IN VITRO STUDIES OF THE ANTIFUNGAL ANTIBIOTIC GRISEOFULVIN*

FRANK J. ROTH, JR., PH.D., BENNETT SALLMAN, PH.D. AND HARVEY BLANK, M.D.

The therapeutic efficacy of orally administered griseofulvin for the treatment of superficial fungous infections due to dermatophytes seems established. This antibiotic was first isolated by Oxford and coworkers (1) from *Penicillium griseofulvin* in 1939. In 1946, Brian *et al.* (2) observed that cultures of *P. janczewskii* produced a factor which caused stunting of germ tubes of *Botrytis allii*. Grove (3) subsequently showed that this "curling factor" was identical to griseofulvin and established its chemical structure. The antibiotic was employed agriculturally to prevent botrytis wilt of lettuce and alternaria blight of tomatoes. (4)

In 1958, Gentles (5) reported the value of griseofulvin administered by the oral route in the treatment of experimental dermatophytoses in animals. Guinea pigs experimentally infected with *Microsporum canis* were treated successfully with an oral dose of 60 mg./kg. of the antibiotic. Treatment was begun ten days after infection and clinical benefits were demonstrated within four days after onset of therapy. By the eighth day of treatment nearly all hair follicles were devoid of fungal elements and hair formed subsequent to therapy resisted completely further invasion by the dermatophyte. Other experiments showed that griseofulvin was strikingly effective in curing guinea pigs experimentally infected with *Trichophyton mentagrophytes*. Gentles states (6) that in recent work he has found 15 mg./kg. curative in guinea pigs.

In an amplification of the initial report by Gentles, Martin (8) showed that experimental dermatophytoses in guinea pigs is controlled by oral doses of 25 to 100 mg./kg. of griseofulvin. He observed that the topical application of 1 per cent griseofulvin ointment suppressed the development of cutaneous lesions if applied on the fourth day following experimental infection.

*From the Division of Dermatology, Department of Medicine and Department of Microbiology, University of Miami School of Medicine, Miami, Florida.

This study was supported by grants from the National Institutes of Health and by a U. S. Army Contract.

Presented at the Twentieth Annual Meeting of The Society for Investigative Dermatology, Inc., Atlantic City, N. J., June 7, 1959.

Griseofulvin was demonstrated to be effective in the oral treatment of *Trichophyton verrucosum* infections in calves by Lauder and O'Sullivan (9).

Gentles (7) reported the presence of extractable griseofulvin in the hair of guinea pigs fed 30 to 40 mg./kg. of the antibiotic for twenty-three consecutive days. The hair was extracted and purified by a complex methanol refluxing process. An aqueous extraction purified by treatment with petroleum and benzene followed by methanol and ethanol treatment was also employed. One gram of hair yielded between 5 and 6 mcg. of griseofulvin. He believes that the elimination of the dermatophytes from infected hair is due to the incorporation of griseofulvin in the keratin where it forms a barrier to further invasion by the fungus.

Brian (10) reported that many fungi with chitinous cell walls demonstrated "curling" of their hyphae when exposed to griseofulvin-containing media. Those fungi with cell walls of cellulose failed to demonstrate this effect.

Griseofulvin was not taken up nor translocated by the mycelium of *Phycomyces blakesleeanus* in work reported by Abbot and Grove (11). Although this fungus has a chitinous cell wall, no "curling" was observed in either the mycelium submerged in a medium containing griseofulvin or in the aerial hyphae. Further evidence shows that no antibiotic is adsorbed on the mycelium or stored by the mycelium. These workers believe that degradation of griseofulvin is probably due to fungal enzyme activity in the medium or at the surface of the hyphae. It would appear doubtful that the mere presence of chitin in the hyphal wall insures the sensitivity of a fungus to griseofulvin.

Our initial report (12) of 31 human cases of dermatomycoses showed the antibiotic to be highly effective when administered in oral doses of 1 gm. per day. The drug appears to be of low toxicity in man and no contraindications were observed. Paget and Walpole (13) reported that large intravenous doses of griseofulvin in rats initiated cytotoxic effects. These cytologic effects resulted in mitotic arrestments and were similar to those produced by colchicine. In our recent report on 200 patients (14), no cytotoxic

effects of griseofulvin were observed as measured by blood and sperm counts.

Williams *et al.* (15) and Riehl (16) have similarly reported the efficacy of griseofulvin in the treatment of human cases of dermatomycoses.

MATERIALS AND METHODS

The fungi employed in this study were grown in flasks of glucose-peptone-yeast extract broth on a Gyrotary shaker at 24°C. The time required for the desired amount of growth varied with the species; 4 to 6 day old cultures proved to be most satisfactory for our purposes. Under these conditions of cultivation, the filamentous fungi develop as discrete hyphal aggregates or pellets. At four to six days all but the slowest growing forms produce pellets averaging approximately 1.0 mm. in diameter. For use the broth was poured from the flask and the pellets were washed twice for fifteen minutes with sterile saline. The pellets were resuspended in a third volume of saline and the flask contents poured into a Petri dish.

Two procedures were used for the determination of the degree of sensitivity of a fungus to griseofulvin: (1). *Test tube assay procedure.*—One volume of double strength glucose-peptone-yeast extract broth was delivered into each tube in the series. The tubes used measured 20 mm. by 125 mm. and had a screw cap. Sterile standard griseofulvin solution was then delivered into the tubes to achieve the desired concentration but not to exceed one volume unit. The preparation of the griseofulvin solution varied with the requirements of the particular assay. For stock solutions with an antibiotic concentration of 25 mcg./ml. or less, sterile demineralized water was used as the solvent. When higher concentrations were needed, the griseofulvin was first dissolved in a small volume of ethyl alcohol or N,N-Dimethylformamide and the solution brought to final volume with water. The stock solutions of the antibiotic were sterilized either by filtration or autoclaving. The deficiency between the quantity of antibiotic solution added and one volume unit was made up with sterile water. In most assays a volume unit was 2.0 ml. to give a total assay volume of 4.0 ml. in the tubes. After thorough mixing, a fungal pellet was introduced into each tube. The pellet was then measured in at least two diameters with a stereomicroscope. Measurements were made twice a day for the duration of the incubation period. The tube method proved to be most useful for screening fungi for sensitivity and for obtaining differential values in the magnitude of 0.05 to 0.1 mcg./ml. (2). *Plate assay procedure.*—In this method, plates measuring 3.25″ by 4″ with nine wells of 7/8″ diameter were employed. The plates were sterilized in 150 mm. Petri dishes along with a disc of wet heavy absorbant paper to retard evaporation. Serial dilutions of the standard griseofulvin were prepared with water and the unit volume of double strength broth added. Into a well of the plate 0.5 ml. of each antibiotic concentration was pipetted. One well received the assay broth only, to serve as a normal growth control. A washed pellet of the fungus to be assayed was introduced into the center of each well. The diameter of each pellet was measured microscopically with a micrometer eyepiece. The plates and tubes were maintained at 30°C. in an incubator with a high humidity to retard evaporation. The measurements of the pellets were repeated twice a day and the physical characteristics of the advancing peripheral hyphae of the pellet noted. The plate assay technic was found to be more sensitive and accurate than the tube method. The difference in antibiotic concentration from well to well can be of the magnitude of 0.01 to 0.02 mcg./ml. More important, the higher magnification permitted with the standard microscope allows more accurate measurements and visualization of abnormalities in the structure of the mycelium. With experience the griseofulvin-induced changes in the peripheral hyphae can be used as an ancillary determinant for evaluating retardation of growth.

The unicellular yeasts, bacteria, and the dimorphic fungi with a yeast growth phase were assayed by a modified tube method and growth measured by photometric determination of optical density.

OBSERVATIONS AND EXPERIMENTAL RESULTS

I. The Effect of Griseofulvin on the in vitro *Growth of Dermatophytes*

Thirteen species of dermatophytes were assayed for sensitivity to griseofulvin by use of both the tube and the plate assay methods. Preliminary studies had indicated that very low concentrations of the antibiotic would be required to secure the inhibition of these fungi. Griseofulvin is soluble in water to the extent of approximately 30 mcg./ml. In that this concentration is far greater than that required for *in vitro* inhibition of dermatophytes, the stock solutions of the antibiotic were prepared with distilled water without other solvents. Serial tube assays were run first, employing a concentration range of 0.05 to 5.0 mcg./ml. of antibiotic in increments of 0.05 mcg. The introduced pellets were measured in cross diameters, twice daily for eight days. From the results obtained it became obvious that the establishment of a

minimal inhibitory concentration value was directly related to and determined by the length of the observation period. The effect of increasing concentration of griseofulvin is one of progressive retardation of the growth rate until a level is reached at which growth is so slow as to defy measurement. Thus the longer the period of incubation and observation, the higher will be the minimal inhibitory concentration value. The failure of griseofulvin to produce an "all-or-none" effect on sensitive fungi all but precludes a sharp end-point determination independent of time. For these reasons, two arbitrary observation time limits were chosen: 72 hours and 160 hours. In addition, we have observed that it is possible to decrease the amount of antibiotic required for inhibition of dermatophytes by limiting or reducing the nutritive value of the assay broth, especially as to its carbohydrate content. An assay medium closely approximating that of the *in vivo* substrate (stratum corneum, hair, nails) would probably provide values more realistic and compatible with the levels required in therapy.

Based upon the inhibitory values obtained in the tube procedure, plate assays were prepared so that the antibiotic concentrations in the wells of the plates encompassed and bracketed the tube assay findings. The plate assays covered a concentration range of 0.1 to 0.5 mcg./ml. in increments of 0.02 mcg. The plates are limited to an observation time of 96 hours, due to evaporation of well contents which would invalidate the concentration values. The minimal inhibitory concentration of griseofulvin for the tested species of dermatophytes is presented in Table 1. The 72-hour value represents the average of both the tube and the plate assay results, while the 160-hour inhibitory concentration is that obtained with the tube assay only.

With the observation that the dermatophytes are inhibited by very low concentrations of griseofulvin, experiments were performed to determine the effect of high concentrations of the antibiotic on the viability and longevity of these fungi. Paired flasks containing 100 ml. of saline with a griseofulvin content of 25 mcg./ml. and pairs containing glucose-peptone broth with identical antibiotic concentration each received fifty washed five-day old pellets of *T. rubrum*. A duplicate set of flasks was inoculated with pellets of *T. mentagrophytes*. One flask of each pair was refrigerated at 2°C., the other flask was incubated at 30°C. On alternate days for thirty days, three pellets were removed from each flask and washed three times for fifteen minutes, each with 10 ml. of sterile saline. After the third washing procedure, the saline was decanted and the pellets planted on plates of glucose-peptone agar. The plates were incubated at 30°C. and observed daily for development of colonies. Pellets kept for as long as thirty days at 2°C. and 30°C. in both saline and broth medium produced normal colonies on the antibiotic-free plates. The pellets that had been submerged in the antibiotic solution for more than ten to twelve days evidenced a prolonged lag period (four to six days) before growth became evident and the initial growth on the agar plates was somewhat retarded and restricted. Once the initial phase of retardation had passed, the growth rate was normal and the colony was typical in all respects. None of the pellets evidenced any signs of growth during the period of residence in the solution of griseofulvin. In a subsequent experiment it was observed that a single washing in 100 ml. of saline for ten minutes was sufficient to rid the pellet of the antibiotic and permit the fungus to resume normal growth.

An attempt was made to induce resistance to griseofulvin in *T. rubrum* by serially passing the fungus through progressively higher concentrations of the antibiotic over a prolonged period of time. Ten washed pellets of the dermatophyte, measuring 3 to 4 mm. in diameter, were placed in a flask containing glucose-peptone

TABLE 1

Minimal inhibitory concentrations of griseofulvin for dermatophytes

Organism	At 72 hr.	At 160 hr.
Microsporum canis	.20–.24*	1.4–1.6*
Microsporum gypseum	.42–.46	1.5–1.7
Microsporum Audouini	.40–.46	2.1–2.3
Epidermophyton floccosum	.38–.42	2.1–2.3
Trichophyton mentagrophytes	.38–.42	1.9–2.1
Trichophyton rubrum	.14–.18	1.3–1.5
Trichophyton tonsurans	.28–.32	1.5–1.7
Trichophyton Megnini	.30–.34	2.2–2.4
Trichophyton gallinae	.40–.44	2.2–2.4
Trichophyton Schoenleini	.34–.38	1.8–2.0
Trichophyton violaceum	.36–.40	1.9–2.1
Trichophyton verrucosum	.28–.30	2.1–2.3
Trichophyton concentricum	.26–.30	2.2–2.4

* mcg. per ml.

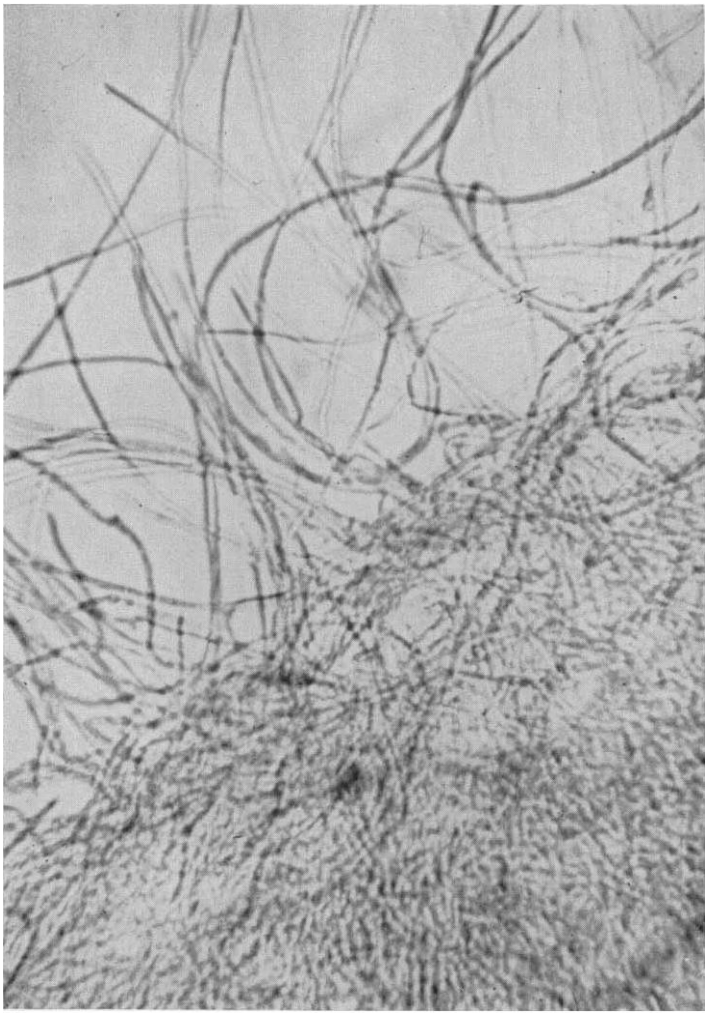

FIG. 1A. Periphery of a normal pellet of *Trichophyton rubrum*, 5 days' growth in gyrotary shaker culture. The marginal hyphae are rarely branched and fringe out from the central mycelial mass of the pellet. (unstained; ×340).

broth with a griseofulvin concentration of 0.5 mcg./ml. All flasks were maintained on a Gyrotary shaker during the course of the experiment. At the end of seven days of rotation all pellets had increased to a diameter of 5 to 6 mm. At this time the hyphal masses were transferred to fresh broth containing 0.6 mcg. of griseofulvin per ml. Every fifth day thereafter the pellets were placed in fresh media and the antibiotic concentration increased 0.1 mcg./ml. At the end of 67 days the fungal pellets were in a broth containing a griseofulvin concentration of 1.8 mcg./ml. During this period of time the pellets had slowly increased in diameter, to measure between 10 and 12 mm.; however, as the antibiotic concentration increased, the growth increment was observed to undergo a progressive retardation. In a broth with 2.0 mcg. of antibiotic per ml. no further increase in pellet diameter was detected. Therefore this griseofulvin concentration was maintained for an additional six transfers of the pellets at the regular five-day interval. At the end of this thirty-day period, the size of the pellets had demonstrated no measurable increase and appeared to be completely stationary. The fungal masses were then washed for six hours in a large volume of sterile saline and plated on antibiotic-free media where

FIG. 1B. Marginal hyphae at periphery of normal pellet, *T. rubrum*. The cytoplasm is fairly homogeneous and evenly distributed throughout the hyphal cell; branching is seldom observed. (unstained; ×770).

they developed normal appearing colonies after an initial dormant period of five to seven days.

The presence of griseofulvin in the medium in which a dermatophyte is growing is readily detected by marked changes in the growth character of the mycelium. These induced abnormalities are depicted in the photomicrographs presented in Figures 1, 2, and 3. Concentrations as low as 0.05 to 0.1 mcg./ml. produce observable changes to the experienced microscopist. The first discernible effect of the presence of griseofulvin at a concentration of 0.1 mcg./ml. (Fig. 2A) is the abundant production of short lateral hyphal projections or branches. The tips of the advancing peripheral hyphae become slightly swollen or clubbed (Fig. 2B). As the concentration is increased to 0.2 to 0.3 mcg./ml. the clubbing tendency is greatly exaggerated and small contortions and knobs make their appearance (Fig. 3A). Still higher levels (0.5 to 0.6 mcg.) result in the hyphae developing coils or curls (Fig. 3B), as reported by Brian and co-workers (10), employing *Botrytis allii*, and later by Napier with *Alternaria solani*, as reported by Gentles (7). At this point the cytoplasm is highly vacuolated and many areas in the hyphae have the appearance of autolyzing "ghost cells" (Fig. 3C). In the region of mature mycelium, swollen balloon-like cells appear and apparently represent giant chlamydospores induced by toxicity (Fig. 3D).

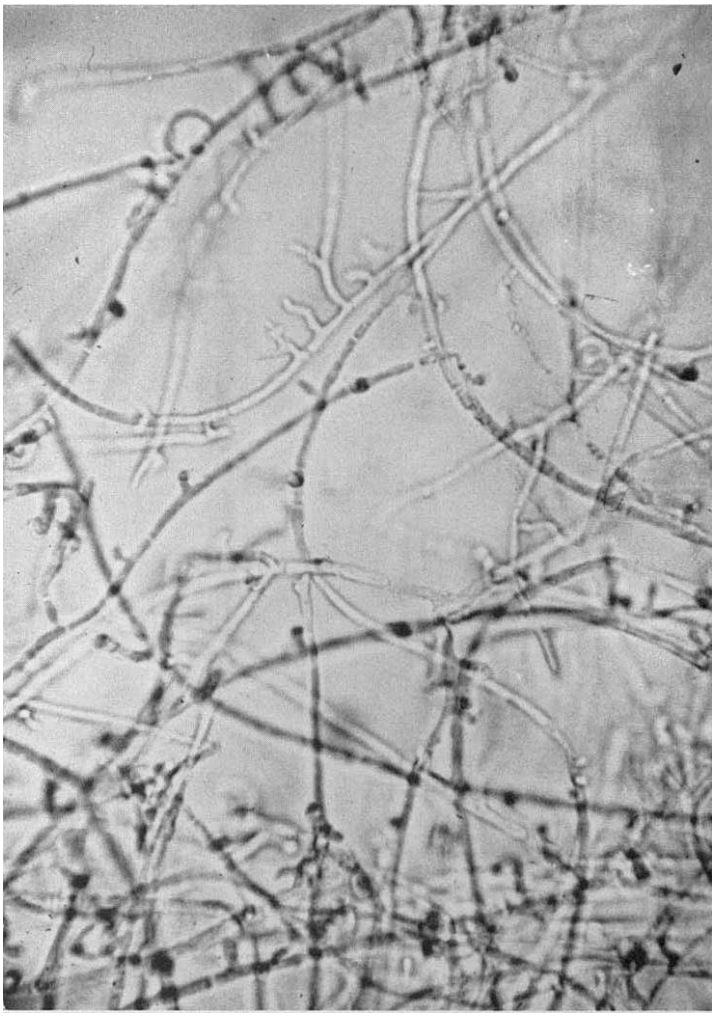

FIG. 2A. Lateral branching and terminal bifurcations observed in marginal hyphae of *T. rubrum* pellet exposed to griseofulvin concentration of 0.1 mcg./ml. for 72 hours. (unstained; ×340).

These abnormal contortions of the hyphae cause the submerged pellet to change macroscopically from a sphere with a fluffy or cottony periphery to one of granular appearance with an irregular knobbed surface.

II. The in vitro *Effect of Griseofulvin on Other Mycotic Agents, Pathogenic Bacteria, and Saprophytic Fungi and Yeasts*

To determine the spectrum of activity of griseofulvin for pathogenic fungi, bioassays were performed, utilizing the etiologic agents of superficial, subcutaneous and systemic mycoses. In the case of those fungi which are dimorphic, the assay was performed at both 30°C. with the filamentous phase and at 37°C employing the yeast or tissue phase. Glucose-peptone broth and brain-heart-infusion glucose broth (both pH 7.0) were prepared by use of a saturated aqueous solution of griseofulvin (30 mcg./ml.) in place of distilled water. The broths were gently heated to dissolve the ingredients and sterilized by filtration. Flasks containing 20 ml. of the glucose-peptone-antibiotic broth were inoculated with three 2-mm. pellets of the fungus to be tested or with a standardized saline suspension of the unicellular yeasts. The vessels were stationed on the gyrotary platform and rotated at 160 rotations per minute. Flasks containing the medium without antibiotic were similarly inocu-

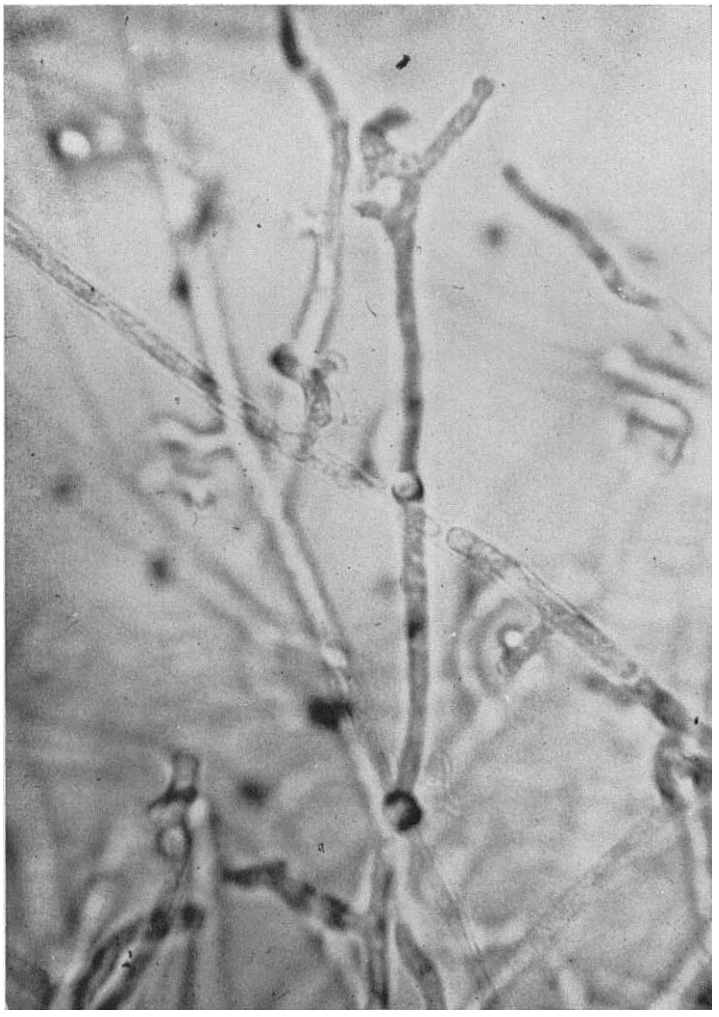

FIG. 2B. Granular cytoplasm and uneven thickness of cell wall noted in higher power view of pellet seen in 2A. The clubbing tendency of the hyphal tips and stunting of lateral branches is seen.

lated to serve as normal growth controls. Standardized inocula of the yeast phase of the dimorphic forms were inoculated into tubes containing 10 ml. of the infusion glucose broth with antibiotic. The tubes were placed in a rack in water bath shaker set at a temperature of 37°C. Tubes of infusion broth lacking griseofulvin were inoculated and run concurrently to ascertain normal growth rates. All cultures were examined twice daily and growth determined as an increase in pellet diameter, an alteration in the nature of the pellet periphery, or in the case of the yeasts and yeast-like forms by an increase in the optical density. Observations were continued up to twenty days for the more slowly growing forms. At the end of the period allowed for growth, the fungus was examined microscopically for abnormal hyphal configurations or other signs of inhibition of growth and toxicity. In no case did the presence of griseofulvin in the media produce a measurably inhibitory effect on the growth rate or significantly alter the morphology of the fungi tested. The mycotic agents tested and found to be unaffected by a concentration of griseofulvin of 30 mcg./ml. are presented in Table 2.

The observation (17, 18) that certain of the broad spectrum antibiotics, especially those of the tetracycline group, markedly enhanced the pathogenicity of *Candida albicans* for diverse

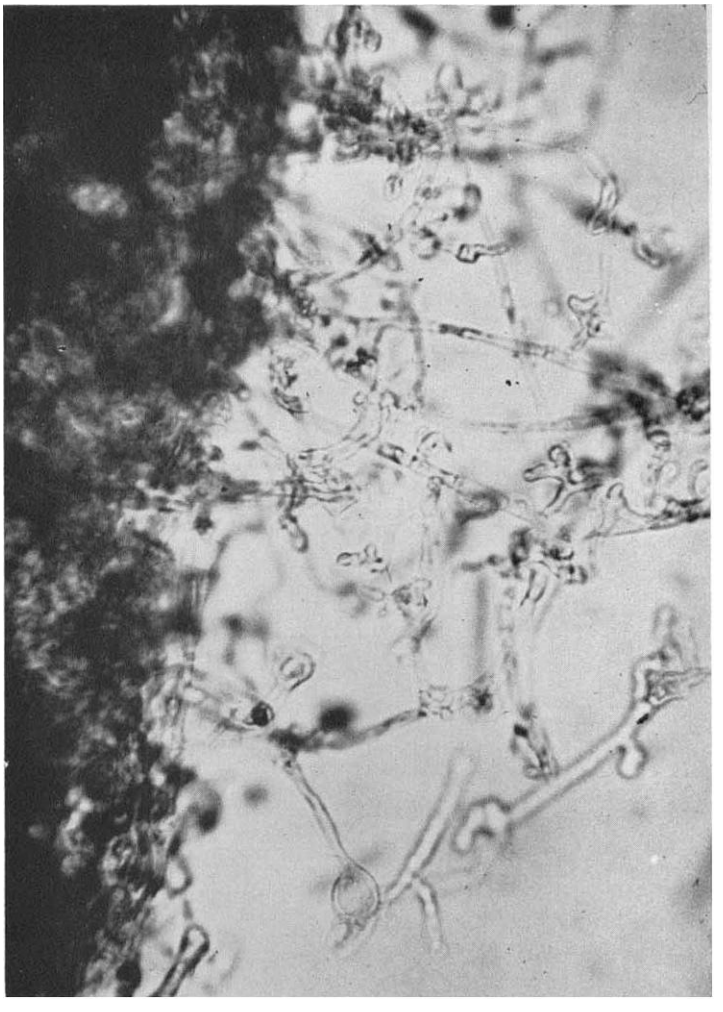

FIG. 3A. Hyphal abnormalities produced in *T. rubrum* pellet growing for 72 hours in a broth containing 0.25 mcg./ml. of griseofulvin. Numerous knobs, contortions and clubbed hyphal tips are in evidence and growth is greatly inhibited. (unstained; ×340).

animal species made it seem advisable to determine whether griseofulvin might have a similar potentiating effect in experimental candidiasis. Six groups of ten adult Swiss albino mice were employed in the study. The mice in the first group each received a saline solution containing 10.0 mcg. of griseofulvin by the intraperitoneal route. The animals in the second, third, and fourth groups were injected with 20, 35, and 50 mcg., respectively. Ten minutes after the injection of the antibiotic, 0.5 ml. of a 1:100 washed cell suspension of a 48-hour culture of *Candida albicans* was injected intraperitoneally into all treated animals. The fifth group of mice received only the yeast cell suspension, while the members of the sixth group were administered only 50 mcg. of griseofulvin intraperitoneally. The injection of the appropriate amounts of antibiotic was repeated for three additional consecutive days. At the end of the eighth day the mice in all six groups appeared to be in normal health and upon sacrifice and examination failed to show any internal lesions or signs of established candidiasis.

The effect of griseofulvin on the *in vitro* growth of various bacteria was investigated. Paired tubes containing 2.0 ml. of double strength nutrient broth were prepared. To one tube of

FIG. 3B. Marked inhibition of growth and gross abnormalities induced in marginal mycelium of pellet of *T. rubrum* by griseofulvin, 0.5 mcg./ml., 60 hours. (unstained; ×340).

each pair was added 2.0 ml. of saline containing 60 mcg. of griseofulvin and to the growth control tube of each pair was added 2.0 ml. of saline alone. Todd-Hewitt media was used in place of nutrient broth for *Streptococcus pyogenes* and *Diplococcus pneumoniae*. Each tube was inoculated with 0.1 ml. of a 24-hour culture of the respective organisms and the amount of growth evaluated macroscopically after eighteen hours' incubation at 37°C. The findings are presented in Table 3. None of the organisms was inhibited in its growth by griseofulvin in a concentration of 15 mcg./ml. Several of the bacteria tested at first seemed to show an apparent growth stimulation but this effect was found not to be significant when tested by quantitative turbidimetric procedure.

A limited survey employing 22 species of common saprophytic fungi failed to uncover any form which demonstrated a significant degree of inhibition in a high (25 mcg./ml.) concentration of griseofulvin. The species subjected to bioassay and found to be resistant to complete inhibitory activity by the antibiotic are listed in Table 4.

A sensitive unicellular microscopic growth form would be of great value in assaying the griseofulvin content of body tissues and fluids. A turbidimetric method would be much more accurate and workable than one based upon the

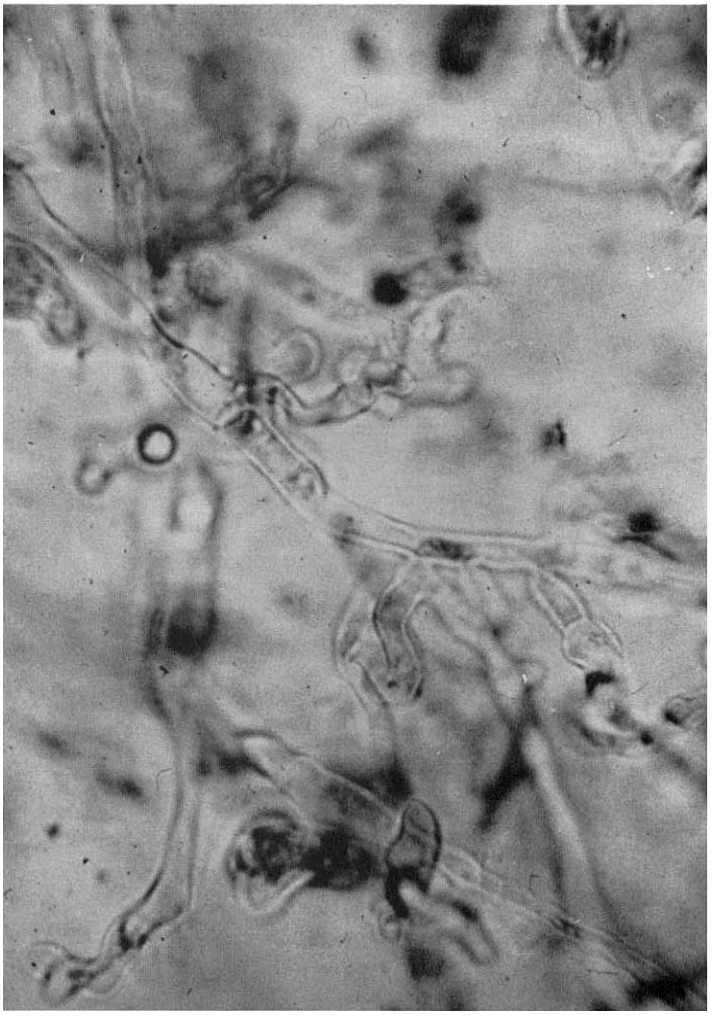

FIG. 3C. Vacuolation and cytoplasmic massing produced in hyphae of *T. rubrum* exposed to 0.3 mcg./ml. of griseofulvin, 72 hours. Some cells appear to have areas devoid of contents and resemble autolyzing "ghost cells". (unstained; ×770).

inhibition of fungal growth or the engendering of abnormalities in hyphal morphology. For this reason 40 species of sexual and imperfect yeasts in 16 genera were tested for sensitivity to griseofulvin. Standardized suspensions of washed yeast cells were inoculated into a series of five tubes of assay broth containing 1, 5, 10, 15, and 25 mcg. of antibiotic per ml., respectively. The inoculated tubes were placed in a rack on a gyrotary shaker platform and incubated at 26°C. The resultant growth was measured turbidimetrically in a Lumetron colorimeter at 24, 48 and 72 hours. The species tested are listed in Table 5. None of the 40 yeasts so tested demonstrated either inhibition or enhancement of growth of significant degree by an antibiotic concentration ranging up to 25 mcg./ml.

III. Effect of Griseofulvin on the Respiration of Trichophyton rubrum

To obtain cellular material for respiration studies, *T. rubrum* was grown in glucose-peptone broth at room temperature on the gyrotary shaker. Most of the strains of the fungus formed hyphal pellets of varying sizes in five days of cultivation. Two groups of pellet sizes were segregated for study: (a) small pellets ranging from 0.4 to 1.0 mm. in size and (b) larger pellets

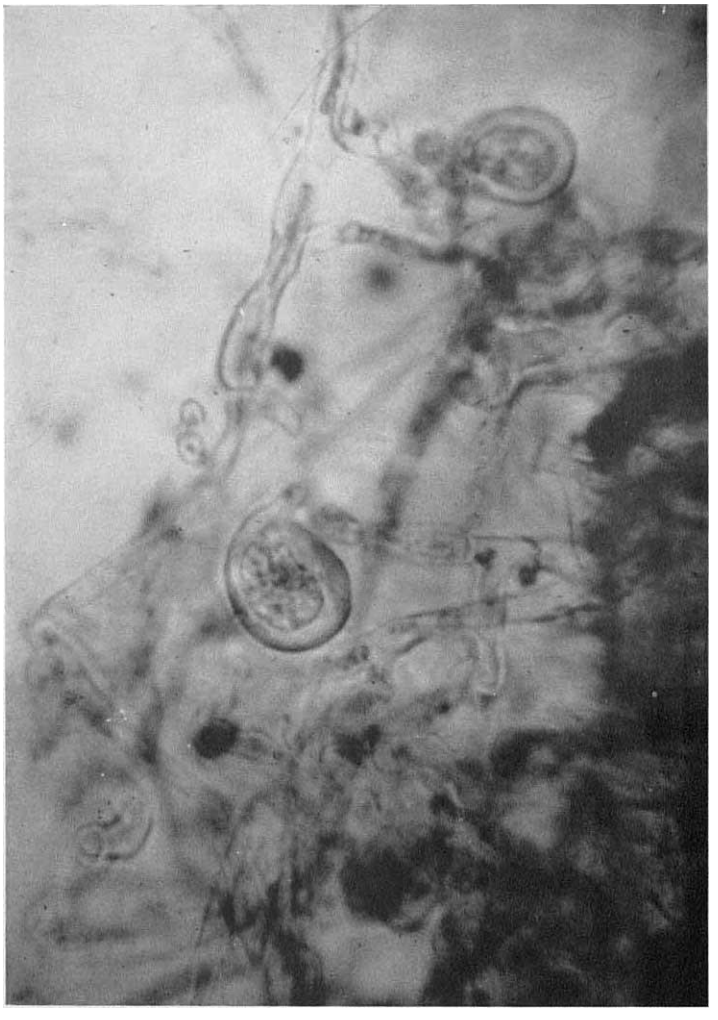

FIG. 3D. Large chlamydospores seen within mature mycelium of fungal pellet of *T. rubrum* exposed for 72 hours to griseofulvin, 0.3 mcg./ml. (unstained; ×770).

1.1 to 2.0 mm. in diameter. In addition to pellet formation, one strain of *T. rubrum* produced diffuse mycelial growth on the shaker in the same time period. QO_2 characteristics of these three types of growth were determined. Prior to Warburg manometric determination, the five-day growth was removed from the culture flasks and washed twice with saline. Standard Warburg technics were utilized, including the use of 20 per cent KOH in the center well for CO_2 absorption. The QO_2 values for the three types of growth are given in Table 6. The larger pellets showed respiratory activity more than six times greater than that of the smaller pellets. The respiratory activity of the diffuse mycelial growth was somewhat greater than that of the small pellets. It was decided to test the effect of griseofulvin on the most active respiring form of the fungus.

The segregated larger pellets were tested for the effect of added glucose and griseofulvin individually on their respiratory activities. Glucose was added in the amount of 15 mg. and griseofulvin, 30 mcg. per flask. Total flask content was 3.0 ml. The pellets were washed twice in saline prior to use. From the upper half of Figure 4 it can be seen that griseofulvin has a very slight inhibitory effect on oxygen uptake when compared with the endogenous respiration (15 ml. QO_2 over a sixty-minute period). It is not likely

TABLE 2

Pathogenic fungi uninhibited by griseofulvin, 30 mcg./ml.

Organism	Temperature of Incubation
Candida albicans	28 C. and 37 C.
Cryptococcus neoformans	28 C. and 37 C.
Sporotrichum Schenckii	28 C. and 37 C.
Hormodendrum compactum	28 C.
Phialophora verrucosa	28 C.
Nocardia asteroides	28 C.
Nocardia brasiliensis	28 C.
Blastomyces dermatitidis	28 C. and 37 C.
Blastomyces brasiliensis	28 C.
Histoplasma capsulatum	28 C. and 37 C.
Geotrichum candidum	28 C.
Aspergillus fumigatus	28 C.

TABLE 3

The effect of griseofulvin on the in vitro *growth of various bacteria*

Organism	Growth in broth	Growth in broth containing griseofulvin (15 mcg/ml)
Aerobacter aerogenes	++	+++
Bacillus subtilis	++	++
Bacillus cereus	++	++
Corynebacterium diphtheriae	++	++
Escherichia coli	++	+++
Klebsiella pneumoniae	++	++
Staphylococcus aureus (coagulase positive)	++	++
Staphylococcus albus	++	++
Proteus vulgaris	++	+++
Pseudomonas aeruginosa	++	++
Salmonella typhimurium	++	++
Salmonella typhosa	++	++
Sarcina lutea	++	++
Serratia marcescens	++	+++
Shigella paradysenteriae	++	+++
Diplococcus pneumoniae	++	++
Streptococcus pyogenes, gp. A	++	++
Streptococcus pyogenes, gh. C	++	++

that the observed, very limited effect of griseofulvin on the respiration of the dermatophyte could account for the marked growth inhibition obtained with much smaller amounts of the antibiotic. The addition of glucose to the washed fungal cells does not increase respiration, indicating that the fungal cells of the large pellet

TABLE 4

Effect of griseofulvin (25 mcg./ml.) on the growth of various fungi

Alternaria tenuis	Slight inhibition
Aspergillus candidus	No inhibition
Aspergillus glaucus	Slight inhibition
Aspergillus niger	No inhibition
Aspergillus terreus	No inhibition
Curvularia geniculata	Slight inhibition
Fusarium oxysporum	No inhibition
Helminthosporium anomalum	Slight inhibition
Helminthosporium sativum	Slight inhibition
Hormodendrum olivaceum	Slight inhibition
Mucor genevensis	No inhibition
Oospora variabilis	No inhibition
Paecilomyces divaricatum	No inhibition
Penicillium nigricans	No inhibition
Penicillium janthinellum	No inhibition
Penicillium chrysogenum	No inhibition
Phoma humicola	Slight inhibition
Rhizopus nigricans	No inhibition
Scopulariopsis communic	No inhibition
Scopulariopsis brevicaulis	No inhibition
Trichoderma koningi	No inhibition

size contain sufficient readily available carbohydrate reserves to account for all the cellular energy needs of the organism, at least during the period of observation. Although it appears from Figure 4 that the respiration inhibition of *T. rubrum* is practically as great following the addition of glucose as is that following griseofulvin, replicate experiments continually showed the slight inhibitory activity of griseofulvin, whereas the glucose inhibition was sufficiently erratic to be ascribed to experimental variation.

To determine whether the failure of griseofulvin to affect the respiration of *T. rubrum* might be due to the failure of cellular penetration by the antibiotic, large hyphal pellets were homogenized for a brief period in a Virtis homogenizer. The homogenized cells were then treated as the intact pellets had been. The results are visualized by graphs in the lower half of Figure 4. It can be seen that the endogenous respiration following homogenization is reduced approximately by half. The intact cell is apparently a major requirement for reasonably normal metabolic activity. Griseofulvin addition showed no activity at all on the cytoplasmic energy centers, indicating in conjunction with its small activity on the intact hyphal cells, that if the antibiotic

TABLE 5

Yeasts uninhibited or enhanced in growth by griseofulvin concentration of 25 mcg./ml.

Candida flareri	*Pichia belgica*
Candida guilliermondi	*Pichia chodati*
Candida krusei	*Saccharomyces bayanus*
Candida krusoides	*Saccharomyces carlsbergensis*
Candida macedoniens	*Saccharomyces cerevisiae*
Candida monosa	*Saccharomyces ellipsoideus*
Candida parakrusei	*Saccharomyces fragilis*
Candida parapsilosis	*Saccharomyces italicus*
Candida pseudotropicalis	*Schizosaccharomyces ostosporus*
Candida tropicalis	*Schizosaccharomyces pombe*
Candida zeylandoides	*Rhodotroula glutinus*
Cryptococcus nigricans	*Rhodotroula mucilaginosa*
Debaryomyces klockeri	*Torulaspora rosei*
Debaryomyces membranefaciens	*Torulopsis pulcherrima*
Endomyces magnusii	*Trigonopsis variabilis*
Endomycopsis lindneri	*Zygosaccharomyces acidifaciens*
Eremothecium ashbyii	*Zygosaccharomyces casei*
Hansenula saturnus	*Zygosaccharomyces delbruckii*
Mycoderma lafarii	*Zygosaccharomyces lactis*
Mycoderma vini	*Zygosaccharomyces marxianus*

affects energy mechanisms at all it is at a more superficial locus, most probably in the cell wall area. The addition of glucose to the homogenate enhanced respiration slightly, indicating that the carbohydrate reserves present in the large pellet cells were probably removed in small measure by rupture of the cell and by the two saline washings.

IV. The Bioassay of Griseofulvin in Body Fluids and Tissues

During the course of this study direct bioassays of 126 sera obtained from patients and normal volunteers on various dosage levels of griseofulvin were performed. In addition, 85 urine samples from the same subjects were subjected to bioassay, utilizing pellets of *T. rubrum* in both the tube dilution method and the well-plate technic.

TABLE 6

Endogenous respiratory quotients of trichophyton rubrum *in various growth stages obtained in shaker culture*

	Air* QO_2
Diffuse mycelial growth	0.75
Small hyphal pellets (0.4–1.0 mm.)	0.39
Large hyphal pellets (1.0–2.0 mm.)	2.49

* QO_2 values are calculated on a dry weight basis.

In our experience these procedures are at best insensitive and inaccurate methods for the determination of blood and excretion levels of the drug. In the blood, and to a lesser extent in the urine, are found normal substances and factors which interfere with growth of dermatophytes to thus complicate assessment of true griseofulvin activity. From our data as obtained by bioassay, it would appear that body fluid and urine levels are rarely in excess of 1 to 2 mcg./ml. and most commonly are considerably lower (0.2 to 0.5 mcg./ml.). Therefore in the direct assay of serum and urine one must employ concentrations of the fluids which in themselves possess a significant capacity to inhibit fungal growth and evoke some morphologic alterations in hyphal structure.

In a limited study the abnormal changes in the microscopic character and structure of the mycelium as induced by griseofulvin were employed as the criteria for determination of the antibiotic concentration. This approach has some merit but was found to be laborious and somewhat cumbersome. A further difficulty was encountered in correlating consistent and defined changes in fungal morphology with small differences in the concentration of the antibiotic in the sample. The microscopic method requires a considerable amount of experience and is subject to significant variation as to individual observers. In addition, the inhibitory action of normally occurring factors in body fluids is difficult to assess and distinguish from those induced by the griseofulvin itself.

A comparison of the concentration levels obtained by bioassay with those reported by chemical extraction and spectrophotometric procedures would suggest that a significant amount of the antibiotic in body tissues and fluids may occur in a form lacking biological activity for the dermatophytes. If, however, the

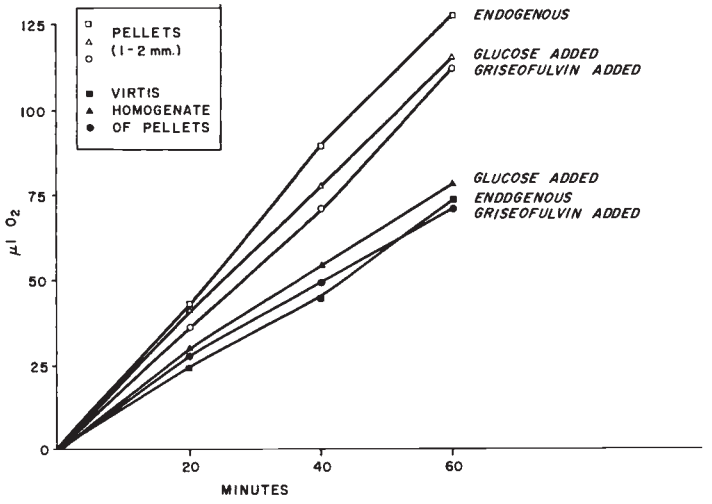

FIG. 4. Effect of griseofulvin on oxygen uptake by *Trichophyton rubrum*

low levels detected by measurement of the body fluids' capacity to inhibit sensitive fungi are valid they may reflect only the low aqueous solubility of griseofulvin. Similarly, the absorption of the orally administered antibiotic may be restricted and the drug once absorbed from the gastrointestinal tract may be quickly incorporated in or fixed by diverse body tissues.

With the observation that the action of griseofulvin on dermatophytes was apparently limited to that of fungistasis, a re-evaluation of the persistance of viable though arrested fungi in the skin of patients undergoing therapy was undertaken. It was reasoned that the keratin of the stratum corneum of these individuals could contain a sufficient depot of antibiotic to thus continue to inhibit static fungal elements even when grown on a favorable medium. Such a condition might lead to erroneous information concerning the duration of treatment or dosage needed to affect a cure as determined by obtaining a negative culture. To test this concept, six patients with tinea corporis due to *T. rubrum* were treated with griseofulvin. At regular intervals the areas of the lesions were scaped thoroughly and the skin pooled. Half of the sample was plated directly upon routine solid media containing cycloheximide and chloramphenicol. The other half of the material was washed in three separate changes of 5 per cent N,N-dimethylformamide in saline for thirty minutes each. The last wash was decanted and the skin in the tube overlayed with routine glucose-peptone broth containing the mentioned antibiotics. It was felt that the washing procedure employed a solvent for griseofulvin and subsequent use of a liquid medium would be sufficient to wash out or lower the concentration of the antibiotic so that an inhibitory carry-over effect would be eliminated. The results secured have not been of sufficient magnitude to justify a definitive conclusion. However, an approximate equal number of positive cultures have been secured by each method and both have been positive 25 per cent of the time. At this point it would not appear that an extraction of griseofulvin from clinical material is necessary prior to cultivation. The findings merely re-emphasize the need for multiple cultures and employment of diverse media to best insure the isolation of these mycotic agents.

The direct bioassay of serum from individuals receiving orally administered griseofulvin indicated that the blood level was very low or that the antibiotic might be present, at least in part, in a form not possessing *in vitro* inhibitory activity for dermatophytes. To test the possibility that serum may bind or complex with the antibiotic to thereby reduce its activity, 10 ml. of normal human serum was combined with 10 ml. of a saline solution of griseofulvin containing the antibiotic at a concentration of 20 mcg./ml. The serum-antibiotic mixture was placed in a water bath shaker at 37°C. for twelve hours. At the end of this time the mixture was assayed by the tube method against *T. rubrum*. The assay

series was incubated for 72 hours at 30°C. At the conclusion of the incubation it was observed that 0.2 ml. of the serum-antibiotic mixture per ml. of broth was required to inhibit the fungal pellet. This volume ratio represents an antifungal antibiotic concentration of 2.0 mcg./ml. In contrast, the same relative degree of pellet inhibition was secured with 0.2 mcg./ml. of griseofulvin when the assay was conducted directly with the saline solution of the antibiotic. These results indicated that approximately ten times more griseofulvin was needed for the inhibition of *T. rubrum* following an incubation of the antibiotic with 50 per cent serum. As measured by its inhibitory potential for dermatophytes it would appear that griseofulvin is partially inactivated or removed by *in vitro* contact with human serum.

SUMMARY

Thirteen of the recognized species of dermatophytes demonstrated a high degree of *in vitro* sensitivity to griseofulvin. Low concentrations of the antibiotic inhibited their growth and produced bizarre abnormalities in their hyphal structure. Higher levels of the antibiotic resulted in an apparent complete arrestment of the growth of the mycelium.

The action of griseofulvin on dermatophytes appears to be limited to that of fungistasis in that these fungi were able to withstand prolonged exposure to high concentrations of the antibiotic without loss of viability.

Griseofulvin failed to demonstrate significant *in vitro* inhibitory activity against the etiologic agents of histoplasmosis, North and South American blastomycosis, candidiasis, chromoblastomycosis, nocardiosis, geotrichosis, aspergillosis, cryptococcosis and sporotrichosis.

The intraperitoneal administration of griseofulvin to white mice experimentally infected with *Candida albicans* did not result in the death of any of the animals nor appear to enhance the severity of the infection.

All tested species of non-pathogenic ascosporogenous and imperfect yeasts and yeast-like fungi were completely resistant to high concentrations of griseofulvin.

Griseofulvin in high concentration did not affect the growth of a select group of pathogenic bacteria.

Griseofulvin had a very slight but consistent inhibitory effect upon the respiration of intact cells of *Trichophyton rubrum*, whereas the cytoplasmic energy centers were not affected at all.

The addition of griseofulvin to normal serum appeared to result in a decline in the activity of the antibiotic as determined by subsequent bioassay.

The direct bioassay of body fluids was generally unsatisfactory due to the low levels of detectable antibiotic present in such materials and the presence of normal interfering substances.

In the cultivation of skin scrapings from patients on griseofulvin therapy for evidence of biological cure, the antibiotic content of the skin does not appear to be a factor for concern since direct inoculation of skin from such individuals on appropriate media did not result in the inhibition of the resident fungus.

An attempt to induce resistance to griseofulvin in *Trichophyton rubrum* by subculturing the organism in gradually increasing concentrations of the antibiotic over a prolonged period of time was not successful.

ACKNOWLEDGMENTS

We acknowledge from Glaxo Laboratories, England, and from Johnson and Johnson and its subsidiary, McNeil Laboratories, Inc., supplies of griseofulvin. The technical assistance of Miss Jo Ann Counts and Mrs. Robert Longenecker is gratefully acknowledged.

Editor's note: This paper was discussed jointly with that of Stanley A. Rosenthal, Norman Goldfarb and Rudolf L. Baer, "Therapeutic and Preventive Effects of Griseofulvin in Guinea Pigs" which follows immediately.

REFERENCES

1. Oxford, A. E., Raistrick, H. and Simonet, P.: Studies in the biochemistry of microorganisms. LX. Griseofulvin, $C_{17}H_{17}O_6Cl$, a metabolic product of penicillium griseofulvum Diercks. Biochem. J., **33:** 240, 1939.
2. Brian, P. W., Curtis, P. J. and Hemming, H. J.: Biological Assay, Production and Isolation of 'Curling factor'. Trans. Brit. Mycol. Soc., **29:** 173, 1946.
3. Grove, J. F., MacMillan, J., Mullholland, T. P. C. and Rogers, M. A. T.: Griseofulvin, IV. Structure. J. Chem. Soc.: p. 3977, 1952.
4. Brian, P. W.: Antibiotics as systemic fungicides and bactericides. Ann. Applied Biol., **39:** 434, 1952.
5. Gentles, J. C.: Experimental ringworm in guinea pigs: Oral treatment with griseofulvin. Nature, **182:** 476, 1958.

6. GENTLES, J. C.: Personal communication.
7. GENTLES, J. C., BARNES, M. J. AND FANTES, K. H.: Presence of griseofulvin in hair of guinea pigs after oral administration. Nature, **183:** 256–257, 1959.
8. MARTIN, A. R.: The systemic and local treatment of experimental dermatophytoses with griseofulvin. J. Invest. Dermat., **32:** 525–528, 1959.
9. LAUDER, I. M. AND O'SULLIVAN, J. G.: Ringworm in cattle. Prevention and treatment with griseofulvin. Vet. Rec., **70:** 949, 1958.
10. BRIAN, P. W.: Studies on the biological activity of griseofulvin. Ann. Bot. (N.S.), **13:** 59–78, 1949.
11. ABBOT, M. T. J. AND GROVE, J. F.: Uptake and translocation of griseofulvin. Exp. Cell Res., **17:** 105–113, 1959.
12. BLANK, H. AND ROTH, F. J., JR.: The treatment of dermatomycoses with orally administered griseofulvin. Arch. Dermat. & Syph., **79:** 259–266, 1959.
13. PAGET, G. E. AND WALPOLE, A. L.: Some cytological effects of griseofulvin. Nature, **182:** 1320, 1958.
14. BLANK, H., SMITH, J. G., JR., ROTH, F. J., JR. AND ZAIAS, N.: Griseofulvin for the systemic treatment of dermatomycoses. J.A.M.A. (in press).
15. WILLIAMS, D. I., MARTIN, R. H. AND SARKANY, I.: Oral treatment of ringworm with griseofulvin. Lancet, **2:** 1212, 1958.
16. RIEHL, G.: Griseofulvin - ein peroral wirkendes Antimykoticum. Hautarzt, **10:** 136, 1959.
17. SELIGMANN, E.: Virulence enhancing activities of aureomycin on *Candida albicans*. Proc. Soc. Exper. Biol. & Med., **79:** 481, 1952.
18. ROTH, F. J., JR. AND EYLAR, O. R.: A selective enhancement by aureomycin of the pathogenicity of *Candida albicans* for mice. Bact. Proc., p. 87, 1954.