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Uptake of Griseofulvin by the Sensitive Dermatophyte, *Microsporum gypseum*

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ABSTRACT

EL-NAKEEB, MOUSTAFA A. (Rutgers, The State University, New Brunswick, N.J.), AND J. O. LAMPEN. Uptake of griseofulvin by the sensitive dermatophyte, *Microsporum gypseum*. J. Bacteriol. **89**:564–569. 1965.—Actively growing cultures of *Microsporum gypseum* took up large amounts of griseofulvin-(4-methoxy-H^3) from the medium. Initially, most of the material could be extracted with hot water, but there was a continuing increase in firmly bound forms of the antibiotic. The fungus accumulated griseofulvin intracellularly to a level up to 100 times that present in the medium. The process appeared to involve two phases. A small amount of griseofulvin was bound almost instantaneously. This binding was independent of the culture conditions or cell viability. The second stage was prolonged and was governed by the different factors controlling active metabolism, but it proceeded in organisms whose growth had been inhibited by the antibiotic itself or by limited nutrients. This stage required a supply of metabolic energy, since it was temperature-dependent, needed an exogenous energy source, and was completely inhibited by sodium azide or 2,4-dinitrophenol. Uptake was optimal at *p*H 5.5 to 6.5. Synthesis of a transport system is probably required, since uptake is prevented by *p*-fluorophenylalanine. Heat-killed cells did not take up griseofulvin beyond the small amount bound instantly.

Although ample evidence has been presented for the uptake of griseofulvin by higher plants (Brian, 1960; Crowdy et al., 1956), there has been no direct evidence for a comparable binding of the antibiotic by sensitive fungi. Brian (1949), Banbury (1952), and Aytoun (1956) observed that griseofulvin affected only hyphae and hyphal parts with which it was in direct physical contact and not the remote (e.g., aerial) regions of the mycelium. Brian (1960), therefore, proposed that the antibiotic might act from the outside of the cell directly on the cell wall (characteristically distorted by griseofulvin).

Abbot and Grove (1959) could not demonstrate any griseofulvin (or metabolite) in the hyphae of *Phycomyces blakesleeanus*, *Botrytis allii*, or *Mucor ramannianus* after 20 to 50 days contact with the antibiotic. The only indication of fungal uptake of griseofulvin is the statement by Boothroyd, Napier, and Somerfield (1961) that 50% of the griseofulvin added to cultures of *B. allii* could be found in the mycelium.

The fungi used in the studies just cited were only moderately sensitive, or even insensitive, to the antibiotic action of griseofulvin, and it seemed desirable to determine whether or not one of the most highly sensitive organisms, such as the dermatophyte *Microsporum gypseum*, would accumulate griseofulvin within its mycelium.

MATERIALS AND METHODS

General procedure. Three series of cultures were prepared by inoculating 10-ml volumes of arginine-glucose-salts (AGS) medium with *M. gypseum* spores as described previously (El-Nakeeb, McLellan, and Lampen, 1965) and shaking at 28 C for 72 hr. A solution of griseofulvin-(4-methoxy-H^3) (specific activity, 13.6 μc/mg) in dimethylsulfoxide was then added aseptically to cultures of series A and B to give a final concentration of 10 μg/ml. Similar volumes of dimethylsulfoxide were incorporated into series C as a control. All flasks were shaken at 28 C. At the specified time intervals after addition of griseofulvin, cultures from each series were centrifuged and the cells were washed twice with 2 to 5 ml of cold distilled water. The supernatant fluid and washings from series A were used for measuring the radioactivity which remained in the medium. The cells of series B and C were utilized for the dry weight estimations (El-Nakeeb et al., 1965). Those of series A were transferred, directly or after different extractions, onto filter membranes (Schleicher & Schuell Co., Keene, N.H.) which were washed with water, dried at 100 C, and measured for radioactivity.

To measure cell-bound radioactivity, the washed mycelia from series A were usually suspended in 2 to 5 ml of distilled water and heated at 100 C for 5 min. After cooling to room temperature, the tubes were centrifuged and the residues washed with 1 to 2 ml of distilled water. The extraction and washing were carried out three times, and the extracts were combined and counted. Total cell-bound radioactivity is considered to be the sum of that detected in the extracts and in the cell residue. Any variations from this procedure are specified.

Estimation of radioactivity. All samples were assayed for radioactivity by the liquid scintillation method with a dioxane scintillation mixture for the aqueous preparations (El-Nakeeb et al., 1965) and a toluene mixture for the dried mycelia and extracts in organic solvents. The toluene scintillator consisted of 0.3% 2,5-diphenyloxazole and 0.02% 1,4-di-2-(5-phenyloxazolyl)benzene in 100% toluene. Unless otherwise stated, the results were corrected for background activity and for quenching (detected by the internal standard method of Davidson and Feigelson, 1957).

RESULTS

Preliminary studies. Cultures of *M. gypseum* incubated with griseofulvin-H^3 gradually accumulated radioactivity in their mycelia. At least 10% of the added antibiotic was taken up in 48 hr. During this period, the total counts (by direct measurement) fell approximately 20%. A part of this decrease was the result of the formation of quenching agents in the culture fluids and could be corrected by the use of internal standards. It was felt that the poor recovery might also result from trapping by the mycelium of β-particles from H^3 disintegrations. This difficulty should be overcome if the radioactivity could be extracted and counted in solution.

TABLE 1. *Extraction of cell-bound griseofulvin*

Time*	Count/min × 10^{-3} for unextracted cells	Extraction		Original activity remaining in cells
		Solvent†	Temp	
hr			*C*	%
6	6.8	Water	100	13
		Alcohol	90	16
		Alcohol + ether (3:1)	90	19
		Chloroform	20	17
24	20.3	Water	100	20
72	41.7	Water	100	31
		Alcohol	90	35
		Chloroform	20	39

* After addition of griseofulvin.

† Extraction was with 5 ml of solvent for 5 min (single extraction).

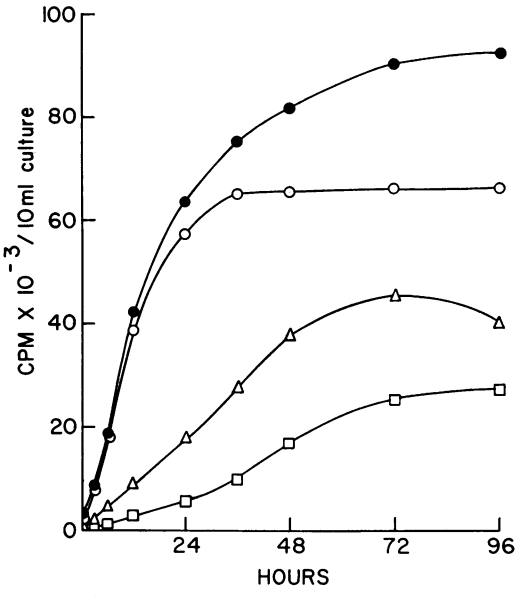

FIG. 1. *Kinetics of uptake of griseofulvin-H^3. Values represent radioactivity taken up by the mycelium from a 10-ml culture:* ○, *water extract of cells;* □, *residue after water extraction;* ●, *water extract + residue;* △, *direct count of cells. The general procedure described in Materials and Methods was used.*

Extraction of griseofulvin-H^3 bound by the cells. The major part of the antibiotic was extracted from the cells by hot water, alcohol, or chloroform (Table 1). Water extraction was selected as the standard procedure, since it was as effective as the other methods and more convenient. A single extraction at 100 C removed between 70 to 85% of the water-extractable activity; the third removed relatively little. Shaking the cells in water at 25 C was less effective. These results form the basis for the routine extraction procedure described in Materials and Methods.

Kinetics of uptake. After the addition of griseofulvin (10 μg/ml) to growing cultures of *M. gypseum*, uptake started quickly and proceeded at a rapid rate for about 24 hr (Fig. 1). During this period, most of the accumulated griseofulvin could be extracted with boiling water. Also, the growth of the organism was completely inhibited. After 24 to 36 hr of incubation, the water-extractable radioactivity in the cells remained almost constant, but there was a continuing increase in forms not removed by water (Table 1). Mycelial growth resumed during the latter period,

accompanied by a reduction in specific activity of the cell material.

A substantial fraction of the radioactivity was not detected when the mycelium was counted directly. With use of the standard extraction procedure, between 92 and 99% of the added radioactivity could be accounted for. Data for total cell-bound radioactivity are probably still minimal values, but appear adequate for present comparative studies.

Effect of griseofulvin concentration. The amount of griseofulvin taken up by the cells was clearly dependent on the level of the antibiotic in the medium (Table 2). Uptake continued until 25 to 33% of the antibiotic had been taken up. The amount of radioactivity in the cells then fell slowly (particularly at 2.5 and 5.0 μg/ml). Destruction of griseofulvin is known to occur during this period (El-Nakeeb, 1963) and complexes with cell products are present both in the cells and in the medium (El-Nakeeb and Lampen, 1964). The resulting decrease in the level of free griseofulvin in the medium may first slow down griseofulvin uptake and eventually initiate release of some of the bound material.

TABLE 2. *Binding of griseofulvin by Microsporum gypseum cultures*

Time*	Griseofulvin	Cell wt	Griseofulvin uptake by cells†			Ratio‡
			Amt/10 ml	Amt/mg of cells	Percentage of added griseofulvin	
hr	*μg/10 ml*	*mg/10 ml*	*μg*	*μg*		
0	100	3	0.7	0.2	0.7	2
6	0	4				
	25	3	3.1	1.0	12.5	46
	50	3	5.4	1.8	10.8	41
	100	3	6.6	2.2	6.6	23
	200	3	8.4	2.8	4.2	15
12	0	6				
	25	4	8.2	2.0	33.0	119
	50	3	12.8	4.3	25.6	116
	100	3	15.3	5.1	15.3	60
	200	3	21.2	7.0	10.6	39
24	0	10				
	25	5	6.2	1.2	25.2	61
	50	3	11.7	3.9	23.4	102
	100	3	22.8	7.6	22.8	99
	200	3	32.4	10.8	16.2	65
48	0	19				
	25	7	5.8	0.8	23.0	42
	50	6	11.3	1.9	22.6	49
	100	4	24.6	6.1	24.6	81
	200	3	46.8	15.6	23.4	102

* After addition of griseofulvin.

† Based on radioactivity in water extract + residue.

‡ Ratio of griseofulvin concentration inside the cell (wet volume) to that in the medium. A value of 10% dry weight was assumed.

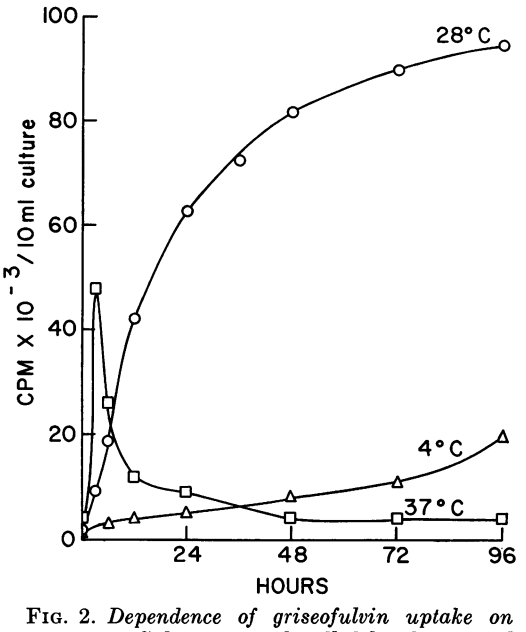

FIG. 2. *Dependence of griseofulvin uptake on temperature. Cultures were handled by the general procedure described in Materials and Methods and incubated at the temperature indicated. At the specified times, the total radioactivity in the cells was determined (water extract + residue).*

The concentration of griseofulvin in the mycelium reached a value up to 100-fold that present in the medium (Table 2).

Effect of temperature. Cultures maintained at 28 C exhibited maximal growth rate and a prolonged antibiotic uptake (Fig. 2). At 37 C, however, there was a rapid uptake of griseofulvin during the first 3 hr; this material was released when autolysis occurred after 6 to 12 hr of incubation. Cultures kept at 4 C showed little, if any, increase in dry weight, but the cells did not disintegrate. These cultures slowly accumulated small amounts of radioactivity (Fig. 2).

Effect of pH. The uptake of griseofulvin (Table 3) proceeded with a maximal rate between *p*H 5.5 and 6.5—a range which also supported the highest rate of growth. A small initial absorption of radioactivity from the medium occurred which was independent of *p*H.

The original *p*H of the AGS medium was 5.4 to 5.6. After 72 hr of growth, the *p*H rose to between 6.2 and 6.6 and increased slightly during further incubation. The *p*H of the AGS medium

TABLE 3. *Effect of pH on the uptake of griseofulvin by Microsporum gypseum*[a]

Time[b]	*p*H[c]							
	4	5	5.5	6.0	6.5	7	8	9
hr								
0	2.2[d]	2.5	2.7	2.4	2.1	2.5	2.8	2.3
6	5.3	6.2	7.2	7.4	7.5	7.5	5.3	3.3
24	20.4	30.0	36.1	38.0	39.2	25.1	2.5	1.9

[a] Groups of 48-hr cultures were centrifuged and washed once with sterile distilled water at 4 C. The washed cells were resuspended in sterile AGS medium buffered to the required *p*H by a mixture of K_2HPO_4-KH_2PO_4 of varying proportions; the final concentration of these salts was 1% (w/v) of the medium. The antibiotic was then added, and the rest of the experiment was carried out as described under General Procedure in Materials and Methods. Values represent total cellular radioactivity (water extract + residue).

[b] After addition of griseofulvin.

[c] The changes in *p*H during the uptake period did not exceed ±0.15.

[d] Uptake expressed as counts per minute × 10^{-3} per 10 ml of culture.

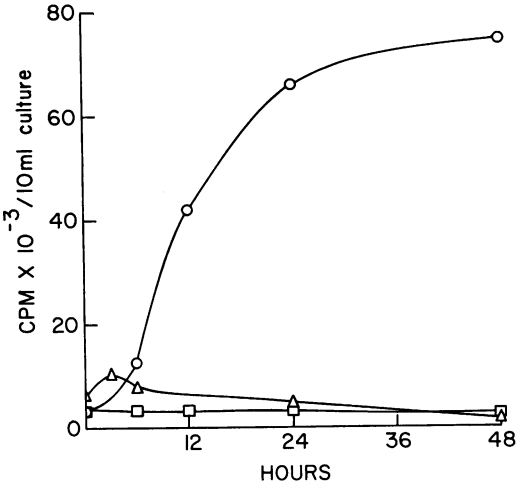

FIG. 3. *Griseofulvin uptake under anaerobiosis and by autoclaved cells. Values represent total radioactivity (water extract + residue) taken up by 10-ml cultures: ○, viable cells incubated under air; △, viable cells incubated under nitrogen gas; □, cells autoclaved at 15 psi for 20 min and subsequently incubated aerobically.*

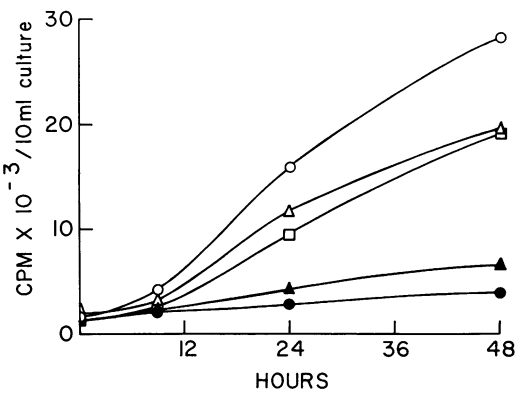

FIG. 4. *Role of nutrients in the uptake of griseofulvin-H³. Three series of 44-hr cultures (similar to those of the general procedure described in Materials and Methods) were centrifuged aseptically at 4 C. The cells were washed twice with ice-cold sterile water and resuspended in fresh AGS complete medium (○); or in medium lacking: △, glucose; □, arginine; ●, glucose and arginine; ▲, glucose and arginine, but supplemented with 0.2% ammonium sulfate. Griseofulvin was then added and the uptake measured as usual. The initial pH of the fresh media was adjusted to 6.5 with K_2HPO_4 (1%, final concentration). The maximal change in the pH of all media did not exceed 0.20 during the entire experiment.*

is, therefore, suitable for both the cultivation of *M. gypseum* and the uptake of griseofulvin.

Effect of autoclaving and of anaerobiosis. Heat-killed cells did not accumulate an appreciable amount of radioactivity (Fig. 3). Viable cultures under anaerobic conditions took up griseofulvin at a rate comparable to that of the aerobic cultures for 3 hr. After that period, the cells started to lyse. Uptake ceased and most of the bound material was eventually released.

Requirement for exogenous energy source. In the absence of both glucose and arginine, there was negligible antibiotic binding (Fig. 4). Addition of ammonium sulfate increased the uptake only slightly. Since ammonium sulfate can serve as an alternative nitrogen source for growth of *M. gypseum*, the reduced uptake in the absence of glucose and arginine is not the result of nitrogen deficiency. If either glucose or arginine alone was omitted, griseofulvin uptake continued at 55 to 70% of the normal rate. Thus, a source of energy is required; this can be supplied either as glucose or arginine, both of which can be utilized as carbon and energy sources for growth.

Effect of inhibitors. Addition of sodium azide (10^{-2} M), 2,4-dinitrophenol (10^{-3} M), or *p*-fluorophenylalanine (10 μg/ml) to *M. gypseum* cultures completely inhibited griseofulvin uptake for 24 hr

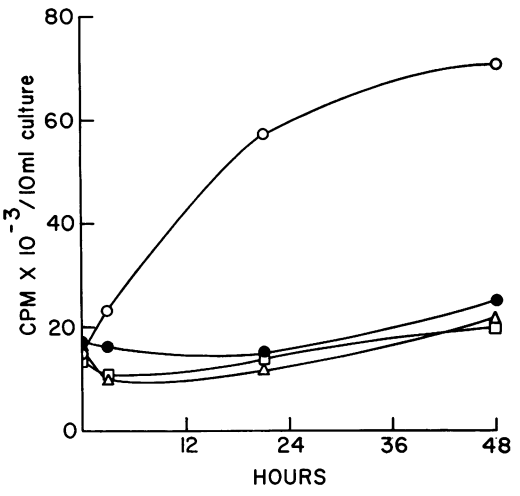

FIG. 5. *Effect of metabolic inhibitors on griseofulvin uptake. The procedure was the same as for Fig. 4, except that the washed cells were resuspended in complete AGS medium buffered by 1% K_2HPO_4 to pH 6.5 and containing:* ○, *no inhibitor;* △, *sodium azide (10^{-2} M);* □, *2,4-dinitrophenol (10^{-3} M);* ●, DL-*p-fluorophenylalanine (10 μg/ml).*

(Fig. 5). A small amount of radioactivity was later taken up in the presence of any of the three compounds. The zero-time "binding" was not affected.

The growth of the dermatophyte (as determined by dry weight) ceased completely for at least 48 hr under the influence of these inhibitors.

DISCUSSION

In the present studies, the uptake of radioactive griseofulvin by *M. gypseum* has been demonstrated by measurement of the total radioactivity of the cell mass. It should be emphasized that almost all of the radioactivity accumulated in the mycelium of *M. gypseum* is present as the chemically unaltered antibiotic complexed with various cell constituents (El-Nakeeb, 1963). Thus, cellular radioactivity is considered to be a valid indication of antibiotic level.

Crowdy et al. (1956) found that the uptake of griseofulvin by plants involved two processes. The first was probably a passive movement not inhibited by the respiratory inhibitors, 2,4-dinitrophenol and sodium azide. The second process was more complicated. There was an initial rapid uptake which was dependent on metabolic energy, followed by a prolonged accumulation that was not affected by the metabolic inhibitors. The uptake of griseofulvin by *M. gypseum* can also be divided into two phases. The first is the immediate removal of small amounts of the antibiotic from the medium under all conditions tested. This initial uptake was independent of *p*H, temperature, nutrients, metabolic energy, and viability (or sensitivity of an organism to griseofulvin; El-Nakeeb, 1963), and may represent a simple absorption of the antibiotic by the lipids of the dermatophyte. This postulate is consistent with the greater affinity of griseofulvin for lipids than for aqueous media (Freedman, Baxter, and Walker, 1962) and the high lipid content of dermatophytes (Wirth, Beesley, and Miller, 1961; Blank, Shortland, and Just, 1962). At least the major portion of the material that is immediately removed appears to be true griseofulvin-H^3 and not a trace impurity in the radioactive preparation. For example, when *Saccharomyces cerevisiae* cells (which give only this nonspecific binding) were added repeatedly to a sample of medium containing griseofulvin-H^3, radioactivity continued to be taken up with each separate addition. From the present data, one cannot state whether or not this immediate binding is a prerequisite for the continued uptake of griseofulvin.

The second phase was a prolonged uptake which was dependent on *p*H, temperature, and antibiotic concentration. It required an external source of energy and was inhibited by 2,4-dinitrophenol or sodium azide. Active growth of the culture was not essential, however. The need for a continuing source of metabolic energy for the uptake of griseofulvin is not unique; it has been reported, for instance, for the absorption of nystatin (Lampen et al., 1959), streptomycin (Hancock, 1962*b*), chloramphenicol (Vasquez, 1963), and oxytetracycline (Arima and Izaki, 1963).

Autoclaving the cultures prior to the addition of griseofulvin completely prevented any appreciable intracellular accumulation of the antibiotic. This is in contrast to the binding of streptomycin (Hancock, 1962*b*) or nystatin (Lampen et al., 1959), which is enhanced when boiled cells are used. Incubation of *M. gypseum* under conditions unfavorable for active metabolism (under anaerobiosis, at high *p*H, or at too high a temperature) greatly retarded griseofulvin uptake. The uptake of streptomycin (Hancock, 1962*a*, *b*) and oxytetracycline (Arima and Izaki, 1963) was also severely affected by these conditions.

The complete inhibition by *p*-fluorophenylalanine of the second phase of griseofulvin uptake indicates that this process probably involves *de novo* protein synthesis (possibly of a transport system), since this amino acid analogue is known

to inhibit the production of active enzymes (Halvorson and Spiegelman, 1952; Cohen and Munier, 1959).

The mycelium of *M. gypseum* concentrated griseofulvin to a level up to 100 times greater than that in the external medium (Table 2). It should be emphasized, however, that the major portion of the intracellular antibiotic is in the form of relatively stable complexes (El-Nakeeb, 1963) and the intracellular concentration of free griseofulvin may well be extremely low. It is possible that no transport of free griseofulvin against a concentration gradient has actually taken place. In no case did *M. gypseum* take up all the griseofulvin added to the medium. The incomplete uptake might be due, at least in part, to a complicated equilibrium between the antibiotic and its complexes inside and outside the mycelium.

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