of distribution of bishydroxycoumarin in rats and humans (8). On the other hand, it is possible that hepatic blood flow rate will affect the elimination kinetics of certain drugs with very short biologic half-lives which are metabolized in the liver.

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Keyphrases

Perfused organ system—drug elimination
Elimination kinetics—perfusion rate effect
Distribution factors, effect—elimination kinetics
Kinetic equations—perfused organ system

Effect of Deuterium Oxide on the Culturing of *Penicillium janczewskii* III. Antifungal Activity of Fully Deuterated Griseofulvin

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The *in vitro* antifungal activity of fully deuterated, partially deuterated, and protiogriseofulvin was compared. The antibiotics were evaluated by plate assay with *Microsporum gypseum* (ATCC 14683) as the test organism. A statistically significant enhancement in antifungal activity was observed in zones of inhibition when fully deuterated griseofulvin was compared with partially deuterated and protiogriseofulvin. No significant difference was apparent in a comparison of biological activity of partially deuterated and ordinary griseofulvin. The enhanced antifungal activity demonstrated by fully deuterated griseofulvin may be due to an increased efficiency of action. The antifungal target site may involve the metabolism of a C—D bond directly or indirectly. Since C—D bonds are generally more stable than C—H bonds, the increased stability of the molecule may alter the rate of griseofulvin metabolism by the fungus.

In the first paper (1) of this series the nutritional requirements for optimal growth of *Penicillium janczewskii* in heavy water were reported. The effects on antibiotic production were noted. The second study (2) described in detail the isolation, purification, and characterization of the deuterated griseofulvin biosynthesized by the organism in a replacement culture. In the present report comparison is made of the *in vitro* antifungal activity of ordinary griseofulvin and the antibiotics obtained from the organism grown in a deuterated environment.

EXPERIMENTAL

Antifungal activity of the antibiotic was determined by plate assay using *Microsporum gypseum* as the test organism. A modification of the USP (3) procedure for microbiological assay of griseofulvin was employed.

Medium I—The medium was composed of dextrose, 40.0 g.; peptone, 10.0 g.; agar, 15.0 g.; chloramphenicol, USP, 0.05 g.; and sufficient distilled water to make 1,000 ml. The pH was adjusted, if necessary, to 5.65 ± 0.05. The medium was placed into 100-ml. vials, fitted with rubber closures, sealed, and capped. The vials were autoclaved at 15 p.s.i. for 15 min. The medium was used immediately after cooling to 50°, or was stored for future use. After storage at room temperature, the solidified medium was melted by gentle warming on a steam bath.

Medium II—The composition of this medium differed from Medium I in that 2 ml. of a 1% aqueous solution of cycloheximide (Actidione, Upjohn) was added for each 100 ml. of the agar medium. The cycloheximide solution was injected through the rubber closure of the vial using a needle and syringe fitted with a Swinney adapter. The membrane filter had a porosity of 0.22 μ.

Preparation of Inoculum—The test organism, *Microsporum gypseum* (ATCC 14683), was grown

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on Sabouraud dextrose agar slants at 25° for 10 days at which time the growth assumed a chamois-like appearance. The slants were stored in a refrigerator at 5° for as long as 2 months.

Three-hundred milliliters of Medium I was placed into a Roux bottle and allowed to solidify. The medium was inoculated by spreading an aqueous suspension of the test organism evenly over the surface of the agar with the aid of sterile glass beads. The culture was incubated at 25° for 2 weeks. At the end of this period, a stock spore suspension was prepared by collecting the surface growth in 50 ml. of sterile water. The spore suspension was stable for at least 2 months at 5°.

Test plates were prepared using a 6-ml. base layer of Medium I covered with a 4-ml. seed layer of Medium II inoculated with varying volumes of spore suspension to give a 1, 2, or 3% suspension. The most appropriate percent of inoculum was determined by observing the largest and clearest zones of inhibition produced by 5 mcg./ml. of standard griseofulvin on the test plate.

Procedure—Six milliliters of Medium I was added to a 20 × 100 mm. Petri dish and allowed to harden for use as a base layer. The Petri dishes were covered and chilled at 5°. Medium II was allowed to cool to approximately 50° and 2 ml. of spore suspension was added for each 100 ml. of medium. The culture and agar were mixed thoroughly and 4 ml. was added to each plate containing the uninoculated base agar. Each plate was tilted back and forth to spread the inoculated agar evenly over the surface. When the agar hardened, six cylinders were placed on the surface so that they were at 60° intervals on a 2.8-cm. radius. Stainless steel cylinders were used having an outside diameter of 8 ± 0.1 mm., and inside diameter of 6 ± 0.1 mm., and a height of 10 ± 0.1 mm.

A standard stock solution of protio-griseofulvin was prepared by dissolving 50 mg. in sufficient *N,N*-dimethylformamide (DMF, Eastman Organic) to give a concentration of 1,000 mcg./ml. A standard curve was prepared by diluting the 1,000 mcg./ml. griseofulvin solution to 64, 80, 100, 125, and 156 mcg./ml. in DMF. One part of each of these solutions was further diluted with 19 parts of 0.1 *M* phosphate buffer pH 8.0, to give final solutions of 3.2, 4.0, 5.0, 6.25, and 7.8 mcg./ml.

Three plates were used for the determination of each point of the curve, with the exception of the 5.0 mcg./ml. solution. On each of the three plates, three cylinders were filled with the 5.0 mcg./ml. solution and the other three cylinders were filled with the particular concentration of the standard curve under test. There were thirty-six 5.0 mcg./ml. determinations and nine determinations for each of the other points on the curve. The plates were incubated for 48 hr. at 30°, after which the diameters of the circle of inhibition were measured. The data obtained were corrected (4) and a potency curve was constructed by plotting the concentration of reference standard protio-griseofulvin *versus* the zone of inhibition on semilogarithmic graph paper. The griseofulvin concentrations were plotted on the logarithmic scale and the diameter of the zones on the linear scale, and the line of best fit was constructed. The procedure was repeated using deuteriogriseofulvin and the curve was plotted on the same semilogarithmic paper (see Fig. 1).

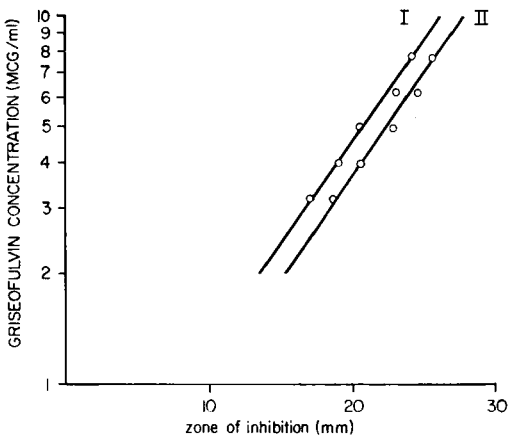

Fig. 1—The relationship of griseofulvin concentration and the diameter of zone of inhibition; (I) ordinary griseofulvin, (II) deuteriogriseofulvin.

Reference standard protio-griseofulvin, protio-griseofulvin isolated in this laboratory, partially deuterated griseofulvin (2), and fully deuterated griseofulvin (2) were assayed according to the method of Knoll *et al.* (4). The samples were diluted with DMF to give a concentration of 100 mcg./ml. These solutions were further diluted with 19 parts of 0.1 *M* phosphate buffer pH 8.0 to give a final concentration of 5.0 mcg./ml. of griseofulvin. A series of 18 assays were compared for each form of griseofulvin. The data are shown in Table I.

RESULTS AND DISCUSSION

The potency curves, Fig. 1, for the ordinary griseofulvin and the fully deuterated griseofulvin were found to be straight lines. The linearity of the curves was tested by calculating the regression equations, and their significance was established by analysis of variance using the *F* test as the test of significance. The value of *F* obtained for ordinary griseofulvin and fully deuterated griseofulvin was found to be significant at the 0.1% level and 1% level, respectively. This denotes that on the basis of probability there is a significant linear relationship over and above any curvilinear relationship.

The zones of inhibition were determined for a predetermined concentration (5 mcg./ml.) of standard griseofulvin, protio-griseofulvin obtained in this laboratory, partially deuterated griseofulvin, and fully deuterated griseofulvin (Table I). They were compared statistically by means of analysis of variance. The valid comparison of fully deuterated *versus* partially deuterated and ordinary griseofulvin, as well as partially deuterated griseofulvin *versus* ordinary griseofulvin are permitted since there are two degrees of freedom in this experiment. The difference between fully deuterated *versus* partially deuterated and ordinary griseofulvin was found to be highly significant ($p = 0.001$), indicating that there is a true difference in the zones of inhibition due to fully deuterated griseofulvin.

The computed value of *F* for comparison of partially deuterated griseofulvin with reference standard griseofulvin was found not to be significant. It was concluded therefore, that there is virtually no

TABLE I—COMPARISON OF THE DIAMETERS OF THE ZONES OF INHIBITION FOR THE GRISEOFULVINS AT A CONCENTRATION OF 5 MCG./ML.

Type of Griseofulvin			
Reference Standard,[a] cm.	Protio-,[b] cm.	Partially Deuterated,[b] cm.	Fully Deuterated,[b] cm.
2.20	2.17	2.13	2.37
2.21	2.21	2.08	2.40
2.21	2.20	2.15	2.34
2.23	2.12	2.14	2.38
2.16	2.16	2.09	2.40
2.23	2.09	2.18	2.41
2.20	2.22	2.29	2.42
2.13	2.18	2.21	2.50
2.20	2.23	2.13	2.36
2.20	2.10	2.21	2.41
2.22	2.11	2.20	2.50
2.20	2.21	2.09	2.42
2.15	2.14	2.12	2.36
2.16	2.16	2.20	2.33
2.09	2.18	2.21	2.40
2.16	2.12	2.16	2.36
2.17	2.17	2.11	2.41
2.15	2.16	2.21	2.36
$\bar{x} = 2.18 \pm 0.04$[c]	$\bar{x} = 2.16 \pm 0.03$[c]	$\bar{x} = 2.16 \pm 0.06$[c]	$\bar{x} = 2.40 \pm 0.05$[c]

[a] Obtained from McNeil Laboratories (Lot No. 2461). [b] Isolated in this laboratory (2). [c] Standard deviation, $n = 18$.

difference between the antifungal properties of the two samples.

It is apparent that there was no difference in zones of inhibition obtained with reference standard griseofulvin and protio-griseofulvin obtained from H_2O cultures in this laboratory. The mean for standard griseofulvin was found to be 2.18 ± 0.04 cm. and the mean for the laboratory-produced ordinary griseofulvin was 2.16 ± 0.03 cm.

The mode of action of protio-griseofulvin is not completely understood. Consideration has been given to the theory that the agent exerts its fungistatic properties by interfering with the synthesis of cell wall chitin, or by interference with nucleic acid metabolism. Recent evidence suggests that griseofulvin stimulates DNA synthesis and causes the formation of abnormal cells, leading to the proposal (5) that the site of action is in the replicatory system of the fungal cell. It is not unlikely that the basic mechanism of antifungal action for fully deuterated griseofulvin is the same as that for ordinary griseofulvin. However, the mean zone of inhibition for deuterio-griseofulvin at a concentration of 5 mcg./ml. was found to be 2.40 ± 0.05 cm. whereas the mean for standard protio-griseofulvin at the same concentration was 2.18 ± 0.04 cm. The data observed over the range of concentrations tested indicate that deuterio-griseofulvin is about 10% more active than protio-griseofulvin on a weight basis. Since the molecular weight of deuterio-griseofulvin is about 5% greater than protio-griseofulvin, comparison on a mole basis would provide an even greater effect for the deuterio-analog.

The enhanced antifungal activity demonstrated by the fully deuterated antibiotic may be due to an increased efficiency of action. This increase in efficiency may be the result of a primary deuterium isotope effect, where the target site may involve the cleavage of a C-D bond directly. A kinetic effect involving a rate-limiting step may be affected. Since C-D bonds are generally more stable than C-H bonds, the increased stability of the molecule may play a role in that the fungus may not be able to eliminate the fungistatic agent as readily. The biological effect could also be due to a secondary isotope effect where deuterium in molecular positions other than a reaction locus are involved.

No increase in antifungal activity was observed with partially deuterated griseofulvin. More extensive deuterium replacement is apparently required in the molecule, or at least at critical loci, before enhanced antifungal activity can be observed.

Deuterium isotope effects in biological systems are extremely complex, and a combination of several effects may well be involved. A better understanding of the mechanism of antifungal activity of ordinary hydrogen griseofulvin will probably be necessary before the effect of deuterium replacement in the molecule can be fully understood. More studies concerning stability, enzyme systems of the fungus, and the effect of the antibiotic upon them, are clearly necessary for a better understanding of the fungistatic properties of fully deuterated griseofulvin.

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Keyphrases

Deuterated griseofulvin—antifungal activity
Antifungal assay—*Microsporum gypseum*
Griseofulvin, protio, fully and partially deuterated—comparison