelial) (yeastlike)	50251	13
Monosporium apiospermum	45221	100
Allescheria boydii	48102	>100
Phialophora verrucosa	45229	13

as 1.56 to 6.25 μg/ml were effective against many fungi. Inhibition of growth of the following bacteria, however, was not obtained even with 100 μg/ml: *Staphylococcus aureus* (No. 45142), *Streptococcus hemolyticus* (No. 44131), *Bacillus subtilis* (No. 45137), *Bacillus cereus* var. *mycoides* (No. 48294), *Salmon-*

TABLE III. Therapeutic Activity of Fungicidin in Mice Infected with *C. neoformans*.

Infection		Therapy			% survival weeks							Findings at autopsy		
Date	No.* of microorganisms	Dates	No. of inj.	Total amt. inj. mg/kg	No. of mice, 20 g	1	2	3	4	5	6	7	Microscopic and cultural Brain	Blood	Gross lesions Liver, spleen, kidney
10/16/50	800,000	10/16/50 through 12/4/50	28†	985	10	100	100	100	100	100	90	90	+ (1/1)	— (1/1)	None (1/1)
10/16/50	800,000	10/17/50 through 12/4/50	30‡	1135	10	100	100	80	70	40	20	10	+ (9/9)	— (9/9)	None (9/9)
10/16/50	800,000		0	0	10	100	90	10	0	0	0	0	+ (10/10)	+ (10/10)	+++ (10/10)
	0	10/16/50 through 11/13/50	25	735	5	100	100	100	100	100	100	100			

* Determined by hemocytometer.
† Mice received 13 inj. the first week, 5 the second, 4 the third, 2 the fourth, and 4 during the next three weeks.
‡ Mice received 11 inj. the first week, 7 the second, 5 the third, 3 the fourth, and 4 during the next 3 weeks.
Infection by intravenous route. Therapy by subcutaneous route. Doses ranged from 250-2000 μg per mouse.
+ Microorganisms typical in morphology. — No microorganisms. +++ Many small white nodular masses on surface of organs.

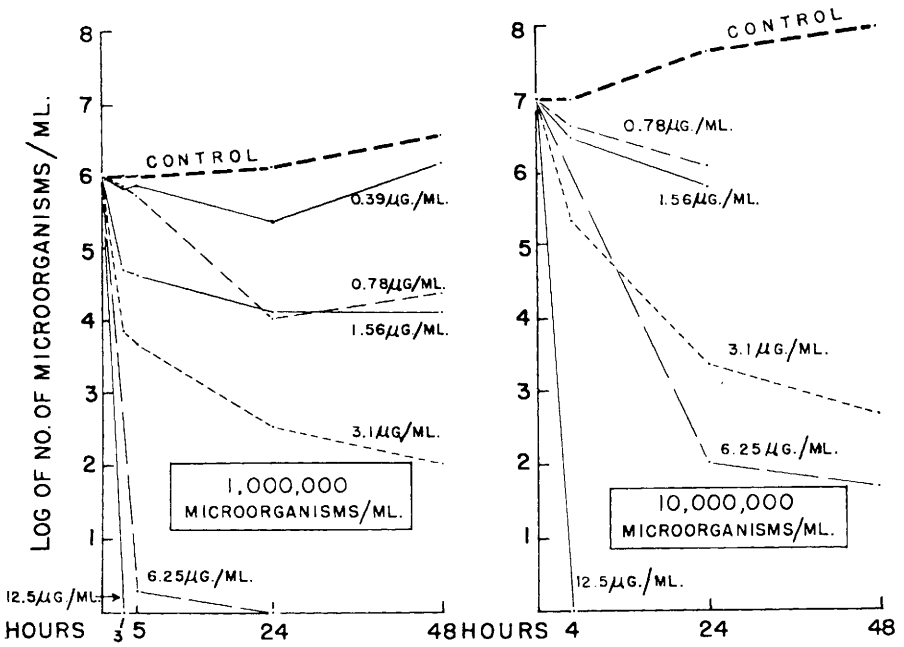

FIG. 3. Effect of fungicidin on multiplication of *C. neoformans*.

rella typhosa (No. 38351), *Shigella paradysenteriae* (No. 45300), *Bacterium mucosum capsulatum* (No. 4767), and *Bacillus circulans* (No. 48205). Growth of a strain of *Mycobacterium tuberculosis* (No. 50539) freshly isolated from a human lung was not inhibited in Dubos medium containing 100 μg of fungicidin ml.

The growth curves in Fig. 3 indicate fungicidin to be strongly fungistatic and fungicidal. In the presence of 1,000,000 microorganisms, 6.25 μg brought about a progressive decline in the population, with sterilization in 24 hours; and, with a ten-fold increase in microorganisms, 12.5 μg produced sterilization within 4 hours. No evidence was obtained that incubation of fungicidin in the presence of defibrinated horse blood or serum modifies its activity.

In vivo activity. Materials and methods. Sterile physiologic salt solution was used for diluting the stock suspension of fungicidin. All tests were made in white mice (Albany strain) of 20-25 grams. Mice were infected by intraperitoneal and intravenous injections of saline suspensions of *C. neoformans* or intraperitoneal injections of *Histoplasma capsulatum*.

Results. Toxicity. The approximate LD 50 of crude fungicidin administered intraperitoneally to mice is between 20 and 26 mg/kg. Injected subcutaneously, however, 2 g/kg did not kill. There was induration and necrosis at the site of injection. No evidence of necrosis was found in the 4 mice receiving 473 mg/kg. Mice receiving a total of 735 mg/kg over a period of 4 weeks showed no ill effects; the fungicidin was administered subcutaneously or intraperitoneally in doses of 200-400 μg, 5 days the first week, 5 days the second week, 4 days the third week, and 3 days the fourth week. In appearance and weight they compared favorably with the control untreated group.

Therapeutic activity. While the data available on the therapeutic activity of fungicidin are limited, Tables III and IV present definite evidence that mice injected with large amounts of *C. neoformans* or *H. capsulatum* and given repeated parenteral doses of fungicidin, have a milder form of infection and life is prolonged beyond that of the controls.

Summary. Two antibiotics of different chemical and biologic properties have been recovered from a soil actinomycete, *Streptomyces* sp.). The one is extracellular and

TABLE IV. Therapeutic Activity of Fungicidin in Mice Infected with *H. capsulatum.*

Infection		Therapy			No. of mice, 20 g	% survival weeks				
Date	No.* of microorganisms	Dates	No. of inj.	Total amt. inj. mg/kg		1	2	3	4	5
10/30/50	6,000,000,000	10/30/50 through 12/4/50	15†	1300	10	80‡	80	80	80	80
10/30/50	6,000,000,000		0	0	10	100	50	0	0	0

* Determined by hemocytometer.

† Mice received 9 inj. the first week, 2 the second, 1 the third, 2 the fourth, and 1 the fifth.

‡ 2 mice died within 48 hr after infection. They were thought to have been injured at time of treatment.

Infection by intraperitoneal route. Therapy by subcutaneous route. Doses ranged from 1-2 mg per mouse.

resembles actidione. The other, designated as fungicidin, is intracellular. It is both fungistatic and fungicidal and apparently lacks antibacterial action. Its activity is not diminished by the presence of horse blood or serum. Fungicidin is distinguished from other antibiotics within our knowledge by its antimicrobial spectrum and its solubility characteristics. The approximate LD 50 for crude fungicidin administered intraperitoneally to mice is between 20 and 26 mg/kg. Injected subcutaneously, its toxicity is considerably lower. Therapeutically, fungicidin appears to be of value in histoplasmosis and cryptococcosis induced in mice.

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Concentration and Distribution of "Encephalin" in the Brain of Humans and Animals. (18398)

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The term "encephalin" designates a substance (or compound of substances?) with sympathomimetic biological action, which has been isolated from human and animal brains (1). It possesses certain epinephrine-like chromogenic and adsorbability properties, but differs from both epinephrine and nor-epinephrine in several respects. The colorimetric readings, obtained in concentrated brain dialyzates against an epinephrine standard were generally in satisfactory quantitative agreement with the pharmacodynamic action of the dialyzates (cat blood pressure, rabbit intestine). Small amounts of nor-epinephrine and epinephrine which have recently been demonstrated in brain extracts and which are believed to derive from sympathetic nerve endings in the cerebral vessels(2) are undoubtedly also present in the encephalin-containing brain dialyzates. Their calculated chromogenic effect would account for only about 1/60 of the total colorimetric readings. Various observations speak against the assumption(2) that the bulk of encephalin might be identical with tyramine. They have been discussed elsewhere(1). One of them is the fact that tyramine does not give the blue color reaction with arsenomolybdic acid which encephalin shares with the known sympathomimetic catecholamines.

Colorimetric assays of encephalin, the re-

1. Raab, W., *Am. J. Physiol.,* 1948, v152, 324.

2. Holtz, P., *Acta physiol. scandinav.,* 1950, v20, 354.