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METABOLISM OF GRISEOFULVIN-^{14}C; STUDIES *IN VITRO**

S. SYMCHOWICZ and K. K. WONG

Department of Physiology and Biochemistry, Biological Research Division, Schering Corp., Bloomfield, N.J., U.S.A.

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Abstract—The metabolism of griseofulvin was studied *in vitro* in selected rat tissues. ^{14}C-Labeled drug was biosynthetically prepared from 1-^{14}C-acetate precursor. Labeled griseofulvin was incorporated into liver slices from the incubation medium and extensively metabolized by this tissue. At least two metabolites, 6- and 4-demethylgriseofulvin, were identified by paper chromatography in the incubation medium as well as in liver slices. Both metabolites were present in free and conjugated forms, but conjugation of the 4-demethyl derivative was more extensive. There was no noticeable metabolism of griseofulvin by the heart, kidney, lung, and skin slices, although the uptake of the drug by these organs from the incubation medium was quite high.

GRISEOFULVIN,† a systemically active antifungal agent, produced by certain strains of *Penicillium*, is widely used in medical and veterinary practice. To date only a few studies of the metabolism of this agent have been reported and, because of its widespread use, further studies should help to elucidate its fate and, possibly, its mode of action. Barnes and Boothroyd[1] have observed only a single metabolite of this drug, 6-demethylgriseofulvin, in rat liver slices. Bedford *et al.*[2] reported disappearance of griseofulvin from incubation medium in the presence of liver slices but not in the presence of slices from other tissues; however, no attempt was made by the authors to study the metabolism of this drug.

^{14}C-Labeled griseofulvin was prepared biosynthetically for the purpose of this investigation, and its metabolism was studied *in vitro*. Studies in rat with this drug are the subject of the following communication.[3]

MATERIALS AND METHODS

Biosynthesis of ^{14}C-*Labeled griseofulvin.* Griseofulvin was produced, in a culture, by a mutant strain of *Penicillium patulum*, following the procedure of Rhodes *et al.*[4] with minor changes. The carbohydrate lactose was replaced in our medium with glucose. In addition, 1-^{14}C-labeled sodium acetate[5] and/or 1-^{14}C-labeled D-glucose (New England Nuclear Corp.) were used as precursors for biosynthesis of the radioactive griseofulvin.

The labeled antibiotic was isolated from the mycelia and extensively purified by a modification of the method of Rhodes *et al.*,[6] which will be described briefly. The

* A preliminary report was presented at the 49th meeting (1965) of the Federation of American Societies for Experimental Biology.

† Griseofulvin (7-chloro-4,6,2′-trimethoxy-6′-methylgris-2′-en-3,4′-dione) is formulated and distributed by Schering Corp. under the trade name of Fulvicin, U.F.

mycelia were extracted for 2 hr with acetone (4 ml/g mycelia) in the presence of calcium hydroxide (0·12 g/g mycelia). The acetone extract was separated by centrifugation and its pH adjusted to neutral with 0·1 N hydrochloric acid. The formed calcium chloride was removed by filtration, and the acetone extract was diluted with water (15 per cent of the volume of acetone) followed by the removal of the organic solvent under a stream of nitrogen. This resulted in the precipitation of griseofulvin from the aqueous phase. The precipitate was collected by filtration and alternately washed with cold methanol and petroleum ether until constant specific activity was achieved.

The purity of the final preparation of griseofulvin was verified by melting point and u.v. spectrum analysis. In addition, the radioactive purity of griseofulvin was determined by ascending paper chromatography in two solvent systems. The biological activity was determined by measuring on agar plates the zone of inhibition against *Trichophyton mentagrophytes*. The pure preparation of ^{14}C-labeled griseofulvin, used throughout this investigation, had a specific activity of 95·8 μc/m-mole.

Experimental procedure with tissue slices. Male rats (Charles River C.D. strain), weighing 150–200 g, were sacrificed by cervical dislocation. Selected organs were removed immediately and placed in ice-cold Krebs-Henseleit medium. Tissue slices were prepared with a Stadie-Riggs tissue slicer. Three to four slices, totaling 0·5 g, were blotted and placed in a small beaker containing 3 ml Krebs-Henseleit medium and 60 μg ^{14}C-labeled griseofulvin, which was previously dissolved in alcohol and diluted with the incubation medium to form a 1% ethanolic solution.

The mixture was incubated in a Dubnoff metabolic shaker at 37° for 2 hr under 95% oxygen and 5% carbon dioxide. At the end of the incubation period, the medium was promptly removed from the beaker and the tissue slices washed with cold fresh Krebs-Henseleit solution, which was then added to the incubation medium for analysis. The washed tissue slices were homogenized (all-glass homogenizer) in 3 ml cold Krebs-Henseleit solution. Aliquots of tissue homogenate or the incubation media were adjusted to pH 1 with 1 N HCl and extracted twice with 10 ml ether. Under these conditions more than 95% of griseofulvin or the 4- and 6-demethyl derivatives were extracted into the organic phase.

In order to determine conjugates of griseofulvin and its metabolites, aliquots of a separate set of incubated samples were adjusted to pH 5 with 0·2 M acetate buffer and incubated with 0·05 ml Glusulase (obtained from Endo Laboratories; each ml contains 100,000 units of glucuronidase and 50,000 units of sulfatase) for 60 min at 37°. These conditions resulted in a maximal degree of hydrolysis, as determined in preliminary experiments. After the incubation, the pH was lowered to 1 with 1 N HCl, and the samples were extracted with ether as described earlier. The conjugated material was calculated by the difference in the content of radioactivity found in the ether extracts of samples with and without prior hydrolysis with Glusulase. The total recovery of radioactivity was determined by measuring the activity remaining in the aqueous phase after ether extractions. All samples were counted in a Packard liquid scintillation spectrometer, with 10 ml scintillation medium (consisting of 4 g PPO and 100 mg dimethyl POPOP per l. toluene) and 5 ml absolute alcohol. Corrections for quenching were made with an internal standard whenever necessary.

In order to characterize the metabolites of griseofulvin, aliquots of ether extracts were spotted on Whatman no. 1 filter paper. Authentic griseofulvin and its 6- and

4-demethyl derivatives were spotted on each paper strip for identification purposes. Ascending chromatography was carried out in the system benzene:cyclohexane: methanol:water (5:5:6:4). The organic and aqueous phases were separated after equilibration overnight in a separatory funnel, and the organic portion to which 0·5% glacial acetic acid was added served as the mobile phase; the aqueous portion served as the stationary phase. The chromatograms were developed in a constant-temperature room (25°) for 7 hr. The positions of griseofulvin and its metabolites were located by u.v. light, and the radioactivity pattern was obtained by scanning the strips with a Vanguard 4π automatic strip counter. Hewlett Packard integrator (model 5202L), attached to the strip counter, was used for quantitative evaluation of the radioactive peaks.

On occasion, another solvent system, *n*-butanol:ammonia (20:1) as the mobile phase and chloroform as the stationary phase, was used for additional chromatographic identification of griseofulvin and its metabolites.

RESULTS AND DISCUSSION

Biosynthesis of ^{14}C-*labeled griseofulvin*

Preliminary experiments showed that with the modified procedure of Rhodes *et al.*[4], highest utilization of the ^{14}C-label for biosynthesis of the griseofulvin was achieved when the radioactive carbon source was introduced into the broth 72 hr after inoculation with *P. patulum*. Sodium acetate-1-^{14}C and glucose-1-^{14}C were about equally effective as precursors for the biosynthesis of labeled griseofulvin (Table 1). Specific

TABLE 1. UTILIZATION OF ^{14}C-PRECURSORS FOR BIOSYNTHESIS OF GRISEOFULVIN

	Glucose-1-^{14}C	Sodium acetate-1-^{14}C	
Radioactivity added (μc)	42·5 (1·5 mg)	43·0 (1·8 mg)	1557 (2·5 mg)
Specific activity (μc/m-mole) griseofulvin	2·17	2·78	95·82
Utilized for biosynthesis (% of amount added)	1·38	1·76	1·66

activity of the formed griseofulvin was proportional to the amount of radioactivity added. The studies also revealed that concentrations of sodium acetate up to 8 mg/ml broth did not affect the growth of *P. patulum*. Higher concentrations of acetate (60 mg/ml), however, completely inhibited the biosynthesis of this antifungal agent.

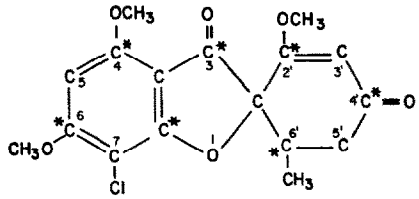
The physical properties of the purified radioactive griseofulvin were compared with standard preparations of griseofulvin, and the results (Table 2) verify the purity of the labeled drug. The position of the radioactive carbon in the griseofulvin molecule (Fig. 1) is assumed to be derived from the head-to-tail linkage of seven acetate (1-^{14}C) molecules, as suggested by Birch and Donovan.[7]

Studies with tissue slices

Preliminary experiments have shown that recovery of griseofulvin-^{14}C (without tissue), subjected to the described experimental procedure, was 94·3 per cent, and the recovery of the ^{14}C-label added to boiled liver tissue was 90·8 per cent. The results in Table 3 show, therefore, that in all samples studied except liver the ^{14}C-label was

TABLE 2. PURITY OF GRISEOFULVIN-^{14}C

	Griseofulvin-^{14}C	Griseofulvin standard
Melting point (C)	218–219°	218–219°
u.v. Absorption (in methanol) max.	291 mμ	291 mμ
min.	270 mμ	270 mμ
R_f, Ascending chromatography		
A. benzene:cyclohexane:methanol: water (5:5:6:4), plus 0·5% acetic acid	0·89	0·89
B. Butanol:ammonia (20:1)	0·91	0·91
Biological activity, 0·1 mg/ml (zone test, *T. mentagrophytes*)	2·3 cm	2·3 cm



GRISEOFULVIN

(7-Chloro-4, 6, 2'-trimethoxy-6'-methyl gris-2'-en-3,4'-dione)

FIG. 1. Structure of griseofulvin. Asterisk denotes assumed position of the radioactive label.

present almost entirely in ether-extractable form. Table 3 further shows that in heart, kidney, lung, and skin slices of rat an appreciable portion of the total radioactivity added to the incubation medium was found in all tissue slices, although the ratio of distribution of the ^{14}C-label between the medium and tissue varied for different organs. This is contrary to the reports of Bedford *et al.*[2] that the concentration of griseofulvin in the Krebs bicarbonate medium did decrease during incubation with liver but not with kidney, lung, or spleen slices.

TABLE 3. THE DISTRIBUTION OF ETHER-EXTRACTABLE RADIOACTIVITY FOLLOWING INCUBATION (2 hr) OF GRISEOFULVIN-^{14}C WITH RAT TISSUE SLICES

Tissue	Incubation medium, A (%)	Tissue slices homogenates, B (%)	Total ether-extractable (A and B) (%)
Heart	49·7 ± 1·9	47·9 ± 1·6	97·6
Kidney	57·2 ± 1·7	35·6 ± 2·4	92·8
Lung	65·5 ± 1·4	32·4 ± 0·9	97·9
Skin	59·4 ± 1·2	31·0 ± 1·3	90·4
Liver	37·3 ± 2·2	31·0 ± 1·9	68·3

Mean ± S.E., four experiments.

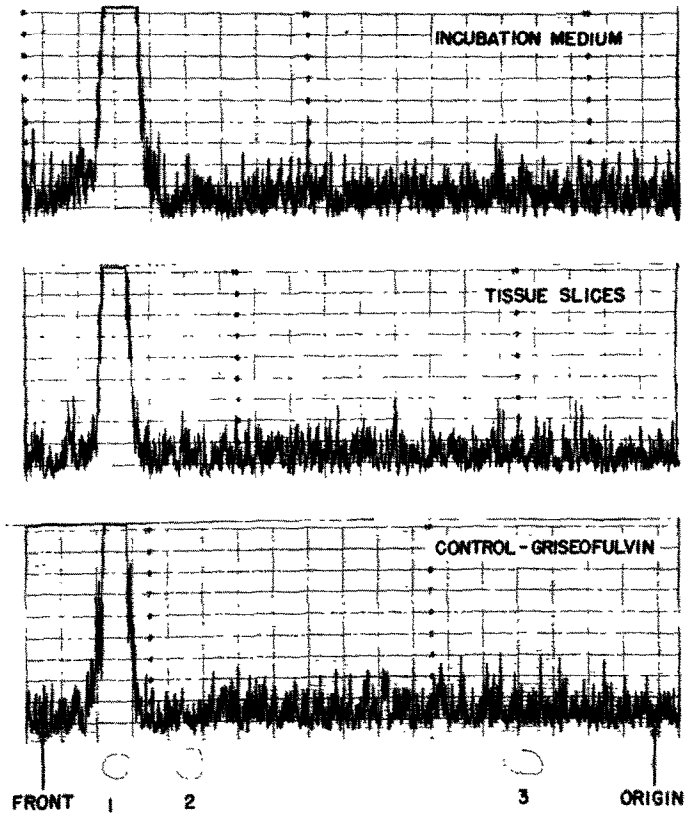


FIG. 2. Metabolic fate of griseofulvin-^{11}C after incubation with rat heart slices. Zones marked 1, 2, and 3 correspond to the position on the paper strip of authentic griseofulvin, 4-demethylgriseofulvin, and 6-demethylgriseofulvin respectively.

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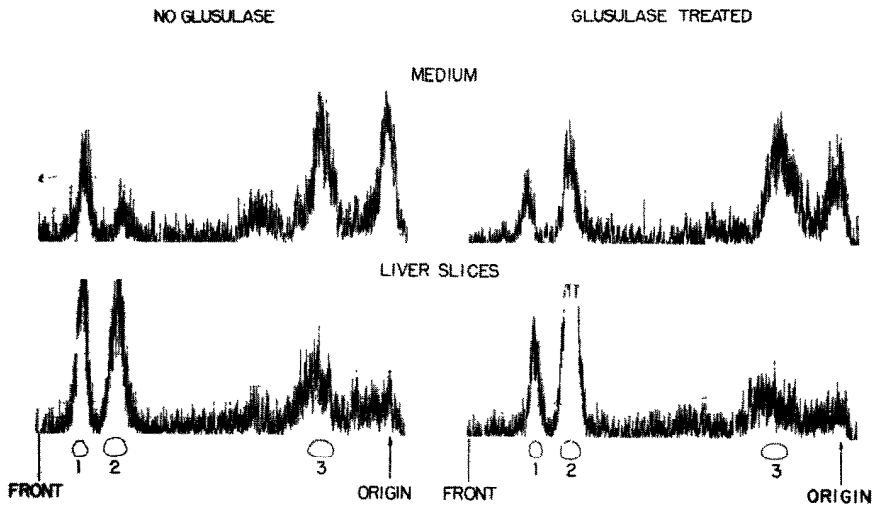

FIG. 3. Metabolic fate of griseofulvin-^{11}C after incubation with rat liver slices. Zones marked 1, 2, and 3 correspond to the position on the paper strip of authentic griseofulvin, 4-demethylgriseofulvin, and 6-demethylgriseofulvin respectively.

The chromatographic analysis of the ether extracts from heart, kidney, lung, and skin revealed that free griseofulvin was the only radioactive component in the incubation as well as in tissue slices. This would indicate that, to any detectable degree, griseofulvin was not appreciably metabolized by these organs under the experimental conditions. This is illustrated (Fig. 2) by a typical chromatographic pattern obtained from ether extracts of the heart. Identical tracings were found with ether extracts from kidney, lung, and skin samples.

As indicated earlier, the radioactive material that could be extracted with ether from liver samples accounted only for about 68 per cent of the total radioactivity present (Table 3). Treatment with Glusulase, however, increased the ether-extractable radioactive material to about 89 per cent (Table 4), which indicates that 21 per cent

TABLE 4. DISTRIBUTION OF ETHER-EXTRACTABLE RADIOACTIVITY FOLLOWING INCUBATION (2 hr) OF GRISEOFULVIN-^{14}C WITH RAT LIVER

	Ether-extractable material at pH 1		Estimated conjugates (%)
	No glusulase (%)	Glusulase-treated (%)	
Incubation medium	37·3	55·0	17·7
Liver slices	31·0	34·1	3·1
Total	68·3	89·1	20·8

Mean, four experiments.

of the ^{14}C-label was present in conjugated form. The incubation medium contained more conjugated material than the tissue slices. The metabolic fate of griseofulvin in the liver is illustrated in Fig. 3, which is typical of the chromatographic pattern obtained in four separate experiments. The positions of authentic griseofulvin and its 4- and 6-demethyl derivatives are marked as 1, 2 and 3, respectively, on the paper strips. Figure 3 shows the presence of several metabolites of griseofulvin, in addition to the free drug. Two of these metabolites correspond to the 4- and 6-demethyl derivatives of griseofulvin. Treatment with Glusulase resulted in an appreciable increase in the relative amount of 4-demethylgriseofulvin, particularly in samples of the incubation medium. The number of observed metabolites was the same in the incubation medium and tissue slices; however, their relative ratio differed. In the incubation medium, after Glusulase treatment, the 6-demethylgriseofulvin was the major metabolite (about 42 per cent), followed by appreciable amounts of 4-demethylgriseofulvin (about 28 per cent) and an unidentified material at the origin (about 18 per cent). In slices, however, 4-demethylgriseofulvin was predominant (about 63 per cent), and the other metabolites were present in small amounts. The Glusulase-treated samples contained about 12 per cent and 18 per cent of free griseofulvin in the incubation medium and tissue slices respectively.

These results demonstrate that liver slices can take up griseofulvin from the incubation medium and metabolize it predominantly by demethylation at the 4- or 6-position, and then conjugate them, possibly as glucuronides, to varying degrees. The different metabolites formed in liver slices were released into the incubation medium at varying rates.

Boothroyd *et al.*[8] have demonstrated that fungi can specifically convert griseofulvin to the 4-, 6-, and 2′-demethyl derivatives. Barnes and Boothroyd[1] found only free 6-demethylgriseofulvin in rat liver slices. Our data clearly show that, in addition to griseofulvin, at least two metabolites (the 4- and the 6-demethylgriseofulvin) were present in liver slices. Both metabolites were evident in the free and conjugated forms. Additional metabolites seen were not identified, but the presence of 2′-demethyl derivative of griseofulvin could not be demonstrated in the ether extracts, when the radioactive peaks were compared by chromatography with the authentic material. It is possible that the unidentified metabolites may be di-demethylated derivatives of griseofulvin, or more extensive degradation products of the molecule.[9]

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