Uptake of ^3H -Griseofulvin by Micro-organisms and its Correlation with Sensitivity to Griseofulvin

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SUMMARY

A correlation was observed between the sensitivity of different micro-organisms to griseofulvin and their ability to take up the antibiotic, especially into their nucleic acid and protein fractions. The insensitive yeasts *Candida albicans* and *Saccharomyces cerevisiae* and the bacterium *Escherichia coli* did not bind an appreciable amount of [4-methoxy- ^3H] griseofulvin. The poorly sensitive filamentous fungi *Aspergillus niger* and *Neurospora crassa* accumulated a considerable quantity of antibiotic, mostly in the water-soluble pool. This was in contrast to the highly sensitive dermatophytes *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. persicolor* in which the nucleic acid and protein fraction contained about half of the total bound griseofulvin (as relatively stable complexes). It is proposed that uptake of griseofulvin is essential for antibiotic action and that the degree of sensitivity shown by an organism is dependent upon the tendency of its macromolecules to complex with the accumulated griseofulvin. Griseofulvin was not degraded by the insensitive or the poorly sensitive organisms tested. Metabolic products of griseofulvin were detected, however, in culture fluids of the sensitive dermatophytes.

INTRODUCTION

Investigations of the response of different micro-organisms to griseofulvin (Brian, 1949; Roth, Sallman & Blank, 1959; El-Nakeeb, McLellan & Lampen, 1965) have singled out the dermatophytes as the most sensitive group. Other filamentous fungi varied in their sensitivity from moderate (plant pathogens) to poor (saprophytes). Yeasts and bacteria were insensitive. The special sensitivity of the dermatophytes stimulated investigations on the uptake of ^3H -griseofulvin by cultures of *Microsporum gypseum* (El-Nakeeb, 1963; El-Nakeeb & Lampen, 1965*a, b*). The principal observations may be summarized as follows. (1) The uptake process involved two phases: the first was rapid and independent of viability of the organisms; the second phase was prolonged and controlled by factors which govern active metabolism (e.g. pH value, temperature, metabolic energy supply) and protein synthesis (probably of a transport system) was required. (2) The fungus concentrated griseofulvin intracellularly to a value about 100-fold that of the medium. (3) The radioactivity which remained in the medium was present either as griseofulvin complexed with cell materials or in degradation products of the griseofulvin. (4) The intracellular ^3H -griseofulvin was bound to fungal constituents and could be extracted (mostly as complexes) with boiling water, hot trichloroacetic acid and hot NaOH. There was essentially no ^3H -label in the nucleic acids and proteins themselves.

Attempts to show uptake of griseofulvin by *Botrytis allii*, *Phycomyces blakeseeanus*, or by *Mucor ramannianus* have been unsuccessful (Abbott & Grove, 1959); but it should be noted that the microspectrophotometric method then used was not sufficiently sensitive to detect small amounts of griseofulvin. Also, these fungi were either poorly sensitive (*P. blakeseeanus*, *M. ramannianus*) or only moderately sensitive (*B. allii*) to griseofulvin. Boothroyd, Napier & Somerfield (1961) stated that about 50 % of the ^{36}Cl -griseofulvin added to *B. allii* cultures could be accounted for in the mycelium. The present study was designed to seek any relationship between the degree of sensitivity of a micro-organism to griseofulvin and the organism's uptake and metabolism of this antibiotic.

METHODS

Organisms and media. The following organisms were used: *Escherichia coli* B, *Candida albicans* 204, *Saccharomyces cerevisiae* LK 2 G 12, *Aspergillus niger* c 116, *Neurospora crassa* A-17, *Trichophyton mentagrophytes* x 8 (two strains: one partially resistant and the corresponding parent sensitive strain), *Trichophyton persicolor* c 654. The sources of the strains, the media and the culture conditions were described earlier (El-Nakeeb *et al.* 1965).

Uptake of griseofulvin. For the filamentous fungi, triplicate series of young rapidly growing cultures (shaken to produce dispersed growth) were prepared. These cultures were generally 48–72 hr old and contained about the equiv. of 3 mg. dry wt. mycelium/10 ml. For each experiment with *Escherichia coli*, *Candida albicans* or *Saccharomyces cerevisiae*, a log phase culture was divided aseptically into three equal volumes. The three series were handled according to the general procedure previously described by (El-Nakeeb & Lampen (1965*a*): microvolumes of a ^3H -griseofulvin solution in dimethylsulphoxide were added to the cultures of series A and B; equal volumes of dimethylsulphoxide were put into series C as a control, and all cultures then shaken at 30°. At specified times samples were withdrawn aseptically (or individual cultures were used) for measurement of griseofulvin uptake (series A) or for determination of mycelial weight (series B, C).

Isolation of intracellular radioactive compounds. Organisms were washed twice with ice-cold distilled water and then successively extracted with boiling water (water-soluble pool) cold trichloroacetic acid, hot trichloroacetic acid (nucleic acid fraction) and finally with hot NaOH (protein fraction) under the conditions adopted for *Microsporum gypsum* (El-Nakeeb *et al.* 1965). Chromatography of the extracts was done by the procedure of El-Nakeeb & Lampen, (1965*b*). Radioactivity was estimated by the liquid scintillation method (El-Nakeeb & Lampen, 1965*a*). All values have been corrected for quenching.

RESULTS

Lack of uptake by insensitive organisms

Escherichia coli, *Candida albicans* and *Saccharomyces cerevisiae* did not take up ^3H -griseofulvin to an appreciable extent (Table 1). The small amounts of radioactivity removed by the organisms from the medium (0.4–0.8 % of the radioactivity added) might have been due to non-specific adsorption, since most of the uptake occurred

almost instantaneously at 4° (0 hr uptake, Table 1). Comparable amounts of ^3H -griseofulvin were adsorbed by autoclaved cultures of *Microsporum gypseum* (El-Nakeeb & Lampen, 1965*a*). The low uptake of ^3H -griseofulvin might result from restricted permeability to the antibiotic or from its rapid degradation by the organisms. The latter possibility can be excluded (at least for *E. coli* and *S. cerevisiae*) since at the end of the growth period (Table 1) about 99% of the added radioactivity was still present as chloroform-extractable undegraded griseofulvin (determined by chromatographic behaviour). It should be emphasized that the griseofulvin added did not inhibit the growth of these organisms (dry wt. increase) or produce detectable morphological changes.

Table 1. ^3H -griseofulvin binding by insensitive organisms

Time (hr)*	Counts/min. $\times 10^{-3}$ /10 ml. culture			Total uptake in mycelium	
	Filtrate	Organism		Added ^3H -griseofulvin %	$\mu\text{g.}/\text{equiv.}$ mg. dry wt.
		Water extract	Residue		
<i>Escherichia coli</i>					
0	975†	6.0	0.5	0.7	0.44
50	962	6.6	1.0	0.8	0.08
<i>Candida albicans</i>					
0	1160	4.4	0.5	0.4	0.08
24	1147	5.2	0.9	0.5	0.03
<i>Saccharomyces cerevisiae</i>					
0	1220	4.7	0.1	0.4	0.04
48	1205	6.3	0.2	0.5	0.02

* The 0 hr sample was taken immediately after addition of 10 $\mu\text{g.}$ ^3H -griseofulvin/ml. to a growing culture (see Methods).

† 0 hr value is counts/min. $\times 10^{-3}$ added to culture.

Uptake of ^3H -griseofulvin by poorly sensitive fungi

The filamentous fungi *Aspergillus niger* and *Neurospora crassa* gradually took up considerable quantities of ^3H -griseofulvin, most of which was extractable by hot water (Table 2); the fraction not extracted was relatively small, but it increased gradually with time of incubation. *A. niger* accumulated more radioactivity per unit weight of mycelium and at a faster rate than did *N. crassa*. The significance of this difference is not clear, since griseofulvin 10 $\mu\text{g.}/\text{ml.}$ had only a slight effect (10–20% inhibition) on the growth (dry wt. increase) of either organism.

Distribution of ^3H -griseofulvin taken up by *Aspergillus niger* and *Neurospora crassa*

Mycelium from 6 and 48 hr cultures was extracted with boiling water, cold trichloroacetic acid, hot trichloroacetic acid, and hot NaOH, and the radioactivity in the extracts was estimated (Table 3). The water-soluble fraction contained 86–94% of the total radioactivity. The rest of the radioactivity was mainly in the protein fraction (hot NaOH extract), with small amounts in the hot or the cold trichloroacetic acid extracts. The distribution of intracellular ^3H -griseofulvin

was very similar in the two fungi. It was, however, completely different from the proportions in *Microsporum gypseum*, in which there was a ratio of about 2:1:1 for the griseofulvin in the fractions water-soluble pool: nucleic acid fraction: protein fraction, respectively (El-Nakeeb & Lampen, 1965*b*).

Table 2. *Uptake of griseofulvin by Aspergillus niger and Neurospora crassa*

Time (hr)*	Counts/min. $\times 10^{-3}$ /10 ml. culture			Total uptake	
	Filtrate	Mycelium		% of added ^3H -griseofulvin	^3H -griseofulvin $\mu\text{g.}/\text{ml.}$ dry wt. mycelium
		Water extract	Residue		
<i>Aspergillus niger</i>					
0	1810†	37	1	2.9	0.59
3	1076	215	6	16.9	1.69
6	1023	252	8	19.8	1.24
24	1006	278	8	21.8	0.68
48	915	345	16	27.5	0.46
<i>Neurospora crassa</i>					
0	1773	28	1	1.6	0.23
3	1701	51	2	3.0	0.14
6	1647	112	6	6.6	0.19
24	1511	208	6	12.1	0.16
48	1401	292	11	17.1	0.16

* After addition of ^3H -griseofulvin 10 $\mu\text{g.}/\text{ml.}$ (see Table 1).

† 0 hr value is counts/min. $\times 10^{-3}$ added to culture.

Table 3. *Conditions required for extraction of ^3H -griseofulvin taken up by Aspergillus niger and Neurospora crassa*

^3H -griseofulvin was added at 0 hr to cultures which were treated in parallel with those used for the experiment of Table 2. After incubation for 6 and 48 hr, triplicate cultures were pooled and the mycelium extracted as described in Methods.

Micro-organism	Time (hr)	Total extracted (counts/ min. $\times 10^{-3}$)	% of total radioactivity in extracts				
			Hot water	Cold TCA*	Hot TCA*	Hot NaOH	Residue
<i>Aspergillus niger</i>	6	274	93.5	2.1	1.8	2.2	0.3
	48	403	86.8	1.8	1.8	9.3	0.2
<i>Neurospora crassa</i>	6	136	90.3	1.5	1.5	5.9	0.8
	48	342	86.4	1.3	2.4	9.5	0.3

* TCA, Trichloroacetic acid.

Culture fluids. The radioactive material remaining in the culture fluids of *Aspergillus niger* and *Neurospora crassa* appeared to be unaltered griseofulvin, it was extracted by chloroform, and on paper chromatography had an R_F value identical with that of authentic griseofulvin.

Hot water extract. With *Aspergillus niger* and *Neurospora crassa* about 90–95% of the radioactivity was present as free griseofulvin. The remaining 5–10% stayed at the origin of the chromatogram. However, after the material had been extracted

from the paper, incubated with an excess of unlabelled grieseofulvin and re-chromatographed, the radioactivity now moved with the added grieseofulvin. The hot water extract fraction probably represented grieseofulvin which was complexed reversibly with fungal constituents in the water-soluble pool.

Nucleic acid fraction. The amount of radioactivity was very small (Table 3), and no attempt was made to determine its nature.

Fig. 1. Complexes of grieseofulvin in the protein fraction from *Neurospora crassa*. (a) One ml. of the 48 hr NaOH extract (experiment of Table 3) was added to a column of Sephadex G-25 (39 × 0.7 cm.; exclusion volume 8.0 ml.) and eluted with water. One ml. fractions were collected and radioactivity (●—●) and extinction (○—○) determined. (b) The fractions of peak II (from a) were pooled and 1 ml. of this material added to a column of Dowex-1-chloride (0.4 × 3.0 cm.). The column was washed with water until radioactivity was no longer removed. N-HCl was then passed through the column. The effluent was neutralized and radioactivity and extinction determined. The recovery of radioactivity in the water wash was 101% of that added to the column.

Protein fraction. The ^3H -label in the hot NaOH extracts from *Aspergillus niger* and *Neurospora crassa* was bound to the fungal proteins. An extract prepared 48 hr after the addition of ^3H -grieseofulvin to a culture of *N. crassa* was subjected to gel filtration on a column of Sephadex G-25 (Fig. 1a). The profile of the eluted radioactivity closely followed that of the peptide peak (tubes 15–20) with very little material at the position of free grieseofulvin (tubes 27–32). Complete separation of the radioactivity from 280 mμ-absorbing material was achieved on a column of Dowex-1 chloride (Fig. 1b). Therefore, the ^3H -label had not been incorporated into the amino acid residues and probably was present as complexed grieseofulvin.

Uptake of grieseofulvin by dermatophytes

As seen previously with *Microsporum gypseum* (El-Nakeeb & Lampen, 1965a), there was a substantial uptake of ^3H -grieseofulvin by the Trichophyton strains, including a partially-resistant culture of *T. mentagrophytes* × 8 (Table 4). The amount of radioactivity concentrated/mg. dry wt. mycelium by *T. mentagrophytes* was much less than with *T. persicolor* (Table 4) or with *M. gypseum*. Even though relatively large amounts of mycelium were present, growth of the sensitive strains of Trichophyton was completely inhibited for at least 24 hr by grieseofulvin 10 μg./ml.

The partially resistant strain of *T. mentagrophytes* showed only a 50 % decrease in dry weight gain of mycelium with this concentration of griseofulvin.

Cellular distribution of griseofulvin in dermatophytes. The three *Trichophyton* cultures (Table 5) accumulated griseofulvin into their nucleic acid and protein

Table 4. *Uptake of ³H-griseofulvin by cultures of Trichophyton persicolor and Trichophyton mentagrophytes* × 8

Time (hr)*	Counts/min. × 10 ⁻³ /10 ml. culture		Total uptake	
	Filtrate	Mycelium	% of added ³ H-griseofulvin	³ H-griseofulvin μg./mg. dry wt. mycelium
<i>Trichophyton persicolor</i>				
0	165 (172)†	7.9	4.7	0.57
6	148	20.6	12.2	4.1
24	131	26.9	15.8	5.3
72	116	35.8	21.1	2.6
<i>Sensitive T. mentagrophytes</i>				
0	162 (178)†	8.5	4.9	0.26
6	138	34.1	19.8	1.04
24	140	38.0	22.0	1.00
120	110	51.6	30.0	0.80
<i>Partially resistant T. mentagrophytes</i>				
0	1523 (1537)†	12	0.8	0.04
6	1369	162	10.5	0.55
24	1228	293	19.2	0.52
72	1012	508	33.1	0.89

* After addition of ³H-griseofulvin 10 μg./ml. (see Table 1).

† Value in parentheses is counts/min. × 10⁻³ added to culture. Note difference in specific activity of added ³H-griseofulvin.

Table 5. *Distribution of ³H-griseofulvin in mycelium of Trichophyton cultures**

Time (hr)†	% of total uptake present in				
	Hot H ₂ O extract	Cold TCA extract	Hot TCA extract	Hot NaOH extract	Cell residue
<i>Trichophyton persicolor</i>					
6	67	1	1	30	1
24	64	1	7	27	2
72	54	1	18	25	2
<i>Sensitive T. mentagrophytes</i>					
6	53	5	11	28	3
24	49	3	15	31	3
120	41	4	9	42	5
<i>Partially resistant T. mentagrophytes</i>					
6	75	3	6	15	1
24	69	2	7	21	1
72	71	2	7	18	2

* Samples from experiment of Table 4. TCA, Trichloroacetic acid.

† After the addition of ³H-griseofulvin 10 μg./ml.

fractions to a much greater extent than did *Aspergillus niger* and *Neurospora crassa* (Table 3). The distribution of the intracellular radioactivity differed, however, among the several dermatophytes tested. The nucleic acid and protein components of the sensitive culture of *T. mentagrophytes* had more associated activity than did the corresponding constituents of the partially resistant culture (Table 5). This might account in part for the difference in sensitivity of the two cultures to griseofulvin. It may also be significant that each of the Trichophyton cultures bound more radioactivity in the protein fraction than in the nucleic acids (Table 5); *Microsporum gypseum* took up large and equal amounts of activity into the two fractions (El-Nakeeb & Lampen, 1965*b*). Only minor amounts of radioactivity were detected in the cold trichloroacetic acid extracts and in the final cell-wall residues of any of the dermatophytes.

Metabolism of ^3H -griseofulvin by the Trichophyton cultures. About 80% of the radioactivity which remained in the culture fluid of *Trichophyton mentagrophytes* and *T. persicolor* was in the form of ^3H -water or other distillable products. The remaining 20% was extractable with chloroform and was examined by paper chromatography. Three components were found: (1) ^3H -griseofulvin (R_F 0.84); (2) a radioactive spot (R_F 0.69); (3) a non-radioactive fluorescent spot (R_F 0.04). In the 6 hr culture about half of this material was free griseofulvin, but after growth for 48 hr essentially all of the radioactivity was in spot (2). It is not certain whether component (2) was a metabolite of griseofulvin or consisted of undegraded griseofulvin bound to cellular products.

The ^3H - in the hot-water extracts of the Trichophyton strains (Table 5) appeared to be present as chemically unaltered griseofulvin. At 6 hr, most of the griseofulvin was free (chromatographically) but complexes were present in subsequent extracts. The hot trichloroacetic acid and hot NaOH extracts contained radioactivity firmly bound to the macromolecular constituents. This was inferred from the results of paper chromatography and gel filtration on Sephadex G-25. These data parallel those presented for the corresponding fractions from *Microsporum gypseum* (El-Nakeeb & Lampen, 1965*b*).

DISCUSSION

From the findings reported here two factors appear to be of importance in determining the sensitivity of an organism to griseofulvin: the ability to take up griseofulvin from the medium and the tendency of the cellular nucleic acid and protein to complex with griseofulvin once it has been taken into the cell. For example, the insensitive organisms *Candida albicans*, *Saccharomyces cerevisiae*, *Escherichia coli* did not accumulate ^3H -griseofulvin (after the initial non-specific adsorption) and did not inactivate it. These organisms may be unable to form a transport system for griseofulvin like that found in *Microsporum gypseum* (El-Nakeeb & Lampen 1965*a*). The second factor is illustrated by the poorly sensitive *Aspergillus niger* and *Neurospora crassa*. These two organisms took up quantities of griseofulvin comparable to those bound by the highly sensitive dermatophytes, but the distribution of accumulated griseofulvin differed greatly between the two groups. *M. gypseum* (El-Nakeeb & Lampen, 1965*b*) and the Trichophyton cultures studied here concentrated about half of the griseofulvin taken up in their protein and nucleic acid fractions. In *A. niger* and *N. crassa*, the corresponding fractions contained not

more than a tenth of the bound griseofulvin and the bulk of the material was extractable with the water-soluble pool. This correlation between high sensitivity to griseofulvin and the concentration of griseofulvin into the nucleic acid and protein fractions is strengthened by the observations on the binding of griseofulvin by RNA from different organisms (El-Nakeeb & Lampen, 1964). Thus, RNA from the sensitive dermatophyte *M. gypseum* formed strong and stable complexes with griseofulvin. Similar preparations from *A. niger* or from yeast had only weak affinity for griseofulvin and any complexes formed were unstable. Although complexing of griseofulvin with nucleic acid and with protein fractions occurred, sensitivity to griseofulvin appears to be more directly correlated with the formation of nucleic acid + griseofulvin complexes. Thus, *A. niger* and *N. crassa* eventually had a moderate amount of griseofulvin complexed with their protein fractions but not with nucleic acid and these organisms are relatively insensitive. Also, the partially-resistant culture of *Trichophyton mentagrophytes* x 8 complexed griseofulvin with its nucleic acid fraction to a lesser extent than did the griseofulvin-sensitive parent strain. The correlation between sensitivity to griseofulvin and its uptake is comparable to that previously reported for penicillin (Eagle, 1954; Rowley, Cooper, Roberts & Smith, 1950) and for streptomycin (Szybalski & Mashima, 1959; Anand, Davis & Armitage, 1960; Hancock, 1962).

Microbial degradation of griseofulvin has been demonstrated only with sensitive organisms; its demethylation was observed by Boothroyd, *et al.* (1961) with the very sensitive *Microsporum canis* and the moderately sensitive *Botrytis allii* and *Cercospora melonis*. We report here degradation by *Microsporum gypseum* and the two *Trichophyton* species, but not by poorly sensitive *Aspergillus niger* and *Neurospora crassa* cultures. Uptake of griseofulvin (which correlates with sensitivity) may be essential if degradation is to take place at a significant rate.

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